

THE CONSTRUCTION AND CHARACTERIZATION OF
NEISSERIA GONORRHOEAE LACKING PROTEIN III
IN ITS OUTER MEMBRANE

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The three major outer membrane proteins (OMP)¹ of *Neisseria gonorrhoeae*, Proteins I, II and III (PI, PII and PIII), have been extensively studied (1, 2), and all have been cloned and characterized (3-6). PIII, first described by McDade and Johnston (7), is constitutively expressed and antigenically invariable. Antibodies to PIII are able to block complement-dependent killing of the gonococcus by human sera (8-10). Furthermore, PIII is closely associated with PI trimers, which makes it difficult to purify PI without PIII contamination (7, 11-14).

PIII was recently cloned by our laboratory and it was found to have significant homology to the COOH-terminal portion of enterobacterial OmpA proteins (3, 4). Other investigators have been able to produce OmpA⁻ strains of *Escherichia coli* (15-17), and we speculated that PIII minus gonococci could also be produced. A gonococcal strain that does not express PIII or produces an altered PIII protein would allow investigators to further elucidate the function of PIII, including its role in PI conformation, function, and membrane insertion. The viability and virulence of PIII⁻ strains could be tested and the necessity of PIII for pathogenesis could be examined. Moreover, gonococcal proteins could be purified without PIII contamination, and therefore, the induction of anti-PIII blocking antibodies by immunizing preparations of gonococcal proteins could be eliminated.

We are interested in the use of purified PI as a gonococcal vaccine because it has moderate antigenic variability (18, 19) and antibodies toward PI exhibit bactericidal and opsonic activity against the gonococcus (20-23). The inevitable presence of minimal PIII contamination in any PI preparation (11, 12, 14) makes it difficult to avoid anti-PIII antibody induction when PI is used as an immunogen. In a previous PI vaccine trial (24), delipidated gonococcal membrane blebs consisting of PI and a small amount of PIII (<10%) were injected into human volunteers with a previous history of gonococcal infections. Sera from these volunteers before immunization had antigenococcal bactericidal and opsonic activity, which decreased after immunization. This is thought to be due to the production of PIII antibodies, which, as stated, block complement-dependent killing of gonococci by human sera (8-10). The

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¹ Abbreviations used in this paper: β la, β -lactamase; GC, gonococcal; OMP, outer membrane protein; ORF, open reading frame; PI-PIII, proteins I-III.

vacinees' sera are currently being analyzed to confirm this. We have recently published studies describing the antibody response to PI inserted into liposomes (25-27). A minor amount of PIII antibodies was induced by these preparations, but the sera still exhibited significant antigonococcal bactericidal and opsonic activity. Nevertheless, it would be advantageous to purify PI without PIII contamination if PI is to be used as a gonococcal vaccine.

This study describes the engineering of a PIII genetic construct by inserting a β la gene into a cloned PIII gene. This construct was used to transform gonococci and to replace the wild-type PIII gene by homologous recombination in order to disrupt PIII production by insertional inactivation. This procedure has been performed in the gonococcus and reported for the IgA protease gene (28) and the *recA* gene (29). Once obtained, these transformants were phenotypically and genotypically characterized, especially for changes in PIII expression.

Materials and Methods

Organisms. Organisms used were gonococcal strains F62, MS11, Pgh 3-2 (kindly provided by Dr. Charles Brinton, University of Pittsburgh, Pittsburgh, PA) and UU1 (kindly provided by Dr. Zell McGee, University of Utah, Salt Lake City, Utah) and *E. coli* strain DH5.

Protein III Gene Construct. The cloning and sequencing of PIII was previously described by Gotschlich et al. (3, 4). The PIII λ gt11 clone 33 was treated with Eco RI to excise the PIII insert. The insert was ligated into Eco RI-cut pMOB45 (30), and tetracycline-resistant, chloramphenicol-sensitive transformants were selected and isolated. A particular isolate was designated pMOB45/33-2 and was used for further constructions. The β la gene is contained on a 2.2-kb Bam HI fragment of plasmid pFA3 (31); the plasmid was purified (32) from *E. coli* and this fragment was isolated by restriction enzyme digestion, agarose gel electrophoresis, and electroelution of the restriction enzyme fragment. Approximately 2.5 μ g of pMOB45/33-2 was digested with Xba I. The unique PIII Xba I site is located at base pair 601, two-thirds into the open reading frame (ORF) of the PIII gene (Fig. 1). Approximately 1 μ g of the purified β la Bam HI fragment was added to Xba I-treated pMOB45/33-2 and the 5' overhanging ends left by the restriction enzyme digestions were filled using the Klenow fragment of DNA polymerase to obtain blunt ends. The fragments were ligated with T4 DNA ligase and transformed into competent cells of *E. coli* strain DH5.

Four colonies were selected that grew on medium containing tetracycline and carbenicillin, and plasmids were isolated from these isolates, respectively designated A-D. Restriction mapping with Eco RI digestion indicated plasmids A, C, and D gave rise to the expected fragments. Further restriction mapping using Pst I and Pvu I gave rise to identical patterns for plasmids A and C but different ones from plasmid D, indicating that plasmid D contained the β la gene in the opposite orientation than that of plasmids A and C. We were able to conclude from these patterns that the β la insert is in the same direction as the PIII gene in plasmid D but is in the reverse direction in plasmid A and C (Fig. 1). Plasmids C and D were isolated in large quantities (32) and then further purified by CsCl equilibrium density gradient centrifugation. Aliquots were methylated using Hae III methylase according to the protocol recommended by the vendor (New England Biolabs Inc., Beverly, MA). This step was performed in order to protect the DNA from one gonococcal restriction enzyme, Ngo II, which is an isoschizomer of Hae III (33, 34). The plasmids were then treated with Eco RI to release the modified gonococcal DNA fragment and then used for transformation.

Transformation. Transformations were carried out as follows. Competent piliated gonococci of strain F62 were grown for 16-18 h on gonococcal agar (35) in 6% CO₂ incubator at 37°C and then resuspended in 1.5% Proteose Peptone No. 3 (Difco Laboratories, Detroit, MI) broth with 30 mM Hepes, pH 7.2, 10 mM MgCl₂, 1% isovitalax (GC-Hepes). The resuspended organisms were diluted to a concentration of 5 × 10⁷ CFU/ml (OD₆₀₀ = 0.05). 2 μ g of either plasmid C or D, Hae III methylated and cut with Eco RI, were added to 1 ml of the diluted organisms and incubated statically at 37°C. After 1 h, 3 ml of GC-Hepes

was added and the tubes with the organisms were rotated at 30 rpm for 5–6 h at 37°C. Control tubes were treated identically, but no DNA was added. Various dilutions of the organisms incubated with the DNA (+DNA) and organisms without DNA (control) were spread on GC agar (35), overlapping a concentration gradient of ampicillin. This gradient was established by concentrically spreading 50 μ l of a 1 mg/ml dilution of ampicillin to the middle two-thirds of each agar plate. After 48 h the plates were examined and healthy, transparent, piliated colonies that appeared at the edge of the ampicillin gradient on plates that were inoculated with +DNA organisms were selected and propagated on GC agar containing 3 μ g/ml of ampicillin. Such colonies did not appear on the control plates. The isolates that grew on the ampicillin plates were then tested with nitrocefin disks (BBL Microbiology Systems, Cockeysville, MD) to detect β la activity, and colonies that tested positive were further investigated. Transformations were also performed using gonococcal strains Pgh 3-2 and UU1, in a similar manner as above, using total chromosomal DNA or electrophoretically isolated restriction fragments of chromosomal DNA from transformants obtained from the above experiment. Chromosomal DNA was obtained using the method of Nakamura et al. (36).

SDS-PAGE and Western Blot. Similar amounts of whole cell lysates of various strains of gonococci were studied by SDS-PAGE using a variation of Laemmli's method (37) as previously described (12). Lysates were made with or without 2-ME to demonstrate that PIII is reduction modifiable (12). Western blots were performed as described by Towbin et al. (38), reacted with PIII affinity-purified rabbit sera, and probed with alkaline phosphatase-conjugated goat anti-rabbit antiserum (Tago, Inc., Burlingame, CA) (39).

DNA Hybridization. DNA hybridization analyses were performed according to the methods reviewed by Meinkoth and Wahl (40) and originally described by Southern (41). Probes included the Eco RI/Cla I fragment from PIII clone 33, which does not include the known repetitive sequence of the gonococcus (4), and the Bam HI fragment from plasmid pFA3 (31), which contains the β la gene.

Whole Organism Absorptions. Transformants and their parent strains were tested for their ability to absorb affinity-purified PIII polyclonal rabbit antisera. 0.1 ml of organisms were diluted in GC-Hepes to obtain an OD₆₀₀ equal to 0.6 (5×10^8 CFU) and serially diluted twofold in V-bottomed microtiter plates. Sera that were to be absorbed were diluted to one-half the dilution that would give an ELISA reading equivalent to 1.0. 0.1 ml of diluted sera was added to the wells, which contained 0.1 ml of diluted organisms, and the plates were then incubated at 37°C for 2 h. After 2 h, the plates were centrifuged at 2,000 rpm and 0.1 ml from each well was carefully transferred to an ELISA plate sensitized with 2 μ g/ml of PIII. The ELISA plates were incubated overnight and the remainder of the procedure was performed as a normal ELISA (26). Results were analyzed by comparing readings of sera absorbed with the organisms to readings of unabsorbed sera.

Phenotypic Characterization of Transformants. Antibiotic sensitivity of various strains was determined by the disc diffusion method at the Clinical Microbiology Laboratory of Memorial Sloan Kettering Hospital, New York, NY. Growth curves were done by inoculating GC-Hepes with 5×10^7 CFU nonpiliated organisms and measuring OD₆₀₀ of each inoculum hourly until the end of exponential growth. The ability of PI to function as a porin in artificial lipid bilayers was analyzed as previously described (42) using isolated PI (26). Testing the sensitivity of organisms to killing by cathepsin G was performed as previously described (43) by Dr. William Shafer (Emory University, Atlanta, GA). To test the competence of the mutant organisms, transformations were performed as above using chromosomal DNA from streptomycin-resistant gonococcal strain MS11, and streptomycin-resistant transformants were detected on gonococcal agar plates containing 1 mg/ml streptomycin.

Results

Gonococci lacking PIII in their outer membrane were obtained by marker rescue. Our procedure entailed transferring a cloned PIII gene to a plasmid vector and then inserting a gene encoding β la into the ORF of the PIII gene. We transformed gonococci with this insertionally inactivated PIII gene construct and isolated organisms that produced β la and were penicillin resistant. Variations of this approach using

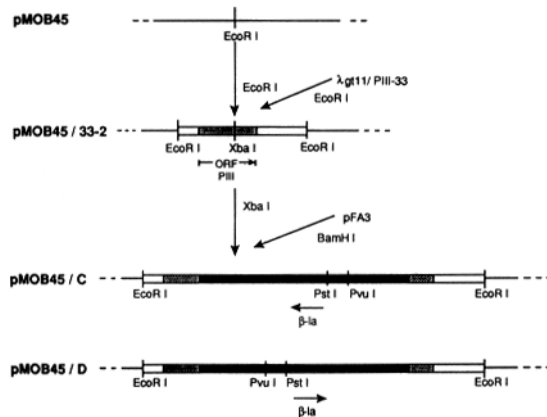


FIGURE 1. Construction of PIII/βla gene. βla, β-lactamase, ORF, open reading frame; pMOB45/C, plasmid C; pMOB45/D, plasmid D.

other gonococcal proteins have been reported previously (28, 29). The PIII clone 33 contains a single Xba I site at base pair 601 and because this site is located approximately two-thirds into the ORF of PIII (Fig. 1) it is possible to disrupt the translation of PIII by inserting a βla gene marker at that site. For these reasons we decided to use a plasmid vector that lacked Xba I sites and a βla gene, pMOB45 (30).

We were able to isolate one colony of a βla producing, ampicillin-resistant gonococcus when piliated F62 gonococci was transformed with Hae III-methylated, EcoRI-treated pMOB45/33-2/D (plasmid D). The PIII/βla insert in this plasmid has the βla gene oriented in the same direction as the PIII gene (Fig. 1). The isolate obtained was further propagated on ampicillin containing GC agar and was designated 2D. Strain 2D lacked a reduction-modifiable protein as seen on SDS-PAGE (Fig. 2) but other protein bands were equivalent. A Western blot of an identical gel (Fig. 3) probed with affinity-purified polyclonal PIII antisera demonstrated that whole cell lysates of 2D exhibited no PIII reactivity, including any truncated version of PIII or a PIII/βla fusion protein. Furthermore, when the mutants were grown in liquid culture there was no evidence that any version of PIII is excreted into the culture supernatant (data not shown). We concluded that this isolate had no immunologically discernable fragments of PIII or PIII/βla fusion proteins.

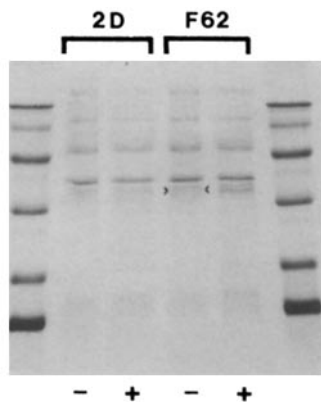


FIGURE 2. SDS-PAGE of F62 and 2D whole cell lysates. (-) Without 2-ME, (+) with 2-ME. Molecular weight standards were human transferrin (80,000), catalase (60,000), ovalbumin (45,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,500) and cytochrome *c* (15,000).



FIGURE 3. Western blot of F62 and 2D whole cell lysates probed with affinity-purified PIII polyclonal antisera. (+) With 2-ME; (-) without 2-ME.

The original phenotype of the isolate was piliated and transparent, but because the strain was propagated nonpiliated organisms were obtained. Furthermore, the PIII/ β la mutation was stable when the mutant was passed on plates that did not contain ampicillin; β la reactivity continued to be demonstrable by nitrocefin disks. There were no gross phenotypic differences between the mutant and wild-type organism (Table I), but the nonpiliated colonies of the mutant appeared to be slightly less cohesive and more fluid as compared with the wild-type. Growth curves of 2D versus F62 (Fig. 4) demonstrated that 2D growth was slightly less than F62 growth. PI isolated from 2D, in the absence of PIII, acts exactly the same in a lipid bilayer as PI from the parent strain; it demonstrates the same voltage-gated porin activity. Cathepsin G, a PMN serine esterase with anti-PIII proteolytic activity, can kill gonococci, even when its proteolytic activity is inhibited (43). There was no difference in killing by cathepsin G of PIII containing gonococci and strain 2D (personal communication, Dr. W. Shafer, Emory University, Atlanta, GA). Therefore, the ability of cathepsin-G to kill gonococci is not related to PIII proteolysis.

The fact that 2D had increased resistance to ampicillin and no longer produced demonstrable PIII implied that the cloned PIII sequence in plasmid D had been reintroduced along with the β la gene into the gonococcal chromosomes by homologous recombination. To confirm this, F62 and 2D chromosomal DNA digested with various restriction enzymes were analyzed using the method of Southern (41). The hybridizations were performed using F62 and 2D DNA digested with Eco RI and Cla I (Fig. 5). The F62 digests reacted with the PIII probe, revealing a 6.7-kb Eco RI fragment and a 3.5-kb Cla I fragment, but did not react with the β la probe.

TABLE I
Phenotypic Characterization: 2D vs. F62

Colony morphology	Minimal difference
Growth	Slightly decreased
Effect of anti-PIII antibody	No effect on killing by immune sera
PIII antibody absorption	2D unable to absorb PIII antibody
Cathepsin G killing	No difference
Porin activity	No difference
Competence	No difference
Antibiotic sensitivity	Ampicillin resistant (3 μ g/ml)

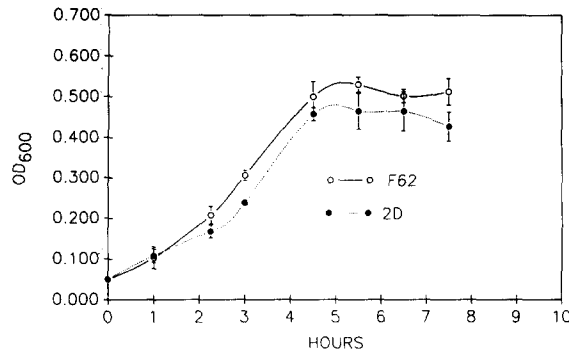


FIGURE 4. F62 and 2D growth curves in GC-Hepes medium.

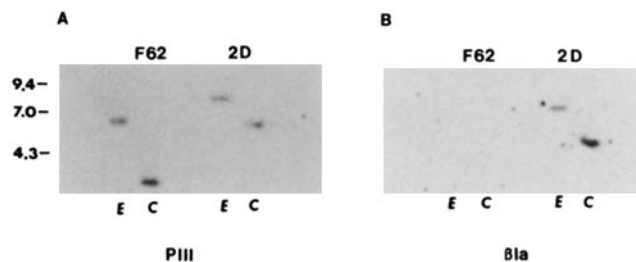


FIGURE 5. Southern blot hybridization of F62 and 2D chromosomal DNA digested with Eco RI (E) and Cla I (C). Probed with (A) [³²P]dCTP-labeled PIII gene, base pairs 0-900 (lacking known repetitive sequence), and (B) [³²P]dCTP-labeled β Ia Bam HI fragment from pFA3.

The 2D digests reacted similarly with either probe, revealing a 8.9-kb Eco RI fragment and a 5.7-kb Cla I fragment. The reactive 2D fragments were 2.2 kb larger than the reactive F62 fragments. 2.2 kb is the size of the Bam HI fragment of pFA3 containing the β Ia gene, which was inserted into the PIII gene. This verifies that homologous recombination did occur between the PIII/ β Ia construct and the gonococcal chromosomal PIII gene.

2D chromosomal DNA was able to transform competent gonococcal strains F62, Pgh 3-2 and UU1. A Western blot of whole cell lysates of Pgh 3-2 and UU1 transformants probed with affinity-purified polyclonal PIII antisera can be seen in Fig. 6. The frequency of homologous transformation of F62 was 2 logs higher than heterologous transformation of UU1 or Pgh 3-2 (10^{-3} vs. 10^{-5} , respectively). Furthermore,

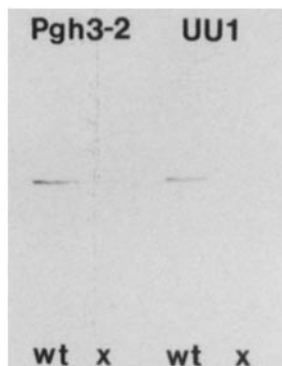


FIGURE 6. Western Blot of Pgh 3-2 and UU1, wild-type (wt), and PIII transformant (x). Probed as in Fig. 3.

the 5.7-kb 2D Cla I fragment, mentioned above, was isolated by agarose gel electrophoresis of Cla I-digested 2D DNA and electroelution of the appropriate gel piece. The DNA thus retrieved was able to transform gonococcal strain F62. We also found that 2D was equally competent as F62 when transformed with chromosomal DNA from streptomycin resistant strain MS11; the frequency of transformation was $2-3 \times 10^{-2}$ for both 2D and F62.

Discussion

As this study demonstrates, we have been able to produce stable, viable gonococcal mutants that lack one of its major OMP, PIII, in its outer membrane. Even though this was done by insertional inactivation within the ORF, no immunologically discernible PIII was produced by the mutant strains. Intact PIII in the gonococci or in outer membrane blebs is resistant to proteolytic enzymes (44), but once PIII is purified it is readily attacked by proteases (14). Most likely, the transformants were producing a shortened PIII protein or a PIII/ β la fusion protein and it is probable that these alterations prevented insertion of PIII into the outer membrane permitting exposure to gonococcal endogenous proteases. This is the most likely explanation why a truncated PIII protein or a PIII/ β la fusion protein was not detected.

We were able to insert a β la gene into the chromosomal PIII gene by homologous recombination as demonstrated by Southern blotting (Fig. 5). The first transformation using a PIII/ β la construct isolated from plasmid D (Fig. 1) propagated in *E. coli* and Hae III methylated was able to transform competent gonococci, but inefficiently. Once 2D chromosomal DNA or specific 2D DNA fragments containing the PIII/ β la gene were used, the frequency of transformation increased up to 10^{-3} . We used Hae III methylated DNA to transform gonococci because other investigators have demonstrated that gonococcal transformation with foreign DNA is extremely difficult, probably because of gonococcal restriction enzymes, and accordingly, the efficiency of transformation can be improved by Hae III methylating the DNA used for transformation (33, 34).

Gonococcal PIII has significant homology to enterobacterial OmpA (4). *E. coli* mutants that lack OmpA are viable but have significant differences from wild-type *E. coli* (45). Alterations of OmpA expression cause the organism to be sensitive to EDTA and detergent. Moreover, OmpA mutations and deletions affect *E. coli* bacteriophage sensitivity (16, 17). We created stable PIII⁻ *N. gonorrhoeae* that are viable and have minimal changes in colony morphology and bacterial growth, similar to OmpA⁻ *E. coli*. OmpA is necessary for mating pair formation in *E. coli* (46-49), and the effect of PIII deletion on gonococcal conjugation is in the process of being evaluated.

Minimal phenotypic changes were observed between 2D and its parent strain, F62 (Table I). The PIII transformation that this strain underwent is stable and does not require the presence of antibiotics. There is a minor decrease in growth in liquid culture (Fig. 4). Gram stain and colony morphology of the two strains were almost identical. Additionally, the lack of PIII in the gonococcal outer membrane does not affect DNA uptake and competence. Purified PI from strain 2D retains similar porin activity as purified PI from wild-type strains containing PIII. These results demonstrate that PI porin function is not affected by the absence of PIII. We are currently examining PIII⁻ strains in various functional models, e.g., bactericidal assays, in-

vasion assays, and these results will be reported in the future. Preliminary results (personal communication, Dr. P. Rice, Boston University Medical Center, Boston, MA) demonstrate that anti-PIII blocking antibodies decreases complement dependent killing of wild-type organisms but do not affect complement-dependent killing of the PIII⁻ transformants.

As mentioned, PI is a promising gonococcal vaccine candidate, but a complicating factor in its use is the inevitable presence of PIII in PI preparations. We have used PI in liposomes to produce bactericidal and opsonic antibodies (25-27) but our preparations still induced some PIII antibodies, even though titers were not high enough to abolish bactericidal and opsonic activity. PIII antibodies have the potential to block complement killing of gonococci (8-10), and therefore, even small amounts of PIII in a gonococcal vaccine preparation can have adverse effects. The results of the PI/gonococcal bleb vaccine trial performed by Arminjon et al. (24) demonstrate this point. When delipidated gonococcal membrane blebs consisting of PI and small amounts of PIII were injected into volunteers with a previous history of gonococcal infections, the antigonococcal bactericidal and opsonic activity of their sera decreased after immunization. We now have organisms that lack PIII and we have isolated PI from these strains without PIII contamination. We are testing the immunogenicity of liposome preparations using this PI. In addition to enabling us to eliminate PIII from purified PI preparations, there is the potential to purify any other gonococcal membrane protein without PIII contamination.

Summary

Protein III (PIII) is a highly conserved, antigenically stable gonococcal outer membrane protein that is closely associated with the major outer membrane protein, protein I (PI). We have previously reported the cloning of the PIII gene. This gene was inserted into the Eco RI site of the runaway plasmid pMOB45. The β -lactamase (β la) Bam HI restriction fragment from the gonococcal plasmid pFA3 was inserted at the Xba I site in the PIII gene. The plasmid construct was Hae III methylated and the PIII/ β la insert was excised with Eco RI and used to transform gonococcal strain F62. One β la⁺, ampicillin-resistant transformant was isolated and designated 2D. A Western blot of 2D whole cell lysate was probed with affinity-purified polyclonal PIII antisera. No PIII reactivity was detected. Southern blot analysis was performed on F62 and 2D chromosomal DNA that were cut with Eco RI or Cla I. A PIII DNA probe hybridized with fragments 2.2 kb larger in strain 2D than strain F62. This corresponds to the size of the β la insert. A β la-specific probe hybridized with the same 2D restriction fragments as above, but did not react with any F62 fragments, confirming that homologous recombination had occurred. There were minimal phenotypic changes between 2D and its parent strain, F62. Chromosomal DNA from 2D was able to transform gonococcal strains F62, UU1, and Pgh 3-2, rendering these PIII⁻. 2D and other PIII⁻ transformants can now be used to study the role of PIII in gonococcal physiology, metabolism, membrane structure, and pathogenesis. Moreover, we now have organisms from which we can purify gonococcal proteins without PIII contamination.

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