Pyocin-Resistant Lipopolysaccharide Mutants of *Neisseria gonorrhoeae*: Alterations in Sensitivity to Normal Human Serum and Polymyxin B

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Pyocins from *Pseudomonas aeruginosa* were used to select several lipopolysaccharide (LPS) mutants of *Neisseria gonorrhoeae* strain FA19. Three classes of LPS mutants were found in the initial group selected for study. The LPS of one class lacked galactose. That of a second group lacked the typical heptose found in the parental LPS, was reduced in glucose, galactose, and N-acetylglucosamine content, appeared to contain a new unidentified sugar component, and consisted of two species of LPS separable on sodium dodecyl sulfate-polyacrylamide gels. The LPS of a third strain lacked the heptose, glucose, galactose, and N-acetylglucosamine found in the oligosaccharide portion of parental FA19 LPS. The minimal inhibitory concentration for polymyxin B of the mutant strains was 3 to 4 times that of the parental strain. The strains lacking only galactose were as resistant as the parent to the bactericidal action of normal human serum, but cells of the other two classes were quickly killed by serum. Gonococcal LPS thus appears to be important in determining phenotypic properties of the cells.

Most strains of *Neisseria gonorrhoeae* causing disseminated infections have been shown to be resistant to the bactericidal action of normal human serum (NHS), whereas many strains isolated from urogenital infections are serum sensitive (30). Several mechanisms have been proposed to account for these differences in sensitivity to NHS. These have included serum blocking factors (20), differences in outer membrane proteins (10), differences in lipopolysaccharide (LPS) target antigens (9, 29; H. Schneider et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, E7, p. 82), and differential activation of the alternative and classical complement pathways (P. A. Rice et al., Proceedings of the European Molecular Biology Organization Workshop on Genetics and Immunobiology of Pathogenic Neisseria, 1980, p. 255). Genetic studies of serum resistance in gonococci (3, 9, 30a, 34) indicate that it is indeed a complex phenotype and that several cell envelope components may be involved.

The possible involvement of LPS in serum sensitivity is of interest to us. The LPS chemo-type of other gram-negative bacteria has been shown to be important in determining several virulence-related properties (17, 23, 24, 38) including sensitivity to NHS. We (9) and others (29) had previously shown that LPS from serum-sensitive and serum-resistant strains of *N. gonorrhoeae* inhibited serum bactericidal activity to a different degree, but were unable to demonstrate a relationship between serum sensitivity and resistance and LPS chemo-type.

The recent discovery of a simple method for selecting LPS mutants of *N. gonorrhoeae* has made it possible to investigate the importance of LPS chemo-type in determining biological properties of gonococci. Morse et al. (21) and Sidberry and Sadoff (32) reported that *Pseudomonas aeruginosa* pyocins would kill most strains of *N. gonorrhoeae* and that LPS was the likely target. Sadoff et al. (25) subsequently found that LPS mutants of a strain of *N. gonorrhoeae* could be isolated by selecting pyocin-resistant survivors. We have utilized this method to select LPS mutants of *N. gonorrhoeae* to study LPS involvement in such properties as serum resistance and antibiotic sensitivity.

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**MATERIALS AND METHODS**

Bacterial strains used and growth conditions. *N. gonorrhoeae* strain FA19, which has been described in a number of previous research reports (2, 3, 9, 27, 39),
was used as the parent strain. This strain resists killing by NHS (9). Growth on solid and liquid GC base medium was as described previously (27). *P. aeruginosa* strains 1 and 103 used for pyocin production were obtained from Frank Young. *Salmonella typhimurium* strain SL1004 was obtained from J. K. Spitznagel.

**Preparation of *P. aeruginosa* pyocins.** Crude and purified pyocin was produced from *P. aeruginosa* essentially as described by Morse et al. (21), with the exception that DNase-RNase was used to remove nucleic acids instead of precipitating them with MnCl₂. Selection of mutants. Suspensions of FA19 cells were seeded onto agar plates, and the surface was allowed to dry. Samples (5 μl) of pyocin suspensions were then dropped onto the plates and allowed to absorb into the plate. The plates were then incubated as usual. Surviving colonies from the area of the pyocin drop were isolated and tested for pyocin resistance by streaking suspensions of the organisms across previously applied streaks of pyocin. Growth in the area of the pyocin streak was monitored. The frequency of mutation to pyocin resistance was determined by mixing 10° pyocin-forming units of *N. gonorrhoeae* suspended in 0.1 ml of growth medium with 25 μl of purified pyocin suspension (3 to 4 μg of protein) and incubating at 37°C for 30 min. This suspension was then plated on agar medium and incubated overnight under the usual growth conditions. The number of surviving colonies was determined, and a representative sample of these colonies was tested for pyocin resistance.

**Characterization of LPS by electrophoresis on polyacrylamide gels.** Crude cell extracts prepared as described by Bailey and Apirion (1) and purified LPS prepared from lyophilized cells by the method of Galanos et al. (7) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with gels and buffers made essentially as described by Lugtenberg et al. (18), except that the acrylamide concentration of the stacking gel was increased to 8% and that of the running gel was increased to 15 or 20%. Bicrylamide was 3% of the total acrylamide concentration. A constant voltage of 100 V was applied to 3-mm gels for 16 to 18 h while 1.5-mm gels were run at a constant power level of 2 W for 10 h. The gels were stained for LPS by the periodic acid–Schiff procedure essentially as described by Dirienzo et al. (5) or by the silver staining procedure of Tsai and Frash (36a), which stains LPS. This latter procedure is preferable to the periodic acid–Schiff procedure for staining LPS since much smaller quantities of LPS can be detected (50 ng versus 5 μg), sharper bands are formed allowing better resolution of differences in LPS, and the procedure is quicker (1 day versus 2 to 4 days). The silver staining procedure also stains proteins in the crude extract preparations.

**Chemical characterization of purified LPS.** The 2-keto-3-deoxyoctonate acid content of the LPS was measured by the thioarbituric acid procedure as modified by Karkhanis et al. (12). Heptose was measured by the cysteine-sulfuric acid procedure as modified by Wright and Rebers (40). Other sugars were measured as acetylated derivatives by gas chromatography essentially as described by Perry et al. (22). Columns packed with SP2340 on 100/120 Supelcoport (Supelco, Inc., Bellefonte, Pa.) were used for the analysis in a Tracor model 560 gas chromatograph equipped with a flame ionization detector and integrator.

**Antibiotic sensitivity testing.** Testing for sensitivity to antibiotics (penicillin, tetracycline, chloramphenicol, erythromycin, streptomycin, rifampin, and polymyxin B), dyes (acridine orange and methylene blue), and detergents ( Triton X-100 and sodium deoxycholate) was done as described previously (6) by utilizing doubling dilutions of inhibitory agents in GC base agar medium.

**Serum sensitivity testing.** The sensitivity of *N. gonorrhoeae* strains to killing by NHS was determined as described by Eisenstein et al. (6) and Cannon et al. (3). *Limulus* lysate clotting. The ability of LPS to clot *Limulus* amebocyte lysate was determined by using materials and instructions obtained from Mallincrodt Inc. (St. Louis, Mo.).

**Genetic transformation.** Genetic transformations were performed as described previously (27), with the exception that limiting cell numbers were used to minimize the occurrence of spontaneous pyocin mutants. Spontaneous streptomycin- and rifampin-resist ant mutants of strain FA500 were selected, and DNA from these variants was prepared as described by Marmur et al. (19), sheared by passing it through a 27-gauge needle 10 times, and used to transform strain FA19. Pyocin-resistant transformants were selected after 5 h of incubation in liquid growth medium by exposing the cells to pyocin and scoring survivors for pyocin resistance. Streptomycin- and rifampin-resistant transformants were also selected to monitor transformation frequencies. Pyocin-resistant transformants were also scored for cotransformation of streptomycin and rifampin resistance.

**RESULTS**

**Selection of pyocin-resistant mutants.** *N. gonorrhoeae* strain FA19 was readily killed by crude preparations of pyocins from *P. aeruginosa* strains 1 and 103. However, when appropriate dilutions of the pyocins were placed on a test lawn of FA19, surviving colonies were found. About 25% of the surviving colonies were found to be resistant to killing by undiluted pyocin preparations. In subsequent experiments where more purified pyocins were used for selection and testing nearly 100% of surviving colonies were found to be pyocin resistant. The frequency of mutation to pyocin resistance for strain FA19 was about 10⁻⁶. The pyocin resistance phenotype appeared to be stable in experiments when random single-colony isolates were tested for pyocin resistance over 12 successive daily transfers. The mutant strains were unable to bind and remove pyocin and thus were pyocin resistant rather than pyocin tolerant (data not shown). Mutant strains selected by pyocin from *P. aeruginosa* strain 1 were also resistant to pyocin from strain 103 and vice versa. Four pyocin-resistant isolates were selected for biochemical and biological characterization.

**Characterization of crude cell lysates by SDS-
PAGES. To determine whether the mutant strains produced altered LPS we analyzed crude cell lysates by SDS-PAGE utilizing a 20% polyacrylamide running gel. These gels are capable of resolving the Salmonella Ra-Re LPS chemotypes (data not shown). The gels were stained by the periodic acid-Schiff staining procedure to locate LPS and with Coomassie blue to locate protein bands. The results of this analysis (data not shown) indicated that the LPS of the four mutant strains was different from that of FA19.

Characterization of LPS by SDS-PAGE. Analysis of purified LPS from these strains on a 15% polyacrylamide gel (Fig. 1) confirmed the differences seen with crude extracts. Staining of this gel with a silver staining procedure (36a) which detects LPS revealed several interesting features of the gonococcal LPS. The LPS of the parent appears to consist of two species. FA5000 LPS also migrated as two species, one of which comigrated with one of the FA19 LPS bands. FA5002 and 5003 LPS migrated as a single species which was different from either of the FA19 bands. FA5100 LPS migrated as a single species to a position similar to that of the second species of FA5000. A sample of S. typhimurium strain SL1004 LPS is included on the gel for comparison. This LPS is reported to consist of lipid A, 2-keto-3-deoxyoctonic acid, and heptose (Rd1 chemotype) (33).

Analysis of crude cell extracts or purified outer membranes on SDS gels prepared by the procedures of Laemmli (14) or Shapiro et al. (31) did not reveal any qualitative protein differences between parent and mutant strains (data not shown).

Chemical analysis of purified LPS. LPS was isolated from each of the strains by the method of Galanos et al. (7). Chemical analysis of the LPS indicated that each of the mutant strains possessed an LPS different from that found in the parent (Table 1). LPS from strains FA5002 and 5003 lacked galactose. That of strain FA5000 was reduced in galactose, glucose, and N-acetylgalcosamine and completely lacked the L-glycero-D-mannoheptose found in FA19 LPS. Analysis of alditol acetate derivatives of acid-hydrolyzed FA5000 LPS by gas chromatography revealed the presence of a new peak in the chromatographic profile (Fig. 2). The retention time for this compound is not the same as that for acetylated D-glycero-D-mannoheptose. The identity of this material has not been determined. Analysis of purified FA5000 and FA19 LPS by SDS-PAGE indicated that two LPS species were present in each strain. Attempts to separate the two species of FA5000 LPS by column chromatography have not been successful; therefore the chemical data shown in Table 1 represent values for a mixture of the two LPS species of each strain. Whole LPS from FA5100 lacked glucose, galactose, and heptose and was reduced in N-acetylgalcosamine content. Core oligosaccharide prepared from the LPS was found to consist only of 2-keto-3-deoxyoctonic acid. These data, along with the electrophoretic behavior of the LPS on SDS gels, lead us to conclude that LPS from strain FA5100 contains only lipid A and 2-keto-3-deoxyoctonic acid in a manner analogous to Salmonella chemotype Re LPS.

LPS inhibition of pyocin killing. Incubation of purified pyocins with LPS from the mutant strains did not inhibit the ability of pyocins to kill strain FA19. LPS from strain FA19 did inhibit pyocin killing of the homologous strain.

Limulus amoebocyte lysate clotting. LPS from the mutant strains retained the capacity to clot Limulus amoebocyte lysate to the same degree as LPS from FA19.

Antibiotic sensitivity of the pyocin-resistant LPS mutants. Each of the mutant strains was found to be two- to fourfold more resistant to polymyxin B than the parent strain (Table 2). The sensitivity of the mutant strains to penicillin, tetracycline, chloramphenicol, erythromycin, streptomycin, rifampin, acridine orange,
TABLE 1. Sugar composition of LPS

<table>
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<tr>
<th>Strain</th>
<th>KDOa</th>
<th>Heptoseb</th>
<th>Galactosec</th>
<th>Glucosec</th>
<th>N-Acetylglucosaminec</th>
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<tr>
<td>FA19</td>
<td>4.5 ± 0.2</td>
<td>4.4 ± 0.1</td>
<td>7.1 ± 0.6</td>
<td>4.5 ± 0.3</td>
<td>16.9 ± 1.0</td>
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<tr>
<td>FA5000</td>
<td>8.9 ± 1.0</td>
<td>0.8 ± 0.4</td>
<td>2.4 ± 0.4</td>
<td>1.7 ± 0.4</td>
<td>12.5 ± 0.4</td>
</tr>
<tr>
<td>FA5100</td>
<td>12.3 ± 1.2</td>
<td>0.0</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>12.0 ± 3.0</td>
</tr>
<tr>
<td>FA5002</td>
<td>4.2 ± 0.1</td>
<td>4.8 ± 0.2</td>
<td>&lt;0.1</td>
<td>3.4 ± 0.2</td>
<td>16.0 ± 5.0</td>
</tr>
<tr>
<td>FA5003</td>
<td>4.1 ± 0.2</td>
<td>4.5 ± 0.1</td>
<td>&lt;0.1</td>
<td>5.3 ± 0.1</td>
<td>13.0 ± 0.5</td>
</tr>
</tbody>
</table>

a KDO (2-keto-3-deoxyoctonate) was measured by the thiobarbituric acid method as modified by Karkhanis et al. (12).

b Heptose was measured as described by Wright and Rebers (40).

c Galactose, glucose, and N-acetyl glucosamine were measured as the alditol acetate derivatives by gas chromatography as described in the text. All values are averages of two to four determinations.

methylene blue, Triton X-100, and sodium deoxycholate was not altered.

Sensitivity to NHS. Strain FA19 is resistant to the bactericidal action of NHS. Since gonococcal LPS has been postulated as a possible target for the bactericidal action of NHS (8, 29), we assessed the serum sensitivity of the mutants. Strains FA5002 and 5003, which lack only galactose in their LPS, remained resistant to serum, whereas strains FA5000 and FA5100 were sensitive to serum (Fig. 3). The serum killing of these strains was similar to that seen for strain F62, a commonly used serum-sensitive strain of N. gonorrhoeae (9). (Strain 5002 was previously mistakenly reported to be serum sensitive [L. F. Guymon et al., Proceedings of the European Molecular Biology Organization Workshop on Genetics and Immunobiology of Pathogenic Neisseria, 1980, p. 33].)

Genetic transformation of the altered phenotype of strain FA5000. Sheared DNA (0.1 μg) from a streptomycin- and rifampin-resistant variant of strain FA5000 was used to transform strain FA19 to pyocin resistance. This was done to determine whether the altered phenotypic properties of FA5000 were the result of a single mutation. Limiting cell numbers of FA19 were used to minimize the possibility of selecting spontaneous pyocin-resistant mutants. The transformation frequency (7.6 × 10⁻⁵) to pyocin resistance was about 10-fold higher than the spontaneous mutation frequency to pyocin resistance (9 × 10⁻⁶) in this experiment. Ten presumptive transformants were selected and

FIG. 2. Gas chromatography elution profiles of alditol acetate derivatives of acid-hydrolyzed LPS of strain FA19 (---) and strain FA5000 (—).
were found to display the same polymyxin B resistance and serum sensitivity phenotypes as the donor. LPS was isolated from one of the transformants and was found to be identical in chemical composition and electrophoretic mobility on SDS-PAGE to LPS isolated from strain FA5000 (data not shown). Therefore, it appears likely that a mutation at a single genetic locus is responsible for the altered chemical and biological properties of strain FA5000. A study of genetic linkage data indicated that this locus is not the same as the sac-1 locus recently described by Cannon et al. (3), nor does it appear to be linked to the nmp-1 locus described by Cannon et al. (2) or to str or rif loci described previously (27) (data not shown).

**DISCUSSION**

This study confirms the observation of Sadoff et al. (25) that pyocin-resistant mutants of *N. gonorrhoeae* are also LPS mutants and that several distinct LPS chemotypes can be found among the mutant strains. In addition we have demonstrated that some important biological properties of the cells are changed as a result of the LPS mutations.

All of the mutant strains we tested were two- to fourfold more resistant to polymyxin B than the parent strain. This result may indicate that the LPS of the mutants has an altered polymyxin-binding capacity or that the exposure of other binding sites on the cells is changed. Studies of other gram-negative cells indicate that LPS is involved in polymyxin binding (28, 36, 37). In contrast to results found with *Salmonella* and *Pseudomonas* core LPS mutants (13, 26) the LPS mutants of *N. gonorrhoeae* were not altered in resistance to other antibiotics, dyes, and detergents tested, nor did they appear to have alterations in outer membrane proteins.

Two of the mutant strains were sensitive to the bactericidal effect of NHS, whereas the parent and the other two mutant strains were resistant. The serum-sensitive mutants both had more extensive LPS alterations than the strains which remained serum resistant. Preliminary studies of the genetics of one of the serum-sensitive mutants indicated that the altered LPS chemotype, serum sensitivity, pyocin resistance, and polymyxin resistance of this strain were due to a mutation at a single site which was distinct from the sac-1 locus recently described by Cannon et al. (3).

These observations lead us to conclude that LPS has an important role in determining the serum resistance phenotype of strain FA19. Other cell wall components are undoubtedly also important. The exact function of the LPS in this phenomenon and the relationship between the LPS mutation site and the sac-1 and sac-2 loci recently described (3, 34) are subjects of current investigations.

Analysis of LPS on SDS-polyacrylamide gels reveals that the parent strain used in this study (FA19) and one of the mutants (FA5000) appear to produce two distinct species of LPS. Strain FA5000 also lacks the heptose typically found in gonococcal LPS (L-glycero-d-mannoheptose) and possesses a new unidentified sugar as a component of the LPS. An apparently identical LPS phenotype has since been found in another independently isolated pyocin-resistant mutant strain of FA19 (Shafer and Guymon, unpublished data). The presence of multiple LPS species in gram-negative bacteria has previously been reported by several investigators (5, 11). Also the loss of the normal form of heptose (L-glycero-d-mannoheptose) and its replacement by a precursor (d-glycero-d-mannoheptose) in an *Escherichia coli* mutant have been reported by Coleman and Leive (4).

The frequency with which spontaneous LPS mutants were isolated is further evidence of the

<table>
<thead>
<tr>
<th>Strain</th>
<th>Minimal inhibitory concn (μg/ml)</th>
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<tbody>
<tr>
<td>FA19</td>
<td>100</td>
</tr>
<tr>
<td>FA5000</td>
<td>400</td>
</tr>
<tr>
<td>FA5100</td>
<td>200</td>
</tr>
<tr>
<td>FA5002</td>
<td>250</td>
</tr>
<tr>
<td>FA5003</td>
<td>250</td>
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</tbody>
</table>

FIG. 3. Survival of *N. gonorrhoeae* in a 1:2 dilution of NHS. Symbols: FA19 (○); FA5002 (○); FA5003 (□); F62, FA5000, and FA5100 (▲).
frequent changes in surface properties which \textit{N. gonorrhoeae} appears to exhibit. Other studies have shown that outer membrane proteins (15, 35, 39) and pili (16) are surface components which show a high degree of inter- and intra-strain variability. This variability is a likely explanation for the difficulty which has been encountered in developing a gonococcal typing system based on surface components.

The further study of these mutants and of LPS mutants of other strains of \textit{N. gonorrhoeae} should prove helpful in defining more precisely the role of LPS in determining the biological properties of the gonococcus. Studies of the relationships between the mutations in these strains and the other loci affecting serum resistance will be useful in understanding the basis of this complex phenomena. The mutants will also be valuable in studies of the structure and chemistry of gonococcal LPS.

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LITERATURE CITED


