

THE DNA SEQUENCE OF THE STRUCTURAL GENE OF GONOCOCCAL PROTEIN III AND THE FLANKING REGION CONTAINING A REPETITIVE SEQUENCE

Homology of Protein III with Enterobacterial OmpA Proteins

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The major outer membrane (OM)¹ proteins of *Neisseria gonorrhoeae* have been extensively studied (1, 2). The overall composition of the cell wall is similar to that found in gram-negative organisms in general (3). The OM of *Neisseria gonorrhoeae* contains numerous proteins, but three occur in large amounts and these are called proteins I, II, and III (PI, PII, PIII) (4). PI is always present, represents the porin molecule of the gonococcus (5–7), and is the protein present in the largest amount (3). The PII proteins, also known as the opacity proteins (8, 9), may or may not be expressed by a gonococcal strain, and this property is subject to phase variation with a frequency of $\sim 10^{-3}$ per cell division (10). PIII was first described by McDade and Johnston (11), and is always expressed by *N. gonorrhoeae*. In contrast with the antigenic variability seen with a number of other gonococcal surface proteins such as pili, PI, or PII, no heterogeneity of molecular weight or of proteolytic peptide patterns of PIII has been observed (12, 13). Studies using cleavable bifunctional reagents (11, 14) indicated that PIII is closely associated with part, but not all of the PI molecules in the gonococcal OM. A portion of the PIII is exposed to the surface in intact gonococci; the protein can be labeled with iodine and reacts with mAbs (15). By mechanisms that are not fully understood, binding of antibodies to PIII interferes with the bactericidal action of complement acting in concert with antibodies to other surface antigens such as lipooligosaccharide of the gonococcus (16–18).

We have recently (19) cloned the structural gene for PIII in the λ gt11 expression vector, and here report the complete structure of the protein deduced from the DNA sequence. In addition, we have found that the flanking region downstream from the PIII structural gene contains a sequence that is repeated at least 20 times in the genome of the parent gonococcal strain.

Materials and Methods

Reagents and Chemicals. Isopropyl- β -D-thiogalactopyranoside (IPTG), and some restriction enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis,

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¹ *Abbreviations used in this paper:* IPTG, isopropyl- β -D-thiogalactopyranoside; OM, outer membrane.

IN); additional restriction enzymes and T4 ligase were obtained from New England Biolabs (Beverly, MA). The enzymes were used according to the recommendations of the vendors. Nitrocellulose BA85 was obtained from Schleicher & Schuell (Keene, NH) and all other reagents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Deoxynucleotides, dideoxynucleotides, and Klenow fragment were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). α -[^{32}P]dATP and radioactive protein A was purchased from Amersham Corp. (Arlington Heights, IL).

DNA Sequencing. *Escherichia coli* strain Y1089 lysogenized with the λ gt11 PIII clone was induced by heating to 43°C and the phage was isolated by flotation in solutions of CsCl (20). The gonococcal DNA insert was excised by digestion with Eco RI and purified by agarose gel electrophoresis and electroelution. The chain termination method of DNA sequencing was used (21) according to the methods described by Sanger et al. (22) for preparing polyacrylamide gels, and substituting inosine triphosphate for guanosine triphosphate in instances where difficulties with band compression arose. Fragments obtained by digestion of the purified insert with restriction enzymes were separated on SeaPlaque agarose obtained from FMC Bioproducts (Rockland, ME) and ligated to the phage vectors mp10 or mp11 (23) as described by Struhl (24). Sequential deletions of the clone were produced by the method of Dale et al. (25) using the Cyclone kit produced by International Biotechnologies Inc. (New Haven, CT).

Immunological Methods. SDS-PAGE was performed on cells lysed in the SDS containing loading buffer (26). Electrophoretic transfer to nitrocellulose was performed according to the methods of Towbin et al. (27), and the Western blots were probed with phosphatase-conjugated reagents (28), or with radioactive protein A obtained from Amersham Corp. An mAb directed to PIII was kindly provided by Dr. J. Swanson (Rocky Mountain Laboratory, National Institutes of Health, Hamilton, MT).

DNA Hybridization Analysis. DNA hybridization analyses were performed according to methods reviewed by Meinkoth and Wahl (29). Hybridizations were carried out at 42°C in 50% formamide and stringent washing conditions were used (1 \times SSC at 68°C). DNA was labeled with ^{32}P by nick translation (30).

Results

The DNA Sequence of the Gonococcal PIII Gene. The PIII structural gene was originally cloned in a λ gt11 vector and the Eco RI insert was sequenced by the dideoxy chain termination method. The insert contained 1,349 residues and within the protein coding region both strands were sequenced (Fig. 1). An open reading frame coding for 236 amino acids was found and it contained the known protein NH₂-terminal sequence (31) following a typical signal peptide sequence of 22 amino acids. This signal sequence included a commonly encountered feature, namely alanine at the -1 and -3 position (32). The predicted molecular weight of the proPIII is 25,544, and 23,298 for mature PIII. Since the predicted mol wt is ~8,000 less than the apparent mol wt of PIII as determined by SDS-PAGE, independent confirmation was sought to establish that the translation ceased at the stop codon at nucleotide 813, rather than at approximately nucleotide 1,000, which would be predicted from the molecular weight estimated by SDS-PAGE. First, advantage was taken of the presence of a single Cla I site in the insert, and the 897 bp fragment produced by Cla I digestion was subcloned into pUC9. By Western blotting it was found that the expressed product had a mobility identical to gonococcal PIII (Fig. 2). Second, the predicted amino acid composition of mature PIII agreed with the values based on amino acid analysis reported by Lytton and Blake (31) (Table I). Last, starting at nucleotide 909, a 13-bp inverted repeat containing a 4-bp loop and ending with 7 thymidine residues was found, and it is very likely that this area represents the transcription

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GAATTCCTATCCGATTTGCCCGCATGTTTCTACAGCGGCCCTGTATGTTGGCAATTCAGCA
  10      20      30      40      50      60
GTTGCTTCTGTATCTGCTGTACAAATCTAATGAGGGAATAAAATGACCAAACAGCTGAAA
  70      80      90      100     110     120
      M T K Q L K
L S A L P V A L L A S G T A V A G E A S
TTAAGCGCATTTTCGTGGCATGCTCGCTCCGGCAGTGTGTCGGGCGAGGGCTCC
  130     140     150     160     170     180
V Q G Y T V S G Q S N E I V R N N Y G E
GTTGAGGTTACACCGTAAGCGGCCAGTCCAACGAAATCGTAGCAACAACATGGAGAA
  190     200     210     220     230     240
C W K N A Y F D K A S Q G R V E C G D A
TGCTGGAAACCGCTACTTTGATAAAGCAAGCCAAAGTCCGCTAGAATGCGGGATGCG
  250     260     270     280     290     300
V A V P E P E P A P V A V V E Q A P Q Y
GTTGCGTCCCGAGCCCGAACCCCGCCTGTCCGCTTGTGGAGCAGGCTCCTCAATAT
  310     320     330     340     350     360
V D E T I S L S A K T L F G F D K D S L
GTTGATGAAACCATTTCCCTGTCTGCCAAAACCGCTTTCGGTTTCGATAAGGATTCATG
  370     380     390     400     410     420
R A E A Q D N L K V L A Q R L S R T N V
CGCCCGAAGCTCAAGACAACCTGAAAGTATGGCGCAACCGCTGAAGTCAAGCAACCAATGTC
  430     440     450     460     470     480
Q S V R V E G H T D F M G S E K Y N Q A
CAATCTGTCGGCTCGAAGGCCATACCGACTTTATGGTTCTGAAAAATACAATCAGGCT
  490     500     510     520     530     540
L S E R R A Y V V A N N L V S N G V P A
CTGTCCGAAACCGCGCATACGTAGTGGCAAACAACCTGGTCAGCAACGGCGTACCTGCT
  550     560     570     580     590     600
S R I S A V G L G E S Q A Q M T Q V C Q
TCTAGAATTTCTGCTGTGGCTTGGGCGAATCTCAAGCGCAAAATCAAGTTTGGTCA
  610     620     630     640     650     660
A E V A K L G A K A S K A K K R E A L I
GCCGAAGTTGCCAAACTGGTGGGAAAGCCCTCAAAGCCAAAAACGTAAGGCTCTGATT
  670     680     690     700     710     720
A C I E P D R R V D V K I R S I V T R Q
GCATGTATCGAACCTGACCGCGGTAGATGTGAAAATCCGCAGCATCGTAACCCGTCAG
  730     740     750     760     770     780
V V P A R N H H Q H *
GTTGCGCGGCACGCAATCATCACCAACACTAAGGCTAGGTAATATCTGCGATGCAATG
  790     800     810     820     830     840
AGGTTAGCGAATTTTGTACCGGGTACTGTTGCAATATTCGTGAAACGTCGGCCGGTATCG
  850     860     870     880     890     900
ATGATGTGAAACAAACCCCGCTTTTGGGGGTTTGTTTTTTGGGTGGTTTTCTGAAACG
  910     920     930     940     950     960
GCTATCGTCAGAAATCGGGGTGCAGGTTCCGATTCGGATTCAGATTCATGTTTGTGCCA
  970     980     990     1000    1010    1020
TTGCCCGCTTTTATAGTGGATTAACAAAAATCAGGACAAGGCGCAGCAAGCGCAGACAGT
  1030    1040    1050    1060    1070    1080
ACAATAGTACGGCAAGGCGAGGCAACGCCGTACCGTTTAAATTTAATCCACTATATCGG
  1090    1100    1110    1120    1130    1140
TTGAAACTCTGATTTAAGGCGGTAGGATGTGGTTTGGCCATAGCAAGGGAATCCTTTC
  1150    1160    1170    1180    1190    1200
TGTATCAAGCCCGAAAGGATAATTCATACAAATTCACGCCCTTCCCCCTCATTTGGGAA
  1210    1220    1230    1240    1250    1260
ATGGATGGAATCGTCCCGATGTGTGCGGCACGTATGCCGATATGGTTTTATCATCAT
  1270    1280    1290    1300    1310    1320
CCCTTTCGGTTGAAACCCCGGGAATTC
  1330    1340
    
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FIGURE 1. Nucleotide sequence and deduced amino acid sequence of PIII. The underlined portion of the sequence represents a 13-bp inverted repeat with a 4-bp loop, which at 3' end has four additional T residues. It is likely that this structure serves as the transcription terminator.

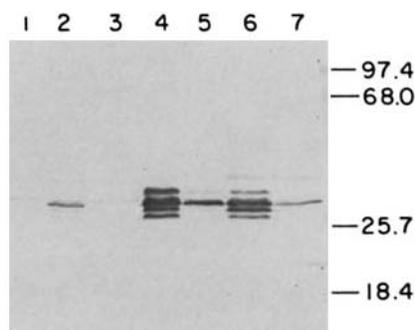


FIGURE 2. Western blot demonstrating that clones of the PIII gene in pUC9 in *E. coli* strain JM103 containing either the full insert 1-1,349 or the fragment from nucleotide 1-897 produced a normal sized immunologically active PIII product. In addition, this figure demonstrates that the expression of the cloned PIII in λ gt11 is stimulated by IPTG. Cultures of the lysogens were grown to an OD_{600} of 0.5 at 32°C, heated to 43°C, and then grown for an additional 60 min at 37°C with or without 5 mM IPTG (35). The blots were reacted with an mAb directed to PIII. (1) λ gt11, no IPTG; (2) PIII, no IPTG; (3) λ gt11, with IPTG; (4) PIII, with IPTG; (5) gonococcus strain R10; (6) PIII 1-1,349 bp in pUC9; (7) PIII 1-897 bp in pUC9. Note that the clones when overproducing give rise to four immunologically active products. A similar pattern has been seen with *E. coli* strains overproducing OmpA, and the forms have been identified as proOmpA, mature OmpA, processed OmpA still associated with inner membrane, and a nascent OmpA molecule (58).

TABLE I
Amino Acid Composition of PIII

Amino acid*	Amino acid analysis		DNA sequence 23,300 mol wt
	30,300 mol wt [‡]	23,300 mol wt [§]	
G	20	15	13
A	34	26	26
V	29	22	26
L	17	13	11
I	9	7	7
S	18	14	17
T	11	8	7
F	7	5	4
Y	8	6	6
C	2	2	4
M	4	3	2
P	16	12	8
D + N	26	20	20
E + Q	37	28	31
H	4	3	4
R	20	15	15
K	17	13	12
W	ND [†]	ND	1

* The one-letter amino acid code is used.

[‡] Values are based on amino acid analysis by Lytton and Blake (31).

[§] The data are recalculated to add up to a mol wt of 23,300 to allow comparison to the predicted composition.

[†] Tryptophan content not determined.

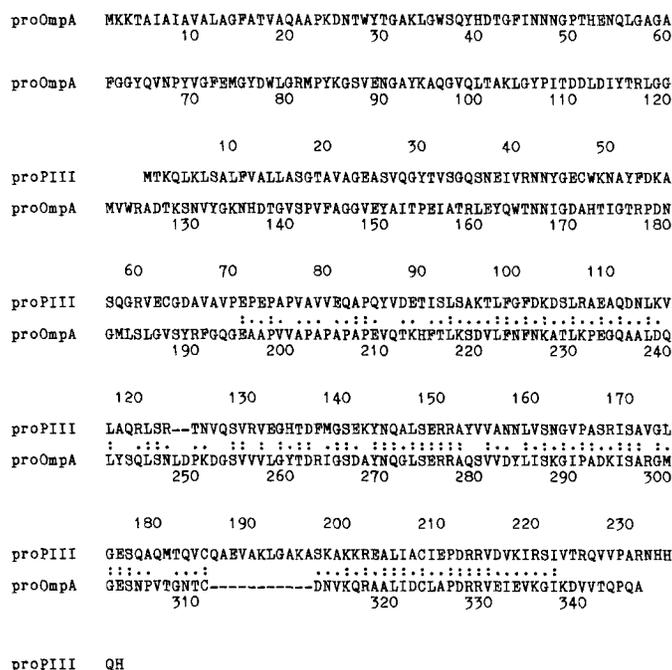


FIGURE 3. Homology of PIII and *E. coli* OmpA. Double dots indicate identities, single dots indicate conservative substitutions.

terminator (33) (Fig. 1). Nonglycosylated proteins that have similarly unusual electrophoretic characteristics have been described. For example the dopamine/cAMP-regulated neuronal phosphoprotein DARPP-32 migrates with an M_r 32,000 and by amino acid sequence has a molecular weight of 22,591 (34).

Since the expression of the cloned PIII gene in λ gt11 was markedly enhanced by addition of IPTG to the medium (35) (see Fig. 2), we conclude that transcription was initiated from the β -galactosidase promoter and we did not seek to identify a promoter sequence in the PIII clone.

When the predicted amino acid sequence of PIII was compared with the protein sequences in the Dayhoff data base using the algorithm of Wilbur and Lipman (36), highly significant homology to the OmpA protein of *Shigella dysenteriae* (37), *E. coli* (38-40), and *Enterobacter aerogenes* (41) was found. This homology also applies to the OmpA proteins of *Salmonella typhimurium* (42) and *Serratia marcescens* (43). The homology of PIII to *E. coli* OmpA is illustrated in Fig. 3. ProPIII contains a proline-rich stretch from residue 70 to 84. The OmpA genes show this feature as well. In OmpA, the five proline residues alternate with six residues of valine or alanine, while in PIII, between the five prolines there are in addition to the six residues of alanine or valine also three glutamates and one glutamine. Thereafter there is strong homology between PIII and OmpA extending through the remainder of the protein, except for the 14 carboxy-terminal residues. A gap of 11 residues has to be introduced into the OmpA immediately after the cysteine at position 311 to preserve the homology. Assuming that the cysteine residues in both proteins are disulfide bonded, PIII has an

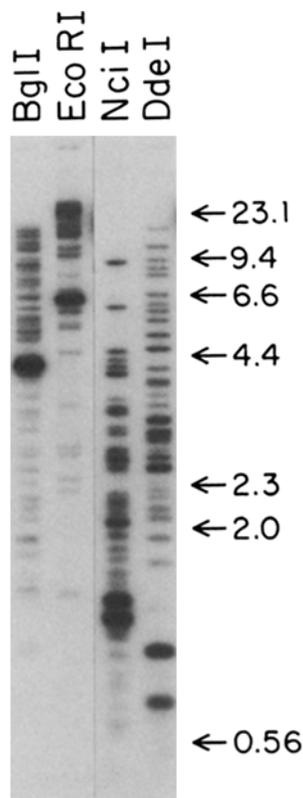


FIGURE 4. DNA hybridization observed when genomic DNA of gonococcal strain R10 was probed with the complete 1,349 bp fragment. Note that the signal due to the PIII structural gene is visible in all lanes, as a single band in the Eco RI and the Bgl I digests, and as more than one band in the Nci I and Dde I digests. From the sequence (Fig. 1) it is known that there is a Nci I site at position 859 and Dde I sites at positions 463 and 810. The expected intervening Dde I fragment of 347 bases was run off the gel.

additional 11 amino acids in this disulfide loop. It is noteworthy that PIII, in contrast to all OmpA proteins, has an additional pair of cysteines with 15 intervening amino acid residues at positions 47 and 63.

Identification of a Repetitive Sequence in the Flanking Region. When the PIII gene was labeled by nick translation and used to probe genomic DNA of the parent gonococcus, the very complex pattern seen in Fig. 4 was obtained. When digestion was performed with Eco RI or Bgl I a single major band was seen with numerous additional signals. The digests obtained with Nci I and Dde I have more than one major band since the insert contains these restriction sites (for details see Fig. 4). The hybridization studies with these digests indicates that some sequence within the insert is repeated at least 20 times in the genome. The bands due to DNA other than the structural gene of PIII were eluted when the blots were washed at 80°C.

The location of the repetitive sequence in the cloned DNA was determined by using the phage clones obtained in the course of sequencing and additional ones that were derived specifically for this purpose. The hybridization studies were

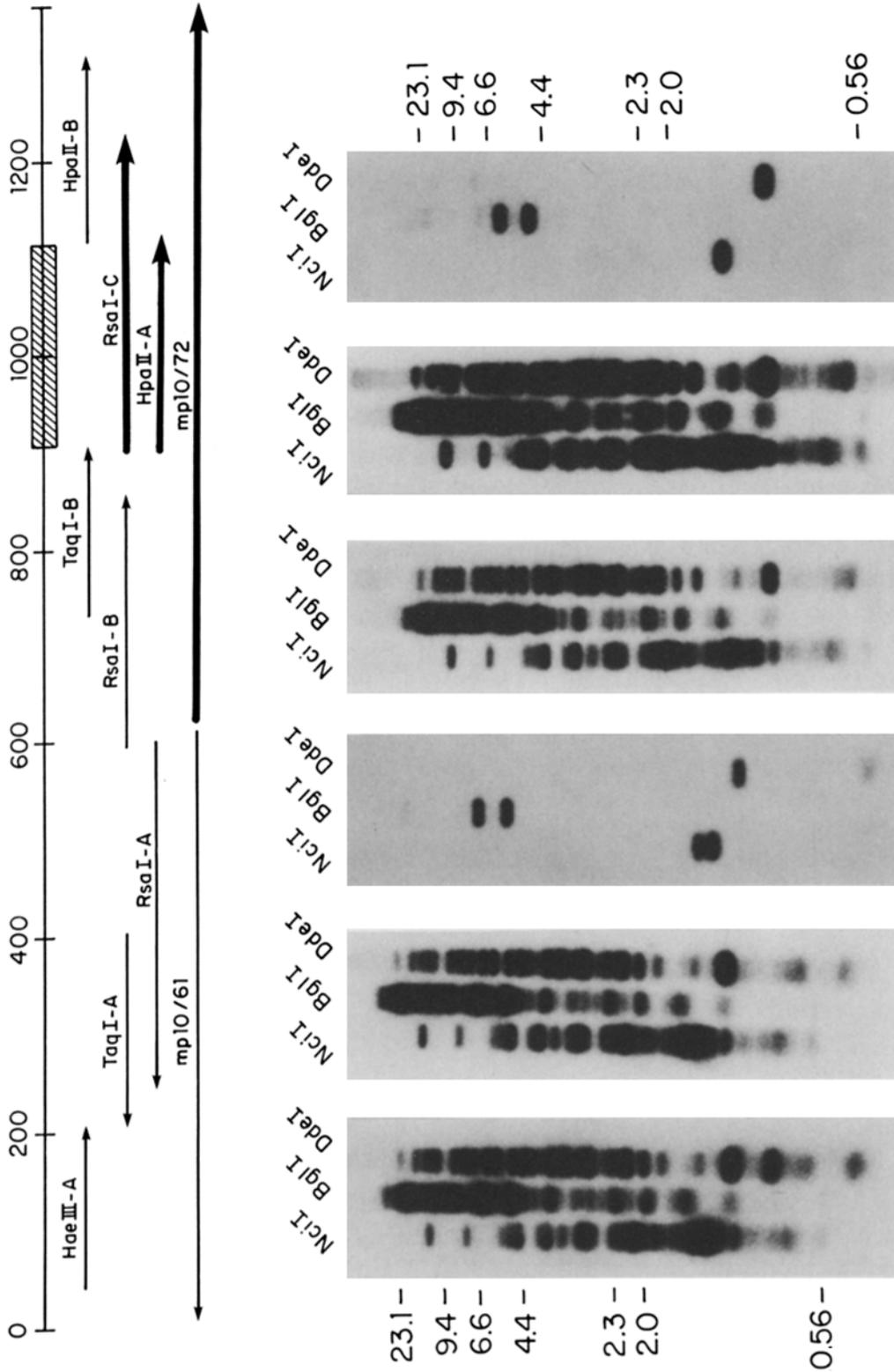


FIGURE 5. The location of the repetitive sequence within the cloned DNA fragment. Strain R10 genomic DNA was digested with Bgl I, Nci I, and Dde I. After electrophoresis and transfer to nitrocellulose the blots were cut into strips and hybridized to 150 ng/ml of single-stranded DNA of the subclones indicated in the figure and washed with 1X SSC at 68°C. Bound single-stranded DNA was detected by hybridization with replicative form of mp10 that had been labeled by nick translation. The numbering at the top represents the sequence of the insert. The arrows indicate the part of the sequence contained in each of the M13 clones. The heavy arrows represent clones that contained the repetitive sequence while the light arrows indicate clones lacking this sequence. The patterns obtained allow the conclusion that the repetitive sequence is somewhere between nucleotide 898 and 1,113. The autoradiographs shown are part of a single experiment in which the Bgl cut was incomplete; in other hybridization studies only a single band hybridizing with the PIII structural gene was seen.

performed by exposing nitrocellulose strips bearing genomic DNA cut with restriction enzymes to single-stranded DNA of selected phage clones. The strips were washed under stringent conditions, and the retained phage DNA was detected with nick-translated replicative form of mp10. The results are outlined in Fig. 5 and indicate that the repetitive sequence is located somewhere between nucleotide 898 and 1,113 in the sequence.

Discussion

Structural and functional aspects of *E. coli* OmpA have been extensively studied. The protein is able to bind to LPS, resulting in a change in conformation (44). It is apparent that this protein is necessary for mating pair formation in conjugation (44–47). Deletion mutants of OmpA cause the organisms to be sensitive to EDTA and detergent. Deletion of OmpA and lipoprotein expression simultaneously results in major ultrastructural changes; the organisms assume a coccoid form and the OM has a tendency to shed in the form of blebs (48). In fact, they rather look like pathogenic *Neisseria* (49). OmpA serves as a receptor for colicins K and L (50, 51), and as a host of phages (47, 52). This property has been exploited effectively to map in detail the surface exposure of OmpA by determining the sequence changes that occur in phage resistant mutants. On the basis of these studies, it has been postulated that there are four surface exposed domains centering on amino acid residues 46, 91, 131, and 175 (53, 54). OmpA is resistant to proteases in the intact organism but susceptible when envelopes are used. For instance, pronase cleaves the protein at position 198 (38). Taking into account the periplasmic location of the protease-susceptible sites and that no phage-resistant mutants have shown any sequence changes in the carboxy-terminal portion of the molecule following the mutable site near residue 175 (54), it has been proposed that this portion of the protein is located in the periplasmic space (53).

PIII is resistant to proteases in the intact gonococcus or in isolated OM blebs (55), which, by electron microscopy, appear to be sealed vesicles. However, the purified protein is very readily attacked by proteases (31). If one assumes that the close structural similarity of PIII and OmpA indicates that the homologous portions of the PIII are similarly disposed in the periplasmic space, it becomes evident that only a relatively small segment of the protein (residues 23–65, approximately) is available to be exposed to the surface. This area does contain three tyrosine residues at positions 30, 44, and 52 in accord with the ability to surface label PIII (13). It also contains a potential 15-residue disulfide loop that is not seen in any of the OmpA proteins.

When hybridization studies were performed it was evident that some portion of the cloned insert occurred at least 20 times in the genome of the parent gonococcus. Further hybridization studies summarized in Fig. 5 located the repetitive sequence to the 3' flanking region between residues 898 and 1,113. The signals with the repetitive sequence were detected under stringent washing conditions (1× SSC, 68°C), and did not elute until the wash temperature was raised to 80°C. In a very recent publication, Correia et al. (56) have identified a repetitive sequence in the genome of the gonococcus. Its existence was suspected from results obtained by two-dimensional heteroduplex S1 nuclease mapping of

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      1010      1020      1030      1040      1050      1060      1070
AGATTGATGTTTGTGTCGATTGCGCGGCTTTATAGTGGATTAACAAAAATCAGGACAAGGCGACGGAAGC
Gonococcal Repeat*          TATAGTGAATTAACAAAACCGGTAC
                               C G                      T A G

      1080      1090      1100      1110      1120      1130      1140
CGCAGACAGTACAATAGTACGGCAAGGCGAGGCAACGCGTACCGGTTAAATTTAATCCACTATATCCG
Gonococcal Repeat          GTACCGG-TTTTGTTAATTCACTATA

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FIGURE 6. Comparison of the PIII flanking sequence with a gonococcal repeat sequence. Asterisk indicates the consensus repeat sequence reported by Correia et al. (56) and includes on the line below the alternative bases they found. The underlined portions of the sequence highlight a 13-bp inverted repeat that is part of the consensus sequence.

genomic DNA (57). They obtained DNA sequences of three random pieces of genomic DNA selected on the basis of colony hybridization, and identified a 26-bp consensus sequence, which is shown in Fig. 6. It is evident that this sequence is present in the area where this study identified a repetitive element. It is also noteworthy that an analogous sequence recurs in an inverted orientation 53 bp downstream. The underlined sequences represent a 13-bp inverted repeat located within these sequences. Correia et al. (56) in one instance also found these sequence elements as an inverted repeat with 440 intervening bases. The function of the repetitive sequences is not known, and Correia et al. (56) suggested that they may serve as recombination sites.

Summary

The insert of a λ gt11 clone expressing gonococcal protein III was sequenced. The deduced amino acid sequence showed a coding frame of 236 amino acids with a typical 22-amino-acid signal peptide, followed by the known NH₂-terminal sequence of PIII. The mature protein has a molecular weight of 23,298. It was found that PIII had extensive and very striking homology to the carboxy-terminal portion of enterobacterial OmpA proteins. The homology encompasses the OmpA domain that is believed to be located in the periplasmic space. If the disposition of PIII across the OM is analogous, then the surface-exposed domain consists of <40 amino acids. These include a potential 15-amino-acid disulfide loop, a feature not found in OmpA proteins.

Hybridization studies with the sequenced insert indicated that it contained a repetitive sequence that occurred at least 20 times in the genome. By additional hybridization studies the area containing the repetitive sequence was narrowed to a region of 43 bp. This region contained an exact copy of the consensus sequence of a 26-bp repetitive sequence recently described (56). An analogous sequence recurs in an inverted orientation 53 bp downstream.

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