CLINICAL MICROBIOLOGY REVIEWS, Apr. 1989, p. S60–S63
0893-8512/89/0S0S60-04$02.00/0

Vol. 2, Suppl.

Protein III: Structure, Function, and Genetics

MILAN S. BLAKE,[1]* LEE M. WETZLER,[1] EMIL C. GOTSCHLICH,[1] AND PETER A. RICE[2]

Laboratory of Bacteriology and Immunology, The Rockefeller University, New York, New York 10021,[1] and The Maxwell Finland Laboratory for Infectious Diseases, Boston University, Boston, Massachusetts 02215

Protein III (PIII) was originally described by McDade and Johnston (14). In their studies of the gonococcal outer membrane, they observed that all gonococcal strains contained a similar protein whose apparent molecular weight in sodium dodecyl sulfate-polyacrylamide gel electrophoresis increased upon treatment with a reducing agent. Furthermore, they and others (14, 17) provided evidence, using bifunctional cross-linking reagents, that PIII was closely associated with the protein I (PI) porin within the gonococcal outer membrane. It was further shown that PIII was structurally and immunologically conserved among several strains of gonococci (11, 12). In studies in which several proteolytic enzymes were used to investigate the surface orientation of gonococcal outer membrane proteins, PIII was found to be resistant to protease treatment (2). However, Shafer and Morse, using lysosomal cathepsin G, showed that PIII in gonococcal outer membranes could be cleaved by this enzyme (20). In addition, the rate at which PIII was degraded by cathepsin G could be correlated to the lipooligosaccharide expressed by a particular strain of gonococcus; i.e., the PIII associated with low-molecular-weight lipooligosaccharide was cleaved more rapidly than was the PIII associated with a higher-molecular-weight lipooligosaccharide. Surface-labeling experiments and the ability of PIII to react in vivo with a monoclonal antibody also indicated that PIII had surface-exposed domains (22). Our interest was piqued by several unanswered questions. We wanted to know (i) how this highly conserved, surface-exposed protein could exist in an organism that expended a great deal of its genome and energy in antigenic variation; (ii) how, if at all, PIII affected the functions of PI; and (iii) how PIII could be eliminated as a contaminant in preparations of PI.

PURIFICATION

To study PIII more directly, we developed a method for purifying it (13) and began to examine its biochemical and immunochemical characteristics. Early on, we observed that to purify PI, it was essential to separate PI from PIII in the initial extraction step. Otherwise, PIII remained tightly associated with PI throughout the subsequent steps of purification. We found that if the pH of the extraction buffer was altered, different proteins would be solubilized. If the extraction was performed at pH $\leq$ 4, PI and PII were quantitatively released, but PIII remained with the cell debris (1). PIII was solubilized in rather pure form by reextraction of the cell debris with a pH 10.5 buffer. It was completely released with minimal PI contamination. The PIII was then purified by cation-exchange chromatography on CM-Sepharose, followed by gel filtration on Sephacryl-200 (13). The resulting product retained all the biochemical and immunochemical characteristics of the native PIII, except that, unlike the native molecule, purified PIII was highly susceptible to proteases. The amino acid composition and amino-terminal sequences of PIII isolated from several strains of *Neisseria gonorrhoeae* were determined and found to be identical. Furthermore, these data suggested that PIII was distinct from gonococcal PI or PII. Polyclonal rabbit antiserum was raised to the purified PIII and used in whole-cell enzyme-linked immunosorbent assay (ELISA) inhibition studies. These data confirmed the results of others (12, 22), which suggested that PIII was surface exposed and indicated that approximately 70% of the antibodies to the purified molecule were adsorbed by intact gonococci. It was also observed that the class 4 protein of *N. meningitidis* was recognized by these antisera.

CLONING OF THE STRUCTURAL GENE

The structural gene of PIII has been cloned in the expression vector λ gt11 (7). Unlike clones of gonococcal PI, the cloned PIII gene produced a full-sized, immunologically reactive product in *Escherichia coli* following isopropyl-β-D-thiogalactopyranoside (IPTG) induction as shown by Western immunoblot analysis. The cloned protein displayed the typical increase in apparent molecular weight in sodium dodecyl sulfate-polyacrylamide gel electrophoresis upon reduction. The PIII gene has been sequenced by the chain termination method and found to contain an open reading frame of 236 amino acids (8). This consisted of a typical 22-amino-acid signal peptide sequence followed by the known N-terminal sequence. However, the calculated molecular weights for the pro-protein and the mature protein were 25,544 and was 23,298, respectively. This was almost 8,000 less than expected from its migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. There were several lines of evidence indicating that the PIII sequence terminated at nucleotide 813 (rather than at about nucleotide 1000). First, a subclone in plasmid pUC9 extending from nucleotides 1 to 897 produced a complete PIII product. Second, a typical transcription terminator was found starting at nucleotide 909. Third, the predicted amino acid composition of the mature PIII corresponded closely to that determined by analysis. Several different methods have been used in an attempt to determine the carboxy-terminal residue of gonococcus-expressed PIII by using carboxypeptidase Y digestion (E. J. Lytton and M. S. Blake, unpublished data). No free amino acids were released, suggesting that the carboxy terminus was blocked or unavailable for cleavage.

Recently, Klugman (personal communication) has cloned and sequenced the gene of the *N. meningitidis* class 4 protein by using similar cloning and sequencing strategies that were used with PIII. His results showed that the sequences of the two proteins (PIII and class 4) were almost identical, the most pronounced differences being within the proline-rich area.

* Corresponding author.

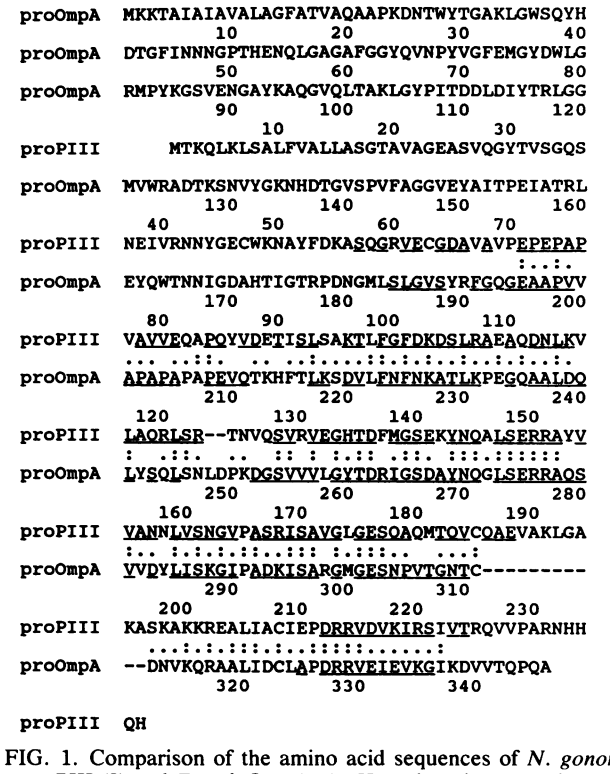

FIG. 1. Comparison of the amino acid sequences of *N. gonorrhoeae* PIII (8) and *E. coli* OmpA (4). Homology between the two proteins is indicated by a colon, and conservative substitutions are indicated by a period. Furthermore, each of these two proteins has been compared with the sequence of *P. aeruginosa* protein F (6), and where their respective sequences compare significantly, the sequence is underlined.

PARTIAL HOMOLOGY WITH PROTEINS FROM OTHER GRAM-NEGATIVE ORGANISMS

The translated sequence of PIII was compared with those of other known protein sequences and found to have homology with enterobacterial OmpA proteins (3). PIII also has significant sequence homology with the protein F gene of *Pseudomonas aeruginosa* (6). (Fig. 1). The similarity between OmpA and PIII begins in a proline-rich region, where OmpA has five proline residues interspersed with valines or alanines. The same feature is seen in PIII, except that in addition to the six valines or alanines, the area also contains three glutamates and one glutamine. Thereafter, the homology is very striking, with the exception of the terminal 14 amino acids in PIII. The distance between the homologous cysteine residues is 11 amino acids longer in PIII. In the nonhomologous region of PIII, just to the amino-terminal end of the proline-rich region, there are two cysteine residues at positions 47 and 63. This disulfide loop is not seen in any of the other known OmpA proteins. However, in the *P. aeruginosa* protein F sequence, there is an area containing four cysteines just following the proline-rich region (6). We were interested in how these cysteines related to each other, and we took advantage of two methionyl residues at positions 139 and 182 to determine whether the two cysteine residues at positions 186 and 209 were disulfide bonded to those at positions 47 and 63. Under nonreducing conditions the molecule was chemically cleaved by cyanogen bromide at the methione residues (9). These fragments were then analyzed by gel electrophoresis, by the methods of Swank and Munkres (21). When cyanogen bromide-generated PIII fragments in a nonreduced form were compared with those which had been reduced prior to electrophoresis, no difference was seen and the number of fragments observed was the same. This suggested that the cysteine residues at positions 47 and 63 formed one 15-amino-acid disulfide loop, while the cysteine residues at positions 186 and 209 formed another. Likewise, we have compared the cyanogen bromide-generated fragments from the purified cloned product with those generated from the purified gonococcal product. All the peptides had similar migrations, with the exception of one in each case. The peptide from the gonococcal PIII was isolated by using reverse-phase high-pressure liquid chromatography on a diphenyl column and subjected to amino acid sequencing. This revealed the sequence beginning at residue 183; it also showed that this peptide contained the carboxyl terminus. The observation that the peptide fragment from the gonococcal PIII migrated with an apparent molecular weight that was approximately 2,000 higher than that of the fragment from the cloned product, as well as our inability to obtain a free residue upon carboxypeptidase Y digestion, suggests that an additional substance may be covalently bound to the carboxyl terminus. Such a terminal addition does not seem to be the case with the *E. coli* OmpA protein. However, the carboxyl terminus of PIII diverges at position 224 from the sequence of the *E. coli* OmpA.

PIII STRUCTURE

Morona et al. (15, 16) studied the surface-exposed regions of the OmpA protein of *E. coli*. Utilizing several bacteriophages which use OmpA as a receptor (5, 23), they obtained a large number of phage-resistant OmpA mutants. From the changes in the sequence of the OmpA gene in these mutants (15, 16) and by comparison of enzymatic cleavage of OmpA between intact organisms and cell envelopes (4), a model of the OmpA protein within the membrane has been proposed (15). Changes in the OmpA sequence in the phage-resistant mutants were clustered in four major areas, which could be envisioned as four surface-exposed loops. A large periplasmic portion of the protein that begins at about residue 198 of OmpA is predicted from the proteolytic resistance of OmpA, except when studied in isolated envelopes. In this case, proteolytic cleavage occurs at residue 198 of OmpA. Thus, the homology of the PIII sequence with that of OmpA begins where it is postulated that the OmpA molecule is within the periplasmic space. If the analogy and the model of the two proteins is in fact the case, only residues 23 to 70 would be exposed on the surface of the gonococcus, while the rest of the PIII molecule would be periplasmic. This might explain the resistance of PIII to proteolysis in intact organisms and the drastic change in this resistance during the purification process. If, however, PIII was located in the membrane such that the homologous portion extended externally, antibodies reactive to PIII might occur through exposure to *E. coli* rather than to *N. gonorrhoeae* or *N. meningitidis*. Recently, we used a whole-cell ELISA inhibition study to determine which of these two models applies to PIII. The results of such an experiment are shown in Fig. 2. These data would suggest that at least 40% of the PIII-OmpA cross-reactive antibodies were removed by incubation with intact gonococci. Other data substantiated the hypothesis that portions of the OmpA-like region on PIII were exposed on the surfaces of gonococci (18). Using the primary structure of PIII, we are proceeding to map the surface-exposed portions by immunological and biochemical techniques.

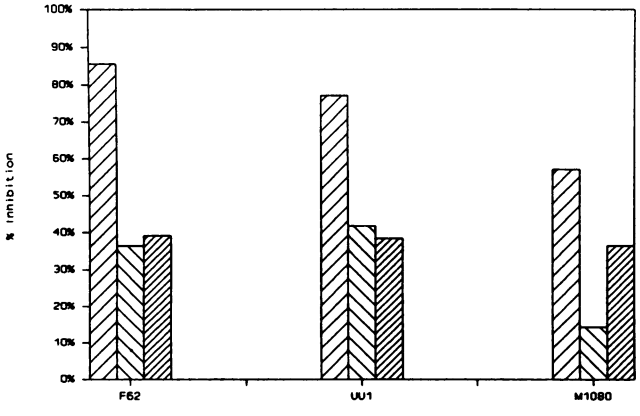

FIG. 2. Whole-cell inhibition ELISA of the reactivity of two hyperimmune PIII antisera (and) and one OmpA antiserum ( ) with purified OmpA. The basis of this ELISA has been described previously (13). Microdilution plates were coated with purified OmpA. Each of the hyperimmune rabbit antisera was then diluted with phosphate-buffered saline to obtain a titer for each serum sample which would give a reading of 1.0 at 405 nm in the ELISA. Bacteria from two different gonococcal strains, F62 and UU1, and one meningococcal strain, M1080, were suspended and diluted in phosphate-buffered saline until the optical density at 500 nm reached 0.6. This bacterial suspension of each strain was dispensed into 0.1-ml aliquots and centrifuged, and the supernatant was removed. The bacteria were suspended in 0.1 ml of the above-mentioned dilution of each serum and incubated for 2 h at 4°C. The bacteria and adsorbed antibodies were then removed by centrifugation. The supernatant containing the antibodies which did not adhere to the bacteria was then applied to microdilution plates which had been precoated with purified OmpA. The rabbit antibodies were then detected by ELISA with a goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase. The percent inhibition was calculated as follows: percent inhibition = [1 − (ELISA value of sera after whole-cell absorption)/(ELISA value of sera not absorbed)] × 100.

PIII FUNCTION

The function of PIII either in pathogenesis or in the physiology of the organism is unknown. There is also no information about the effect of PIII antibodies on the infection at either a mucosal or a systemic level. However, Rice and co-workers (10, 19) showed in very carefully controlled in vitro studies that human complement-fixing immunoglobulin G (IgG) antibodies to PIII interfered with the bactericidal activity of immunoglobulin G antibodies directed to other surface antigens such as lipooligosaccharide. These aspects are described more fully elsewhere in this issue (18). These observations, i.e., that antibodies to PIII block bactericidal antibodies to other gonococcal surface structures, might explain why PIII is so highly conserved, but they raise numerous other questions. Among these are how these naturally occurring blocking antibodies arise. For example, are they elicited by OmpA-bearing *E. coli*, by other neisserial infections, or possibly by both? What are the epitopes recognized by the blocking immunoglobulin G antibodies? How do these antibodies contribute to the pathology of gonorrhea?

MUTANTS LACKING PIII EXPRESSION

Recently, a mutant lacking the PIII protein in the outer membrane was obtained (Wetzler, Gotschlich, and Blake, unpublished data). With these mutants and others presently being constructed within our laboratory, we hope to more fully determine (i) the function of PIII; (ii) epitopes of PIII which are surface exposed; and (iii) the way in which PIII and antibodies to PIII affect the pathogenesis of gonococcal infections. Furthermore, with gonococcal mutants lacking in expression of PIII, we can now purify and more fully understand PI without potential PIII contamination.

LITERATURE CITED

1. **Blake, M. S., and E. C. Gotschlich.** 1984. Purification and partial characterization of the opacity-associated proteins of *Neisseria gonorrhoeae*. J. Exp. Med. **159**:452–462.
2. **Blake, M. S., E. C. Gotschlich, and J. L. Swanson.** 1981. The effect of proteolytic enzymes on the outer membrane proteins of *Neisseria gonorrhoeae*. Infect. Immun. **33**:212–222.
3. **Blake, M. S., E. J. Lytton, M. E. Seiff, and E. C. Gotschlich.** 1988. Studies on gonococcal protein III, p. 85–97. *In* M. A. Horwitz (ed.), Bacteria–host cell interaction. Alan R. Liss, Inc., New York.
4. **Chen, R., W. Schmidmayr, C. Kraemer, U. Chen-Schmeisser, and U. Henning.** 1980. Primary structure of major outer membrane protein II* (ompA protein) of *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **77**:4592–4596.
5. **Datta, D. B., B. Arden, and U. Henning.** 1977. Major proteins of the *Escherichia coli* outer cell envelope membrane as bacteriophage receptors. J. Bacteriol. **131**:821–829.
6. **Duchene, M., A. Schweizer, F. Lottspeich, G. Krauss, M. Marget, K. Vogel, B. von Specht, and H. Domdey.** 1988. Sequence and transcriptional start site of the *Pseudomonas aeruginosa* outer membrane porin protein F gene. J. Bacteriol. **170**:155–162.
7. **Gotschlich, E. C., M. S. Blake, J. M. Koomey, M. Seiff, and A. Derman.** 1986. Cloning of the structural genes of three H8 antigens and of protein III of *Neisseria gonorrhoeae*. J. Exp. Med. **164**:868–881.
8. **Gotschlich, E. C., M. Seiff, and M. S. Blake.** 1987. 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. J. Exp. Med. **165**:471–482.
9. **Gross, E., and B. Witkop.** 1962. Nonenzymatic cleavage of peptide bonds: the methionine residue in bovine pancreatic ribonuclease. J. Biol. Chem. **237**:1856–1860.
10. **Joiner, K. A., K. A. Warren, M. M. Frank, and P. A. Rice.** 1985. Blocking immunoglobulin G enhances complement consumption and deposition on *Neisseria gonorrhoeae*, p. 431–434. *In* G. K. Schoolnik, G. F. Brooks, S. Falkow, C. E. Frasch, J. S. Knapp, J. A. McCutchan, and S. A. Morse (ed.), The pathogenic neisseriae. American Society for Microbiology, Washington, D.C.
11. **Judd, R. C.** 1982. ^{125}I-peptide mapping of protein III isolated from four strains of *Neisseria gonorrhoeae*. Infect. Immun. **37**:622–631.
12. **Judd, R. C.** 1982. Surface peptide mapping of protein I and protein III of four strains of *Neisseria gonorrhoeae*. Infect. Immun. **37**:632–641.
13. **Lytton, E. J., and M. S. Blake.** 1986. Isolation and partial characterization of the reduction-modifiable protein of *Neisseria gonorrhoeae*. J. Exp. Med. **164**:1749–1759.
14. **McDade, R. L., Jr., and K. H. Johnston.** 1980. Characterization of serologically dominant outer membrane proteins of *Neisseria gonorrhoeae*. J. Bacteriol. **141**:1183–1191.
15. **Morona, R., M. Klose, and U. Henning.** 1984. *Escherichia coli* K-12 outer membrane protein (OmpA) as a bacteriophage receptor: analysis of mutant genes expressing altered proteins. J. Bacteriol. **159**:570–578.
16. **Morona, R., C. Kraemer, and U. Henning.** 1985. Bacteriophage receptor area of outer membrane protein OmpA of *Escherichia coli* K-12. J. Bacteriol. **164**:539–543.
17. **Newhall, W. J., W. D. Sawyer, and R. A. Haak.** 1980. Cross-linking analysis of the outer membrane proteins of *Neisseria gonorrhoeae*. Infect. Immun. **28**:785–791.

18. **Rice, P. A.** 1989. Molecular basis for serum resistance in *Neisseria gonorrhoeae*. Clin. Microbiol. Rev. **2**(Suppl.):S112–S117.
19. **Rice, P. A., M. R. Tam, and M. S. Blake.** 1985. Immunoglobulin G antibodies in normal human serum directed against protein III block killing of serum-resistant *Neisseria gonorrhoeae* by immune human serum, p. 427–430. *In* G. K. Schoolnik, G. F. Brooks, S. Falkow, C. E. Frasch, J. S. Knapp, J. A. McCutchan, and S. A. Morse (ed.), The pathogenic neisseriae. American Society for Microbiology, Washington, D.C.
20. **Shafer, W. M., and S. A. Morse.** 1987. Cleavage of the protein III and major iron-regulated protein of *Neisseria gonorrhoeae* by lysosomal cathepsin G. J. Gen. Microbiol. **133**:155–162.
21. **Swank, R. T., and K. D. Munkres.** 1971. Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. Anal. Biochem. **39**:462–477.
22. **Swanson, J. L., L. W. Mayer, and M. R. Tam.** 1982. Antigenicity of *Neisseria gonorrhoeae* outer membrane protein(s) III detected by immunoprecipitation and Western blot transfer with monoclonal antibody. Infect. Immun. **38**:668–672.
23. **van Alphen, L., L. Havekes, and B. Lugtenberg.** 1977. Major outer membrane protein d of *Escherichia coli* K-12: purification and *in vitro* activity of bacteriophage K3 and F-pilus mediated conjugation. FEBS Lett. **75**:285–290.