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Expression of a Cloned Lipopolysaccharide Antigen from *Neisseria gonorrhoeae* on the Surface of *Escherichia coli* K-12

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A gonococcal gene bank maintained in *Escherichia coli* K-12 was screened by colony immunoblotting, and a transformant expressing a surface antigen reactive to anti-gonococcal outer membrane antiserum was isolated. The isolate carried a recombinant plasmid, pTME6, consisting of approximately 9 kilobases of *Neisseria gonorrhoeae* DNA inserted into the *Bam*HI site of pBR322. Surface labeling of *E. coli* HB101(pTME6) confirmed that the antigen was expressed on the *E. coli* cell surface. The antigenic material was resistant to proteinase K digestion and sensitive to periodate oxidation, indicating that the material was carbohydrate. Purified lipopolysaccharide (LPS) from HB101(pTME6) produced a unique band on silver-stained polyacrylamide gels that contained immunoreactive material as seen on Western blots of LPS samples. Only two of three *E. coli* LPS mutant strains carrying pTME6 reacted with the antigonococcal antiserum, suggesting that a certain *E. coli* core structure is necessary for antigen expression. We conclude that pTME6 contains one or more gonococcal genes encoding an LPS core biosynthetic enzyme(s) which can modify *E. coli* core LPS to produce a gonococcuslike epitope(s).

The lipopolysaccharide (LPS) of *Neisseria gonorrhoeae* apparently contains no O antigen (22), possessing only a core oligosaccharide covalently linked to lipid A, and thus is similar to the rough enterobacterial LPS. In contrast to *Escherichia coli* and *Salmonella typhimurium*, which are fairly constant in core oligosaccharide structure (18), the core portion of gonococcal LPS displays considerable heterogeneity (1, 3).

Numerous studies have demonstrated that LPSs isolated from different strains of *N. gonorrhoeae* produce different banding patterns on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22, 28). These differences in SDS-PAGE mobility have been shown by Schneider et al. to be linked to differences in antigenic expression and are thought to be due to variation in the number of glycose units in the core (28). Strains of *N. gonorrhoeae* which are resistant to the lethal effects of pyocins have alterations or mutations of their LPS (26). Connelly and Allen (6), working with a pyocin-sensitive strain of *N. gonorrhoeae* and its pyocin-resistant variant, discovered that along with the change in pyocin sensitivity, the variant LPS also showed altered SDS-PAGE mobility and lost antigenic reactivity to sera directed against the parent strain. Resistance to killing by normal human serum of some strains of *N. gonorrhoeae* has been shown to be due in part to a lack of certain LPS antigens on the cells (27). Studies with gonococcal LPS mutants have demonstrated that while small changes in glycose structure had no effect on serum resistance, larger alterations of core structure corresponded to a loss of serum resistance by these variants (11).

These studies indicate that heterogeneity of core oligosaccharide both between and within strains of *N. gonorrhoeae* is correlated with changes in antigenic expression, pyocin resistance, and resistance to the bactericidal action of serum and therefore may be linked to the virulence of *N. gonorrhoeae*. While not yet conclusively proven, the heterogeneity of gonococcal LPS may be due to structural differences in the core constituents. While the genetics and biosynthesis of lipopolysaccharide of *S. typhimurium* and *E. coli* have been well characterized (12, 18, 19, 25), the mechanisms of antigenic heterogeneity and phenotypic variation of gonococcal LPS remain to be elucidated. Knowledge of LPS structure and biosynthesis would be useful in understanding the importance of LPS in the virulence of *N. gonorrhoeae*.

Previously in our laboratory, we created a gonococcal gene bank in *E. coli* HB101 (8). Screening the gene bank for gonococcal surface antigens, we obtained a number of clones, one of which produced an unusual Western blot pattern. We report here that this recombinant plasmid, pTME6, carries one or more gonococcal genes coding for an enzyme(s) involved in LPS biosynthesis.

Gonococcal strain RUN4383 was used as the source of DNA for construction of the gene bank from which the gonococcal insert of pTME6 was obtained. The *E. coli* K-12 strain HB101 was used to maintain plasmids pTME6 and pBR322; it possesses no O antigen but does have a complete LPS core structure. *E. coli* C600, NS1, and NS4 were obtained from Barbara Bachmann. Strain C600 is an *rfbD1* mutant that also lacks rhamnose (4, 23), whereas NS1 and NS4 are *lpcA1* and *lpcB4* mutants, respectively (31). *E. coli* cells were grown on MacConkey agar (Difco Laboratories, Detroit, Mich.) or in Luria broth. Ampicillin (30 μg/ml) was added to cultures as needed. *N. gonorrhoeae* strains were cultivated on GC medium base (Difco Laboratories) containing Kellogg supplement (14). Gonococcal cultures were incubated at 37°C in a 5% CO_2 atmosphere.

Rabbit anti-gonococcal outer membrane antiserum was a gift from Erik Sandström and Joan Knapp and was absorbed with 5 volumes of *E. coli* before use. ^{125}I-labeled donkey anti-rabbit F(ab')$_2$ fragments or ^{125}I-labeled donkey anti-rabbit whole antibody (Amersham Corp., Arlington Heights, Ill.) was used as the secondary antibody in all immunoblots.

E. coli cells containing pBR322 or pTME6 were removed from a MacConkey-ampicillin plate, suspended in 1 ml of phosphate-buffered saline (PBS), centrifuged, and suspended in 1 ml of PBS to a constant optical density at 600 nm

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(OD_{600}). To surface label these cells, anti-gonococcal outer membrane antiserum was added to a final dilution of 1:250, and the cells were incubated at room temperature for 30 min. Cells were centrifuged, washed once with PBS, and suspended in 1 ml of PBS to which ^{125}I-labeled anti-rabbit whole antibody was added (2×10^5 cpm/ml). The cells were incubated for 30 min at room temperature and washed two times in PBS. The final pellet was suspended in 200 μl of PBS, and the counts were determined in a Beckman gamma counter.

Chromosomal DNA from three gonococcal laboratory strains, RUN4383, F62, and RUG5014, was isolated as described previously (8). Plasmid DNA from *E. coli* transformants was isolated by isopycnic CsCl density gradient centrifugation (10). Agarose gels containing DNA were photographed and then bidirectionally blotted onto nitrocellulose (BA85; Schleicher & Schuell, Keene, N.H.) (29), probe DNA was labeled in vitro by nick translation (7), and hybridization of probe DNA to DNA bound to the filters was performed (34) at 42°C. Hybridized filters were washed, dried, and autoradiographed at −70°C with Kodak XRP film (Eastman Kodak, Rochester, N.Y.).

Cells from one to two plates were harvested and outer membrane fractions were prepared as described previously (5). The protein content of outer membrane samples was measured by the method of Lowry et al. (17), with bovine serum albumin (BSA) as the standard. LPS from *E. coli* strains was isolated by a slight modification of the phenol-chloroform-petroleum ether method of Galanos et al. (9). Gonococcal LPS was isolated by the hot phenol-water method of Westphal and Jann (35) as modified by Johnston et al. (13). Precipitated LPS from *E. coli* or *N. gonorrhoeae* was dissolved in deionized water and dialyzed against 1 mM EDTA for 1 to 2 days. The LPS was repeatedly sedimented at 105,000 × *g* for 3-h intervals until the OD_{260} and OD_{280} of the supernatant fluid were below 0.05. The pellet was suspended in deionized water, lyophilized, and stored at −20°C.

SDS-PAGE was performed on 13% acrylamide gels with 3% stacking gels by the Laemmli method (16). For electrophoresis of purified LPS samples, 13% polyacrylamide separating gels without SDS were prepared as described by Schneider et al. (28). Three percent stacking gels contained 0.1% SDS. Electrophoresis was carried out in SDS-PAGE running buffer at 5 to 7 mA per gel for 16 h. Silver staining of polyacrylamide gels to detect LPS was carried out by the method of Tsai and Frasch (33).

In some experiments, outer membrane preparations or whole-cell lysates were treated with 25 μg of proteinase K in 20 ml of deionized water at 60°C for 1 h. Samples were then mixed with an equal volume of 2× SDS-PAGE sample buffer and boiled for 5 to 10 min. Some outer membrane samples or whole-cell preparations were treated with 5 nmol of sodium metaperiodate in 0.05 M sodium acetate (pH 5.5) at 4°C for 16 h in the dark. An equal volume of 2× SDS-PAGE sample buffer was added to samples, and incubation was continued for 2 h at room temperature in the dark. The sample buffer contained enough glycerol to completely neutralize any excess periodate. Samples were boiled for 5 to 10 min and subjected to SDS-PAGE.

Samples were prepared for Western blotting (immunoblotting) by transferring protein or LPS from SDS-PAGE onto nitrocellulose (BA83; Schleicher & Schuell) (32) with a Transphor electroelution unit (Hoefer Scientific Instruments, San Francisco, Calif.). Filters were blocked by constant shaking for 1 h at 37°C with PBS containing 5% BSA and 0.1% sodium azide (PBS-BSA), incubated for 60 min at room temperature with rabbit anti-gonococcal outer membrane antiserum diluted 1:300 in PBS-BSA, and washed for 10 min in PBS plus 0.1% Triton X-100, followed by 10 min in PBS. Washed filters were incubated for 60 min at room temperature with ^{125}I-labeled donkey anti-rabbit whole antibody diluted to 2×10^5 cpm/ml in PBS-BSA, washed as before, air dried, and autoradiographed at −70°C with XRP film.

Clones expressing gonococcal antigens were identified in a gonococcal gene bank by colony immunoblotting (8). Western blots of whole-cell lysates of these clones identified five isolates which had an unusual pattern of immunoreactivity; all five clones contained identical gonococcal DNA inserts by restriction endonuclease analysis (data not shown). One plasmid isolate, pTME6, was picked for further study. To ensure that the antigen expressed by pTME6 was surface exposed, surface labeling was performed. Cells containing the recombinant plasmid bound five to six times more radioactivity than control cells harboring the vector alone (data not shown).

To confirm that the inserted fragment of pTME6 was gonococcal in origin, Southern hybridizations were performed (Fig. 1). By agarose gel electrophoresis (Fig. 1A), there were six *Sau*3A1 bands visible in pTME6 (lane 10) which were not part of the pBR322 vector DNA (arrows). Bands below 660 base pairs (bp) were not visible on this gel with ethidium bromide staining. The multiple bands on the uncut lanes of the plasmids represent monomers, multimers, and nicked plasmid species. The lower-M_r band on lane 2 of the chromosomal DNA is the 2.6-megadalton cryptic plasmid carried in that strain. The gel was bidirectionally blotted onto nitrocellulose, and the filters were hybridized with ^{32}P-labeled pTME6 (Fig. 1B) or ^{32}P-labeled pBR322 (Fig. 1C) under high-stringency conditions. The six bands unique to pTME6 are visible in lane 10, as are those corresponding to pBR322 DNA. Since *Sau*3A1 fragments of the gonococcal DNA were ligated into the *Bam*HI site of pTME6, digestion by *Sau*3A1 cleanly separated the inserted DNA from the vector. The six bands which were unique to pTME6 were also present in gonococcal chromosomal *Sau*3A1 fragments. No hybridization to gonococcal DNA sequences was observed in a Southern blot with ^{32}P-labeled pBR322 as the probe (Fig. 1C). The total M_r of these six bands was approximately 9 kilobases.

Western blots of outer membrane preparations. Outer membrane isolates of strains HB101(pBR322), HB101 (pTME6), and RUN4383 containing approximately 25 μg of protein were either digested with proteinase K, oxidized with sodium metaperiodate, or left untreated. Samples were subjected to SDS-PAGE and Western blotted with rabbit anti-gonococcal antiserum (Fig. 2). Lane 1b shows the distinctive pattern produced by HB101(pTME6); the lower-M_r smear reacted strongly with the antigonococcal antiserum, with no discrete upper-M_r bands apparent. The antigonococcal antiserum did not react with native *E. coli* outer membrane (lane 1a) but reacted extensively with gonococcal outer membrane (lane 1c). The gonococcal outer membrane blot pattern contained a lower-M_r smear similar to that of the *E. coli* transformant. In addition, several higher-M_r bands corresponding to outer membrane proteins were visible. After treatment of the outer membranes with proteinase K, the immunoreactive smears present in both the HB101(pTME6) and RUN4383 samples were still present (lanes 3a and 4a), which indicated that the material in the smear was not protein. Oxidation of the outer membranes

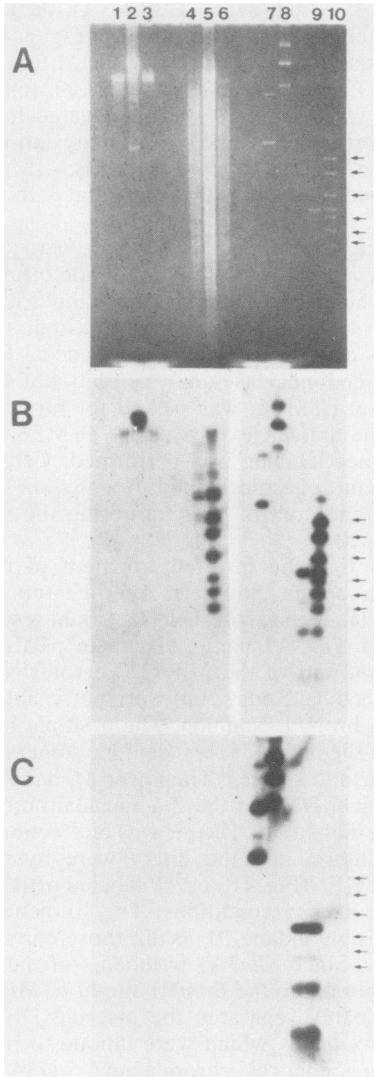

FIG. 1. Agarose gel electrophoresis of *N. gonorrhoeae* chromosomal DNA and plasmid DNA. DNA samples were either undigested (lanes 1 to 3, 7, and 8) or digested with *Sau*3A1 (lanes 4 to 6, 9, and 10) and electrophoresed on a 1.2% agarose gel. DNA samples were chromosomal DNA from *N. gonorrhoeae* strains RUN4383 (lanes 1 and 4), F62 (lanes 2 and 5), and RUN5014 (lanes 3 and 6) and plasmids pBR322 (lanes 7 and 9) and pTME6 (lanes 8 and 10). (A) Ethidium bromide-stained agarose gel. (B) Southern transfer of gel in panel A probed with ^{32}P-labeled pTME6. Six bands unique to the gonococcal insert of pTME6 (arrows) comigrated with *Sau*3A1-digested chromosomal bands (lanes 4 to 6). The higher-molecular-weight bands hybridizing in lanes 4, 5, and 10 represented incomplete digestion with *Sau*3A1. (C) Southern transfer of gel in panel A probed with ^{32}P-labeled pBR322. The probe did not hybridize with the gonococcal insert of pTME6 or to chromosomal DNA.

with sodium metaperiodate, which cleaves carbon-carbon bonds in sugar residues, completely destroyed the immunoreactive smears (lanes 3b and 4b). Sodium periodate oxidation did not degrade proteins, as indicated by the presence of bands on the gonococcal outer membrane (lane 4b). Therefore, the immunoreactive smears in HB101(pTME6) and RUN4383 appear to be carbohydrate.

Immunoblots of purified LPS. To investigate the possibility that the antigen detected on Western blots of HB101(pTME6) outer membrane was LPS, LPS was purified from HB101(pTME6), HB101(pBR322), and RUN4383. Various amounts of LPS (0.5, 1.0, and 1.5 μg) were subjected to duplicate SDS-PAGE. One gel was silver stained, and the other was Western blotted (Fig. 3A and B, respectively). Silver-stained gels of LPS indicated that the samples were essentially free of protein since no bands stained even with 5 μg of LPS (data not shown). Differences in the LPS banding patterns were discernable among each of the three samples. *E. coli* HB101(pBR322) LPS consisted of two bands, the upper band being the largest and most intensely stained. *E. coli* HB101(pTME6) LPS also gave these two bands, but in addition a higher-M_r band was also apparent. *N. gonorrhoeae* RUN4383 LPS produced one large band which migrated to a position between the upper two bands of HB101(pTME6).

The immunoblot of the LPS samples revealed that the gonococcus-specific antiserum reacted with both HB101(pTME6) and RUN4383 LPS but not with HB101(pBR322) LPS, indicating that the *E. coli* transformant contained a gonococcuslike antigen in the LPS. The immunoreactivity of the clone LPS was found on the highest-M_r band that was unique to that strain.

Western blots of *E. coli* LPS mutants transformed with pTME6. To verify that the antigen was a component of the *E. coli* LPS and not a copurifying contaminant, three *E. coli* mutant strains were obtained. The rough strains, NS1 and NS4, had different degrees of LPS core biosynthesis as indicated by compositional analysis (31). Strain C600 possessed a complete core but no O antigen (4, 23). Plasmids pTME6 and pBR322 were transformed (20) into these strains, and outer membrane samples were subjected to SDS-PAGE and then Western blotted to detect antigen

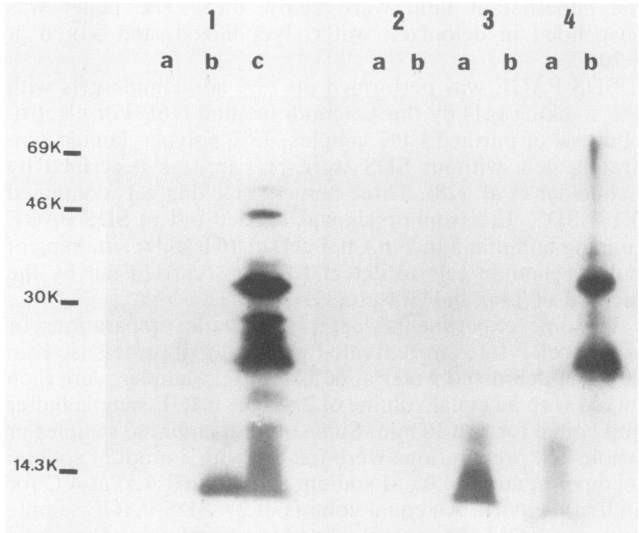

FIG. 2. Effects of proteinase K digestion and periodate oxidation on *E. coli* HB101(pTME6) antigen expression. Outer membranes (25 μg of protein) from *E. coli* HB101(pBR322) (lanes 1a, 2a, and 2b), HB101(pTME6) (lanes 1b, 3a, and 3b), and *N. gonorrhoeae* RUN4383 (lanes 1c, 4a, and 4b) were either left untreated (lanes 1); digested with proteinase K (lanes 2a, 3a, and 4a), or oxidized with periodate (lanes 2b, 3b, and 4b) before 13% acrylamide SDS-PAGE. Western blots were performed with rabbit anti-gonococcal outer membrane antiserum and ^{125}I-labeled anti-rabbit whole antibody and autoradiographed with Kodak XRP film. Marker sizes (in kilodaltons) are shown to the left.

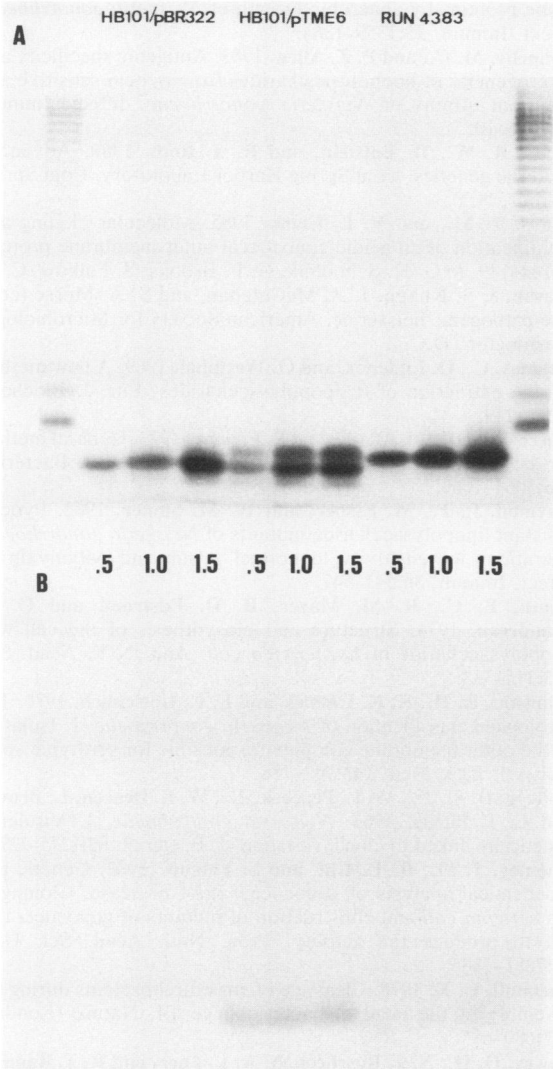

FIG. 3. Comparison of LPS isolated from *E. coli* HB101 containing pBR322 or pTME6 with *N. gonorrhoeae* RUN4383 LPS. (A) Silver-stained polyacrylamide gel of purified LPS; 0.5-, 1.0-, and 1.5-μg samples of LPS from HB101(pBR322), HB101(pTME6), and RUN4383 were subjected to SDS-PAGE and silver stained. Right-end lanes contain 10 μg of *Salmonella minnesota* LPS. (B) Western blot of purified LPS. A gel identical to that in panel A was Western blotted with rabbit antigonococcal antiserum and ^{125}I-labeled anti-rabbit whole antibody. Autoradiograph shows the pattern of immunoreactivity.

expression (Fig. 4). The gonococcal antigen was expressed in only two of the three *E. coli* LPS mutants carrying pTME6 (lanes 2). The untransformed samples (lanes 1) and the samples of pBR322-transformed strains (lanes 3) showed no immunoreactivity. The differences in antigen expression among the *E. coli* strains were presumably due to structural differences in the LPS core moieties.

Proteinase K-treated whole-cell lysates of the LPS mutant strains were subjected to SDS-PAGE and silver stained (Fig. 5). There were no observable differences between the LPS banding pattern of NS4(pTME6) and those of NS1(pTME6) and C600(pTME6) (compare lane e with lanes c and g). All three transformants produced the unique higher-M_r band seen with HB101(pTME6) LPS (lane i). Presumably, the

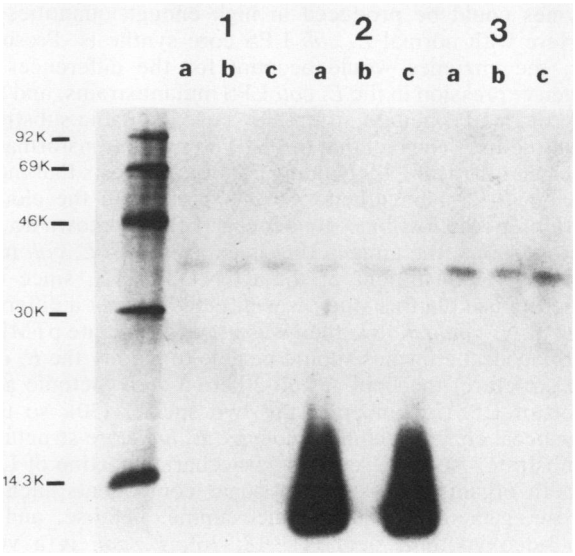

FIG. 4. Expression of pTME6 antigen in *E. coli* LPS mutants. Three *E. coli* strains with various mutations in LPS biosynthesis were transformed with either pTME6 (lanes 2) or pBR322 (lanes 3) or left untransformed (lanes 1). *E. coli* strains used were NS1 (lanes a), NS4 (lanes b), and C600 (lanes c). Outer membranes were isolated from each sample, subjected to SDS-PAGE, Western blotted with rabbit antigonococcal antiserum and ^{125}I-labeled antibody, and autoradiographed with Kodak XRP film. Marker sizes (in kilodaltons) are shown to the left.

LPS differences which prevent antigen expression of NS4(pTME6) are minor and do not alter the M_r enough to be seen as changes in band position. Western blotting of NS1(pTME6), NS4(pTME6), and C600(pTME6) LPS with a second rabbit antigonococcal antiserum with different specificity reacted with the upper LPS band in all three strains (data not shown).

We believe that the gonococcal insert of pTME6 contains genes that encode enzymes involved in LPS biosynthesis in *N. gonorrhoeae*. When these genes are transformed into *E. coli* via the multicopy plasmid pBR322, the gonococcal

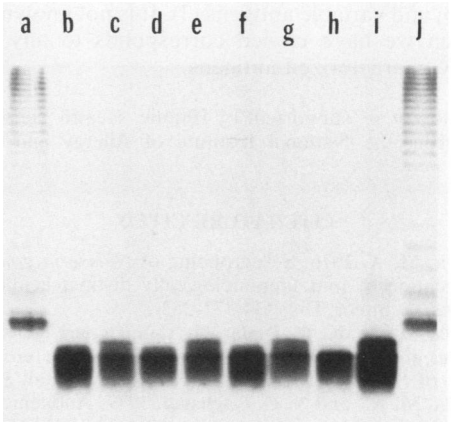

FIG. 5. Silver-stained polyacrylamide gel of LPS from *E. coli* mutants. Strains NS1 (lanes b and c), NS4 (lanes d and e), and C600 (lanes f and g) were transformed with either pBR322 (lanes b, d, and f) or pTME6 (lanes c, e, and g). The LPS banding patterns were compared with those of HB101(pBR322) (lane h) or HB101(pTME6) (lane i). Lanes a and j contain 10 μg of *S. minnesota* LPS.

enzymes could be produced in high enough quantities to interfere with normal *E. coli* LPS core synthesis. Presumably, the enzymes would account for the differences in antigen expression in the *E. coli* LPS mutant strains, and the NS4 rough LPS mutant strain may lack a suitable substrate core structure. The fact that three LPS mutant transformants produced identical LPS banding patterns suggests that more than one LPS biosynthetic gene is present in the cloned insert of pTME6. Since gonococcal LPS is known to be heterogeneous, the antigen that is expressed in *E. coli* may be a minor constituent of the RUN4383 LPS, since the antiserum used in this study was directed against a different strain of *N. gonorrhoeae* than was used to generate pTME6.

Gonococcal enzymes should be able to modify the *E. coli* core structure; the lipid A and 2-keto-3-deoxyoctonic acid region of LPS is similar in the two species (30), so that gonococcal enzymes could recognize *E. coli* core structures as substrate. Also, the core oligosaccharide portion of LPS on both organisms has similar sugar components such as glucose, galactose, *N*-acetylglucosamine, heptose, and 2-keto-3-deoxyoctonic acid (11, 18, 36). *E. coli* is a very efficient expression system for foreign genes and has been used successfully as a host for other *N. gonorrhoeae* genes (8, 15, 21). Gonococcal LPS-biosynthetic genes could alter the *E. coli* core in a number of ways. The gonococcal gene products could add a sugar component(s), phosphate, or ethanolamine side groups, change bonds between glycose molecules (e.g., alpha to beta), or change the bond positions in the core (for example, changing a straight sugar chain to a branched chain). A combination of alterations could also be occurring. Biochemical analysis of core structure will be needed to identify the alterations. Heterogeneity of the core is probably due to incomplete modification of *E. coli* by gonococcal enzymes, resulting in a mix of altered and unaltered LPS molecules.

Various portions of LPS are known to be antigenically heterogeneous among strains or species, most notably the O antigens of members of the family *Enterobactericeae*. In addition, the lipid A of *Haemophilus influenza* has recently been shown to be antigenically heterogeneous among strains (2). Growth conditions and the age of a culture can also have an effect on the structure of LPS (24, 25). The *N. gonorrhoeae* core oligosaccharide varies among strains and has been characterized antigenically into groups of common, serogroup, and variable antigens (3). It is not known whether the antigen we have cloned corresponds to any of these previously characterized antigens.

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