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Selection and Immunochemical Analysis of Lipooligosaccharide Mutants of *Neisseria gonorrhoeae*

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The identification of enterobacterial mutants that contain alterations in the lipopolysaccharide (LPS) oligosaccharide core structure facilitated the development of the model of the physicochemical and immunochemical structures of enteric LPS. Results of recent immunochemical studies have suggested that the structural model of the lipooligosaccharides (LOSs) of *Neisseria gonorrhoeae* may differ from the enteric LPS model. The difficulties in the analysis of the wild-type gonococcal LOS have precluded understanding of the precise nature of the LOS structure. This study was undertaken to isolate a series of mutants of *N. gonorrhoeae* 1291 that had sequential saccharide deletions in the LOS. Results of preliminary studies suggested that the pyocin, designated pyocin C, allowed selection of gonococci with such mutant LOS structures. Results also indicated that the receptor for pyocin C binding was an LOS component. Pyocin C selection led to the isolation of five strains with LOS patterns on sodium dodecyl sulfate-polyacrylamide gels which differed from the LOS of parent strain 1291. In this system, the M_r of the parent LOS was 4,715, while the LOSs from the mutant strains demonstrated progressive saccharide deletions, with M_rs of 4,230, 4,089, 3,627, 3,262, and 3,197. Protein patterns of these mutants on sodium dodecyl sulfate-polyacrylamide gels were qualitatively similar to those of the parent strains. Results of studies with five monoclonal antibodies specific for neisserial LOS indicated that shared as well as unique epitopes were present on the mutant LOSs. Results of ketodeoxyoctonate analysis of the mutant LOSs indicated that the majority of the ketodeoxyoctonate residues may be substituted on C-4 or C-5. Chemical and immunological analysis of such LOS mutants should expedite the development of the model for the structure of gonococcal LOS.

Study of the immunochemical structure of the lipopolysaccharides (LPSs) of members of the family *Enterobacteriaceae* and the subsequent development of an LPS model was facilitated by the identification of LPS mutants with sequential deletions in saccharide units (18). Nonenteric gram-negative bacteria, including *Neisseria meningitidis*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae*, have a principal surface glycolipid analogous to enteric LPS. These structures have been designated lipooligosaccharides (LOSs). They are characterized by a complex antigenic structure in the absence of repeating O side chains. Studies to elucidate the relationship between the chemical structure of the LOS and its antigenic structure have been hampered by the absence of a model for these LOSs.

Pyocin, a bacteriocin produced by *Pseudomonas aeruginosa*, has been shown to bind to the surface and inhibit the growth of *N. gonorrhoeae* (21). Results of previous studies have suggested that LOS receptors on the outer membrane of *N. gonorrhoeae* are the principal binding sites for pyocins (6, 23, 34). Connelly et al. (7) and others (10, 11, 20, 30) have demonstrated that resistance to pyocin 103 is correlated with a structural alteration that involves *N*-acetylglucosamine residues in gonococcal LOS. Results of previous work by Guymon and associates (10, 11) have indicated that pyocin resistance occurs approximately once in every 10^6 organisms. This indicates that the gonococcal LOS chemotype can mutate at a high frequency and that certain strains of gonococcus may be able to produce more than one type of LOS (7, 10, 11, 20, 30).

In this study, pyocin C, an R-type pyocin (6), was selected to isolate LOS mutants of *N. gonorrhoeae* 1291, an LOS prototype strain (4). Five LOS mutants were selected based on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) characteristics and subjected to immunochemical analysis. Detailed physicochemical analysis of these mutants could facilitate development of the structural model for gonococcal LOS.

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MATERIALS AND METHODS

Bacterial strains. *N. gonorrhoeae* 1342, 1291, 4505, 8551, PID2, 3893, and JW31R have been described previously (1, 2, 20). All gonococcal strains studied were unpiliated. *N. gonorrhoeae* WS-1 was obtained from William Shafer (School of Medicine, Emory University, Atlanta, Ga.). This strain contains an LOS which contains only ketodeoxyoctonate (KDO) and lipid A (27). The *P. aeruginosa* strains used for the production of pyocins were obtained from Jerold Sadoff (Walter Reed Army Institute of Research, Washington, D.C.) (28). *Salmonella minnesota* LPS mutant strains Ra through Re were the gift of Herman Schneider (Walter Reed Army Institute of Research, Washington, D.C.). All strains were stored at −70°C in Mueller-Hinton broth containing 10% glycerol.

Bacterial cultural conditions. *N. gonorrhoeae* was grown on GC medium base (Difco Laboratories, Detroit, Mich.) supplemented with 1% (vol/vol) IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.). *P. aeruginosa* was also grown on supplemented GC medium base. Cultures for pyocin isolation were grown in tryptic soy broth (Difco).

Pyocin isolation. Pyocins were isolated from cultures of *P. aeruginosa* by the procedure described by Morse et al. (21).

Pyocin assay. A lawn of *N. gonorrhoeae* was made by the addition of 10 μl of a 10^8 inoculum onto the surface of GC medium base plates (15 by 100 mm). Purified pyocin prepa-

ration (10 μl) was dropped onto the lawn, and the plate was incubated overnight at 37°C in a 5% CO_2 incubator. A zone of lysis was observed, and survivors within the zone were isolated as individual colonies.

SDS-PAGE. The LOS and LPS mutants were subjected to electrophoresis on a 14% polyacrylamide gel by the method described by Mandrell et al. (19). Silver staining was performed by the method described by Tsai and Frasch (31). Protein analysis was done on a 15% polyacrylamide gel by the method described by Laemmli (16). These gels were stained with Coomassie brilliant blue.

M_r determination. The calculated M_r for each gonococcal mutant and parent strain 1291 was determined by comparing the R_f values on SDS-PAGE of the LOS mutants with that of LPS with a known M_r from *S. minnesota* mutants (26) by linear regression analysis (29).

LOS preparation and purification. LOS was isolated by the method described by either Dareau and Hancock (8) or Westphal and Jann (33). The proteinase K (PK) method described by Hitchcock and Brown (13) was used for studies in the initial analysis of the LOS mutants by SDS-PAGE.

Pyocin C-LOS inhibition assay. To establish the inhibition assay, the titer of pyocin C was determined so that the highest dilution that still produced lysis of the gonococci could be determined. LOSs, in serial twofold dilutions from 50 to 6.25 μg/ml (final concentration), were incubated with pyocin C at a predetermined titer at 37°C for 30 min. A lawn containing 10^8 organisms of strain 1291 was made on GC medium base plates, and 10 μl of the pyocin C-LOS mixture was applied. The plates were incubated for 18 h at 37°C in 5% CO_2, and the range of inhibition of lysis was determined. The titer refers to the last dilution in which inhibition of lysis occurred.

Monoclonal antibodies. A catalog of 33 monoclonal antibodies with specificity for neisserial LOS was developed in our laboratory. These were reacted with the mutant LOS preparations. Four monoclonal antibodies from this catalog were extensively studied in this investigation. These included monoclonal antibodies 6B4 and 3F11, which have been described previously (3, 19), and monoclonal antibodies 6B7 and 4C4, which were made to *N. meningitidis* group A strain A1, which has an L11 LOS. Monoclonal antibody 44/179 was a gift from Birkmeyer (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). This antibody was raised to a *N. gonorrhoeae* strain. In addition, the LOS preparations were studied with monoclonal antibody 1C9, which has been shown to react with an epitope in the KDO-lipid A backbone region of enterobacterial LPS. This antibody was developed after immunization of BALB/c mice with the Re mutant of *S. minnesota*.

Immunodot and Western blot assays. The immunodot assay was performed by the method described by Hawkes et al. (12). LOS (3 μg) was applied to the nitrocellulose in these experiments. Western blot analysis was performed by the method described by Knecht and Dimond (15).

KDO analysis. KDO analysis was performed on both the *N. gonorrhoeae* and the *S. minnesota* mutants by the thiobarbituric acid (TBA) method described by Osborn (22) and the semicarbazide method described by Droge et al. (9). In the TBA method, timed hydrolysis curves were generated with 0.025 and 0.050 M H_2SO_4 at 15, 30, and 60 min for the measurement of KDO. All studies were performed at 100°C in a heating block. Optimal hydrolysis conditions were determined, and all calculations were extrapolated to zero hydrolysis time. In the semicarbazide analysis, samples of LOS and LPS were hydrolyzed in 0.1 N acetic acid (pH 3.4) at 100°C in a heating block for 60 min. Lipid A was removed by centrifugation, and 100 μl of the supernatant was reacted in the test system. Correlation coefficients of 11 standard curves were measured for both TBA and semicarbazide analyses. The range of these coefficients was from 0.89 to 0.98, with a mean correlation coefficient of 0.93.

RESULTS

Selection of pyocin and gonococcal strains for study. Pyocins were isolated and purified from nine different *P. aeruginosa* strains. To select a suitable pyocin for further study, the nine pyocin preparations were reacted with the six *N. gonorrhoeae* LOS prototype strains and the pyocin 611 selected LOS mutant JW31R (20). The results of this interaction are shown in Table 1. Pyocin C was the only pyocin which lysed all of the LOS prototype strains, as well as strain JW31R. The lysis of JW31R by pyocin C suggests that this pyocin may select for deep rough LOS mutants. *N. gonorrhoeae* 1291 was selected for study because its LOS was the least complex of those of prototype strains. SDS-PAGE and silver staining techniques indicated that strain 1291 has a single major LOS band.

Pyocin lysis studies. The number of surviving colonies that was present within the lytic zone after pyocin C interaction with strain 1291 was dependent on the individual pyocin C preparation and ranged from 5 to 20. Approximately 25 individual colonies of pyocin C survivors were selected and studied in detail. PK lysates of each colony were prepared, and each cell lysate was subjected to electrophoresis on a 14% SDS-polyacrylamide gel and silver stained. The LOSs from the majority of these strains had different R_f values on SDS-PAGE when compared with the R_f value of the LOS of parent strain 1291. Five of these strains possessed LOS banding patterns which differed by sequential saccharide deletions from the major LOS band of parent strain 1291 (Fig. 1). Four of these strains were selected after the pyocin C interaction with parent strain 1291. Mutant strain 1291_b was selected after retreatment of mutant strain 1291_c with pyocin C. The M_rs of the LOSs of all five mutants were lower than that of the parent LOS. The M_rs of the LOS bands were measured for each mutant. A linear regression analysis was established by using the *Salmonella* mutants as standards, and the M_rs of the LOSs of strain 1291 and the mutants was calculated from that regression. The M_r for the LOS units of parent strain 1291 was 4,715, for 1291_a it was 4,230, for 1291_b it was 4,089, for 1291_c it was 3,627, for 1291_d it was 3,262, and for 1291_e it was 3,197. The LOS of strain WS-1, which contains only lipid A and KDO, had an M_r of 2,642 in this system (data not shown).

TABLE 1. Pyocin susceptibility of gonococcal LOS prototype strains

Gonococcal strain	Susceptibility of the following pyocins[a]:								
	A	B	C	D	E	F	G	H	J
1291	S	S	S	R	S	R	S	R	R
1342	S	S	S	R	S	R	S	R	R
3893	S	S	S	R	S	R	S	R	R
4505	S	S	S	R	S	R	S	R	R
8551	S	S	S	R	S	R	S	R	R
PID2	S	S	S	R	S	R	S	R	R
JW31R	R	ND	S	R	R	R	R	T	R

[a] Abbreviations: ND, not done; R, resistant; S, sensitive; T, turbid (partially sensitive).

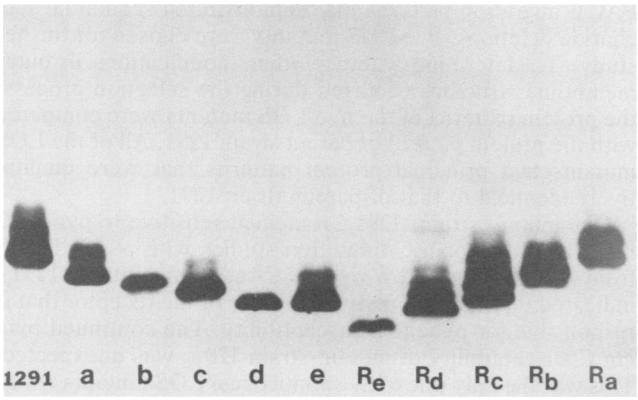

FIG. 1. Silver-stained SDS-polyacrylamide gel of PK lysates isolated from *N. gonorrhoeae* 1291 and 1291 LOS mutants a through e. This gel demonstrates the electrophoretic differences in parent and mutant LOSs. The *S. minnesota* Ra through Re LPS mutants are also shown.

Stability of LOS mutants. PK lysates were prepared from each mutant after five passages on successive days on GC medium base and after storage at −70°C in Mueller-Hinton broth with 10% glycerol. PK lysates of these isolates were subjected to electrophoresis on SDS–14% polyacrylamide gels and silver stained. No differences in LOS banding patterns of the passaged or stored strains were observed when compared with those of PK extracts made from the original colony isolates. In addition, LOS was prepared to strain 1291 and the mutants by the phenol-water method and the method described by Dareau and Hancock (8) from large quantities of organisms grown on solid media. These LOS preparations gave SDS-PAGE banding patterns identical to those obtained with the PK lysates. Based on these observations, it appears that the LOS mutants that we observed are stable.

SDS-PAGE analysis of proteins of LOS mutant strains. Protein patterns of whole-cell lysates of each mutant on SDS-polyacrylamide gels were compared with similar lysates prepared from parent strain 1291 (Fig. 2). The principal proteins in four of the five LOS mutant strains were qualitatively similar to those of parent strain 1291. Some differences in the concentration of P3 were seen in mutant strain 1291_d.

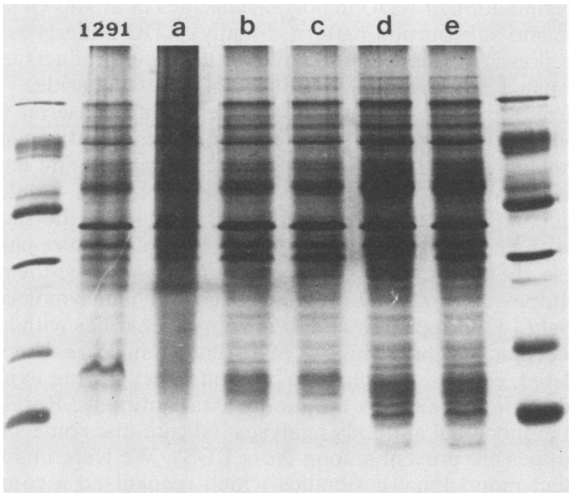

FIG. 2. SDS-PAGE of whole-organism lysates of *N. gonorrhoeae* 1291 and 1291 LOS mutants a through e that demonstrate similar protein electrophoresis patterns. The gel was stained with Coomassie brilliant blue.

TABLE 2. Inhibition of pyocin C by LOSs

LOS	LOS (μg/ml)[a]	
	1291	1291_c
1291	25–50	6.25–12.5
1291_c	12.5–25	12.5–25
1291_a	>50	>50

[a] For mutant strain 1291_a, there was no inhibition of pyocin C lysis at 50 μg of LOS per ml.

Pyocin C-LOS inhibition studies. Studies were undertaken to determine whether LOS could inhibit pyocin C-gonococcal interactions. To accomplish this, each gonococcal LOS mutant was first examined in the pyocin C assay to determine pyocin sensitivity or resistance. The ability of pyocin C to lyse LOS mutant strains varied substantially. Complete lysis of parent strain 1291 and mutant strain 1291_c could be obtained at a dilution of 1:100,000. Undiluted pyocin C was necessary to achieve complete lysis of LOS mutant strains 1291_a, 1291_d, and 1291_e. Mutant strain 1291_b was completely resistant to pyocin C lysis. An inhibition assay was developed to determine whether LOSs isolated from two mutants and the parent strain could inhibit pyocin C lysis. The LOSs were added to pyocin C at final concentrations ranging from 6.25 to 50 μg/ml. The results are given in Table 2. The concentration of LOS needed to completely inhibit pyocin C lysis of parent strain 1291 was between 12.5 and 25 μg/ml. The LOS isolated from strain 1291_c, the pyocin C-sensitive mutant, inhibited pyocin C lysis at lower concentrations (6.25 to 12.5 μg/ml). LOS isolated from 1291_a failed to inhibit pyocin C activity at concentrations as high as 50 μg of LOS per ml.

Monoclonal antibody analysis. Thirty-four monoclonal antibodies with specificity for epitopes on gonococcal or meningococcal LOS were tested for reactivity with the mutant LOSs. Fourteen of these antibodies reacted in the immunodot assay. The results for six representative monoclonal antibodies used to analyze the mutant LOSs by immunodot assay are given in Table 3. Significant antigenic variation existed among the mutant and parent LOSs. No epitopes common to all five LOS mutants were detected by these monoclonal antibodies. Two of the LOS mutants (1291_a and 1291_e) failed to react with any of the 34 monoclonal antibodies. Western blot analysis confirmed the immunodot results with monoclonal antibodies 6B4, 6B7, and 4C4 (data not shown). None of the gonococcal LOSs reacted with mono-

TABLE 3. Monoclonal antibody analysis of *Neisseria* LOS mutants

Monoclonal antibody	Presence of monoclonal antibody epitopes for the following LOS mutants:					
	1291	1291_a	1291_b	1291_c	1291_d	1291_e
44/179	−	−	−	−	+	−
3F11	+	−	−	−	−	−
6B4	+	−	−	−	−	−
6B7	−	−	+	+	−	−
4C4	−	−	−	+	−	−
1C9	−	−	−	−	−	−

clonal antibody 1C9, which recognizes a conserved region on enterobacterial LOS.

KDO analysis. KDO analysis was performed on the *N. gonorrhoeae* and *S. minnesota* mutants (Table 4). The differences seen in TBA and semicarbazide analyses represent reactivities caused by substitutions of KDO at different carbons (32). Results of the semicarbazide studies indicate that there are at least 2 mol of KDO per mol of LOS for strain 1291 mutants a through e. The TBA data indicate that between 45% (1291_e) and 95% (1291_d) of the KDO residues are substituted on C-4 or C-5. The *S. minnesota* mutants were studied in tandem with the gonococcal LOS mutants. The data for mutants Ra and Rc are consistent with previously published results for both the TBA (25) and semicarbazide (9) methods. The results for Re by TBA analysis were consistent with previous data (25), but the semicarbazide analysis consistently gave lower values than expected. Both chemical analyses were performed on the same LPS preparation.

DISCUSSION

The antigenic heterogeneity in the absence of O side chains suggests that the model for gonococcal LOS does not conform to the model for LPS structures obtained by studies of enteric bacteria. The LPS structure of enteric bacteria has been defined by selecting LPS mutants that were obtained by mutagenesis and phage selection (17, 24). Results of the study of these LPS mutants have demonstrated that enterobacterial LPS has a core structure which is relatively conserved (18). A similar series of LOS mutants with sequential deletions in saccharide units would facilitate physicochemical analysis of gonococcal LOS. Pyocins have been used in the past to select for gonococci with mutant LOS structures (11, 20). Other investigators (7, 23, 34) have shown that at least one of the specific receptors for pyocin binding is LOS. As shown in this study, gonococcal clones which survive pyocin lysis have a high probability of possessing altered LOSs, and mutants with sequential saccharide deletions can be selected.

Pyocin C was reacted with *N. gonorrhoeae* 1291, an LOS prototype strain. Numerous colonies with altered LOS structures were selected from the survivors. Based on SDS-PAGE migration patterns that demonstrated sequential saccharide deletions, five LOS mutants were chosen for further study. To determine whether other modifications in outer membrane structure occurred during the selection process, the protein patterns of the five LOS mutants were compared with the protein pattern of parent strain 1291. All of the LOS mutants had principal protein patterns that were qualitatively identical to that of parent strain 1291.

TABLE 4. KDO analysis

Strain	KDO concn (% [wt/wt]) by analysis with:		mol of KDO/mol of LOS by analysis with:		% Substitution of KDO
	TBA	SC[a]	TBA	SC	
N. gonorrhoeae					
1291	4.8	7.8	0.9	1.4	64
1291_a	4.1	14.1	0.7	1.4	70
1291_b	3.4	14.5	0.5	2.3	78
1291_c	3.4	14.3	0.5	2.0	75
1291_d	1.0	15.3	0.1	2.0	95
1291_e	3.6	7.1	0.5	0.9	45
S. minnesota					
Ra	4.0	7.9	0.7	1.3	
Rb	7.2		1.1		
Rc	12.0	15.1	1.5	1.9	
Rd	15.7		1.8		
Re	16.8	6.9	1.6	0.7	

[a] SC, Semicarbazide method.

One mutant strain, 1291_c, remained sensitive to pyocin C retreatment. Pyocin C inhibition studies with purified LOS from this mutant and a pyocin C-resistant mutant, 1291_a, indicated that the LOS may be the site of the receptor that is responsible for pyocin C susceptibility. The continued pyocin C susceptibility of mutant strain 1291_c was unexpected. This was the only one of five gonococcal LOS mutants which remained sensitive to retreatment with pyocin C. This indicates that there is more than one pyocin receptor on the oligosaccharides of the LOS. Because sugars were deleted in the LOS mutants, these receptor regions became reexposed and rendered the mutant pyocin C susceptible, despite the fact that mutants 1291_a and 1291_b were resistant.

The data obtained in the KDO analysis support the presence of two residues per LOS chain, as suggested by Jennings and Johnson (14). Differences in results obtained in the TBA and semicarbazide assays reflect differences in the substitution of the KDO residue. Mutant 1291_e contained only one KDO residue per LOS. This suggests that this mutant lost one KDO residue. For an optimal TBA response, the KDO residues must be linked only through the side chain with a free diol group at C-4 or C-5 (32). The semicarbazide method of KDO analysis measured both substituted and unsubstituted KDO residues. One unexpected result was the 0.7 mol of KDO per mol of LPS for the *Salmonella* Re mutant, as determined by the semicarbazide method. The result by the TBA method gave the expected 2 mol of KDO per mol of LPS with this mutant. These results occurred on repeated analyses. Droge et al. (9) have commented on the variation in substitution of KDO from one preparation of LPS to another, making analysis of precise molar relationships difficult. Unger (32) has also stated that KDO determinations are difficult to perform. First, the ketosidic linkages of KDO are extremely acid labile. Second, no entirely satisfactory procedure exists for the quantitative determination of KDO in polysaccharides of unknown structure and substitution pattern. Finally, KDO undergoes side reactions; this leads to unknown or unstable products under the usual conditions of hydrolysis of polysaccharides.

Epitope analysis with 34 different anti-neisserial LOS monoclonal antibodies revealed differences in antigen structure between the LOSs from gonococcal parent and mutant strains. We anticipated that epitope deletions would be sequential; that is, as the number of residues in the saccharide chain decreases, alterations in epitope structure parallel these chemical changes. This has not proven to be the case. Epitopes that were not previously seen on higher-molecular-weight LOSs appeared on LOSs with saccharides with lower molecular weights. This suggests that as sugar residues are deleted, epitopes which were previously cryptic are exposed and made available to the monoclonal antibody. Results of the monoclonal antibody analyses indicate that some shared epitopes are present among these LOSs. We were unable to detect monoclonal antibodies which recognized a common epitope among all five mutants and the parent strain. We believe that such epitopes exist, but these epitopes may be poorly immunogenic or cryptic until they are exposed. Results of these monoclonal antibody studies also indicate

that the rules that govern antigenic expression of gonococcal LOS are substantially different from those that govern enteric LPS antigen expression. Monoclonal 6B4 and 3F11 recognize conserved LOS epitopes on over 200 gonococcal strains that have been tested (19). Results of the present study indicate that these common epitopes are situated on the most distal portions of the saccharide moiety of the LOS. With the enteric LPS, the antigenically conserved regions are found in the deepest segments of the saccharide portion (17), while the more distal regions of the LPS express unique antigenic specificities. Cross-reactivity between gonococcal LOS and core region of the enteric LPS has been demonstrated with polyclonal antisera (5). The epitopes that are responsible for this cross-reactivity, however, have not been elucidated by this monoclonal antibody study. The antineisserial LOS monoclonal antibodies do not react with enteric LPS (data not shown). Studies with monoclonal antibody 1C9, which recognizes the KDO–*N*-acetylglucosamine region of a wide range of different enteric LPSs, failed to react with any of the gonococcal LOSs. This suggests that while the chemical components of this region are similar, the saccharide arrangement may differ substantially.

Physicochemical studies of the LOS from these mutants are now in progress. Results of these studies should allow the development of a model for the structure of gonococcal LOS.

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