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Use of Transformation To Construct *Neisseria gonorrhoeae* Strains with Altered Lipooligosaccharides

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DNA isolated from a nalidixic acid- and rifampin-resistant derivative of *Neisseria gonorrhoeae* serum-resistant strain 302 (MUG116), a strain that reacts with monoclonal antibody (MAb) 2-1-L8, was used to transform *N. gonorrhoeae* DOV, a serum-sensitive strain, to antibiotic resistance and/or reactivity with the MAb. MAb 2-1-L8 binds to a 3.6-kilodalton lipooligosaccharide (LOS). Reactivity with MAb 2-1-L8 transformed as a single marker and was unlinked to either of the antibiotic resistance markers. Immunoblot analysis of LOSs separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed that new LOSs were synthesized in the transformed cells and that these LOSs corresponded to those of the DNA donor. Although multiple LOS components were made by the transformants, the MAb recognized only one. All transformants that were selected for on the basis of strong reactivity with MAb 2-1-L8 were serum resistant; however, the level of resistance correlated with the apparent loss of recipient LOS components. MAb 2-1-L8-reactive transformants that still produced DOV LOS components remained serum sensitive.

Lipooligosaccharides (LOSs) are important in the pathogenesis of *Neisseria gonorrhoeae* (1, 7, 8, 11, 13, 16, 17). LOS mediates most of the toxic damage that occurs in the fallopian tube model (2), has been implicated in the attachment of piliated and nonpiliated gonococci to host cells (17), serves as a key target on the cell surface for bactericidal antibody (9), has been associated with serum resistance (9), and regulates complement activation on the bacterial cell surface (4, 6). Gonococcal LOSs are heterogeneous glycolipids without repeating oligosaccharides (3). Strains differ in the number and M_r of their LOS components. Within a strain, LOS components differ in their relative concentrations and in the antigens they express (8).

The genetic basis of LOS biosynthesis is unknown. Knowledge of the genetics of LOS biosynthesis and expression would make possible precise characterization of their biology. We chose to study the expression of the 3.6-kilodalton (kDa) LOS, which is associated with serum resistance (ser^r) of gonococci and bears the meningococcal L8 LOS serotype antigen (8), because of its biological significance. In this paper we describe the use of genetic techniques to construct a set of strains that vary in LOS repertoire and the effect of this variation on the ability of the constructed strains to resist the killing action of normal human serum (NHS).

MATERIALS AND METHODS

Bacterial strains and culture conditions. *N. gonorrhoeae* DOV and 302 have been characterized (8, 10). These strains and the derivatives we constructed are described in Table 1. Culture conditions have been described previously (10, 14).

Chemical reagents. Chemicals used for the transformation studies were reagent grade or better and were purchased from Sigma Chemical Co., St. Louis, Mo., unless otherwise noted. Chemicals used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories, Richmond, Calif., and silver nitrate was obtained from Fisher Scientific Co., Silver Spring, Md.

Genetic transformation. Procedures for the preparation of transforming DNA and for genetic transformation have been described previously (14). DNA (200 ng) was added to 2 ml of cells (5×10^7/ml). Transformation was terminated after 30 min by the addition of 50 μg of DNase. Cells were allowed to express transforming DNA for 6 h before being plated on an agar medium. Antibiotic-resistant transformants were selected by using GCMB agar (Difco Laboratories, Detroit, Mich.) supplemented with either 1 μg of rifampin per ml, 1 μg of nalidixic acid per ml, or both. Expression of the 3.6-kDa LOS that is associated with ser^r was detected by its binding of monoclonal antibody (MAb) 2-1-L8. MAb 2-1-L8-reactive colonies were identified by a colony-blotting procedure, as follows. Colonies were incubated for 18 h on agar (GCMB with or without antibiotics) and blotted to nitrocellulose filters (BA85; Schleicher & Schuell, Inc., Keene, N.H.). After air drying for 10 min the filters were exposed to a filler solution (0.1% casein, 0.1% bovine serum albumin, 0.04% NaN_3, and 0.002% phenol red, in phosphate-buffered saline [pH 7.5]) and incubated at room temperature for 1 h with gentle shaking. Filters were washed three times in 20 ml of phosphate-buffered saline with shaking for 10 min. The filters were incubated for 2 h in MAb 2-1-L8, diluted 1:1,000 in filler solution, and washed as described above. The filters were incubated in secondary antibody, alkaline phosphatase-conjugated goat-anti-mouse immunoglobulin (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.), diluted into a filler solution (1:500) for 2 h. The filters were washed as described above, with an additional wash in 50 mM Tris hydrochloride (pH 8.0). Colonies that bound the secondary antibody were detected by immersion of the filters in 15 ml of developing reagent (2 mg of fast red per ml and 1 mg of α-naphthol AS MX phosphoric acid, disodium

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TABLE 1. Properties of strains used or constructed in this study

Strain	Serum sensitivity[a]	Antibiotic resistance		Reactivity[b] with MAb 2-1-L8
		Nal[r][c]	Rif[r][d]	
DOV	S	−	−	−
WR302	R	−	−	++++
MUG116[e]	R	+	+	++++
MUG100[f]	R	+	−	++++
MUG101[f]	R	+	−	++++
MUG102[f]	R	+	−	++++
MUG103[f]	R	−	+	++++
MUG121[f]	R	−	+	++++
MUG301[f]	S	+	−	−
MUG302[f]	S	+	−	++
MUG303[f]	S	−	+	−
MUG304[f]	S	−	+	++

[a] S, Sensitive; R, resistant.
[b] MAb 2-1-L8 is a MAb that reacts with the meningococcal L8 LOS serotype antigen: ++++ strains reacted strongly on colony blot, ++ strains reacted weakly, and − strains did not react.
[c] Nal[r], Resistant to 1 μg of nalidixic acid per ml.
[d] Rif[r], Resistant to 1 μg of rifampin per ml.
[e] MUG116 is a spontaneous nalidixic acid- and rifampin-resistant derivative of WR302.
[f] Constructs of strain DOV transformed with MUG116 DNA and selected for antibiotic resistance.

salt, per ml in 50 mM Tris hydrochloride [pH 8.0]) until color development.

SDS-PAGE and immunoblot analysis. Proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.)-treated whole-cell lysates were prepared from 18- to 20-h cultures by the procedure of Hitchcock and Brown (5). These lysates were diluted so that the apparent LOS concentration in 10 μl approximated 1 μg of purified LOS. Lysates were subjected to SDS-PAGE (10), and the LOS was visualized by silver staining (15). Binding of MAb 2-1-L8 to LOS components transferred to nitrocellulose filters (12) was detected immunoenzymatically as described above.

Sensitivity to lysis by NHS. Strains were tested for their ability to resist the killing action of NHS by a modification of the procedure of Schneider et al. (9). Veronal (Winthrop Laboratories, New York, N.Y.)-buffered saline with 0.1% gelatin, 0.2 mM magnesium chloride, and 0.15 mM calcium chloride was substituted for the Geys balanced salts solution used in the original procedure (7).

RESULTS

Transformation of *N. gonorrhoeae*. DNA from strain MUG116 was used to transform strain DOV to Nal[r] or Rif[r] or both (final DNA concentration, 100 ng/ml); transformation frequencies are shown in Table 2. The two antibiotic resistance genes transform the gonococcus at approximately the same frequency (3.3×10^{-4} transformants per ml for nalidixic acid and 3.4×10^{-4} transformants per ml for rifampin), and these represent the frequencies expected for single-gene transformations. The cotransformation frequency for these unlinked antibiotic resistance markers was 8.5×10^{-7} when selected for simultaneously.

Transformation for reactivity with MAb 2-1-L8 was done nonselectively. The transformation frequency obtained was approximately 1 order of magnitude higher than those obtained when selecting for antibiotic resistance (2.6×10^{-3} versus 3.3×10^{-4} to 3.4×10^{-4}), but indicates that MAb 2-1-L8 reactivity is transforming as a single linkage group.

TABLE 2. Transformation of strain DOV with MUG116 DNA

Marker selected	Transformation frequencies[a]	
	Observed	Theoretical
Rif[r]	3.4×10^{-4}	
Nal[r]	3.3×10^{-4}	
L8	2.6×10^{-3}	
Rif[r] Nal[r][b]	8.5×10^{-7}	1.1×10^{-7}
Rif[r] L8[c]	1.9×10^{-6}	8.8×10^{-7}
Nal[r] L8[d]	2.5×10^{-6}	8.6×10^{-7}

[a] Expressed as the number of transformants divided by the number of CFU. Theoretical frequencies were obtained by multiplying the frequencies observed for the single markers.
[b] Both markers were selected for simultaneously.
[c] Rifampin-resistant transformants were identified first and then screened for reactivity with MAb 2-1-L8.
[d] Nalidixic acid-resistant transformants were identified first and then screened for reactivity with MAb 2-1-L8.

The degree of genetic linkage among the three markers tested was determined by comparing the expected cotransformation frequency for each pair of markers with the theoretical frequencies that would be obtained if the markers were unlinked. The data in Table 2 indicate that the three markers are unlinked (less than 1% cotransformation).

Expression of the 3.6-kDa LOS by transformants. DOV transformants constructed by transformation with MUG116 DNA and selected for resistance to either rifampin or nalidixic acid were screened for reactivity with MAb 2-1-L8 by immunoblot analysis. Those that reacted strongly with the MAb were placed in the MUG100 series; those that reacted weakly or not at all were placed in the MUG300 series.

Figure 1 is a photograph of an immunoblot of transformed DOV colonies cultured on rifampin-containing agar and reacted with MAb 2-1-L8. Two types of reactivities are visible. Some colonies bound the MAb homogeneously (arrows A), whereas others bound it only to sectors of the colony (arrows B). In addition, some colonies reacted very strongly with the antibody (dark areas), whereas others bound the antibody weakly (lighter areas). On passage, sectored colonies gave rise to both reactive and nonreactive colonies. Upon further passage, reactive colonies gave rise to reactive progeny and nonreactive colonies gave rise to nonreactive colonies (data not shown). During 10 days of

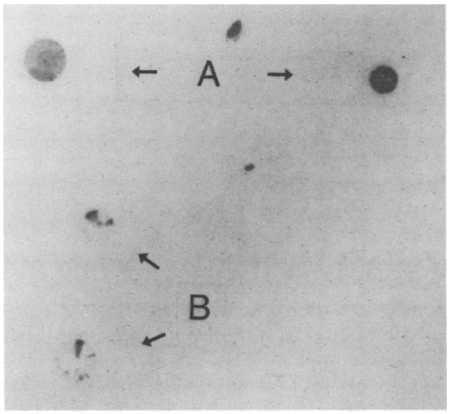

FIG. 1. Immunoblot of Rif[r] transformants of strain DOV. Colonies were blotted onto nitrocellulose and then reacted with MAb 2-1-L8. Arrow A indicates homogeneous reacting colonies. Arrow B indicates sectored colonies.

serial passage of a single colony, reactivity with MAb 2-1-L8 was stably maintained.

SDS-PAGE analysis of LOS. Proteinase K-treated whole-cell lysates of donor, recipient, and transformant strains were examined by using silver-stained SDS-PAGE gels and Western immunoblot analysis. In the MUG100 series of transformants, all five transformants produced a visible LOS band that bound MAb 2-1-L8 strongly on immunoblot (Fig. 2, lanes 3 to 7); of the MUG300 series, only MUG302 and MUG304 produced an LOS that reacted with MAb 2-1-L8; however, this band was not visible on silver staining (Fig. 2, lanes 8 to 11).

The LOS profiles of the parental strains are quite different (Fig. 2, lanes 1 and 2). The LOS profiles of the MUG100 series transformants resemble that of the MUG116 donor with respect to the number and mobility of LOS components; however, these strains produce different amounts of each LOS component. MUG102 most closely resembled the MUG116 donor strain with respect to the number and mobility of the individual components.

LOSs made by the MUG300 series were more variable than those of the MUG100 series. The MUG300 series made LOSs of both donor and recipient origin, as well as LOS molecules with M_rs different from those of either parental strain. The LOS profiles of MUG302 and MUG304 were nearly identical, even though the strains were resistant to different antibiotics. Both series made enough of the 3.6-kDa LOS component to permit its detection by immunoblot binding of MAb 2-1-L8, but not enough to be visualized by silver staining of the SDS-PAGE gel.

The reactivity with MAb 2-1-L8 of each SDS-PAGE-separated LOS component was determined by immunoblotting a duplicate of the silver-stained gel to nitrocellulose (Fig. 2B). As expected, MAb 2-1-L8 bound to the 3.6-kDa LOS produced by the MUG116 donor strain, the five MUG100 series strains, MUG302, and MUG304. It bound to none of the LOS components of the DOV recipient, MUG301, or MUG303. The strongest binding was to LOS of MUG116 and MUG102.

Sensitivity of strains to bactericidal activity of NHS. The ability of strain 302 to resist the killing action of NHS is well established (8). We analyzed the DOV transformants for this phenotypic characteristic (Table 3). MUG102 was quantitatively more resistant to NHS killing than was the donor strain, MUG116; the remainder of the MUG100 series strains were equivalently resistant (difference in $\log_2$ concentration of serum producing equivalent reduction in CFU was <2). MUG301 and MUG304 were equivalent in serum sensitivity to the DOV recipient; MUG302 and MUG303 were equivalently killed by a $\log_2$ difference in serum of >2 than DOV (more ser^S).

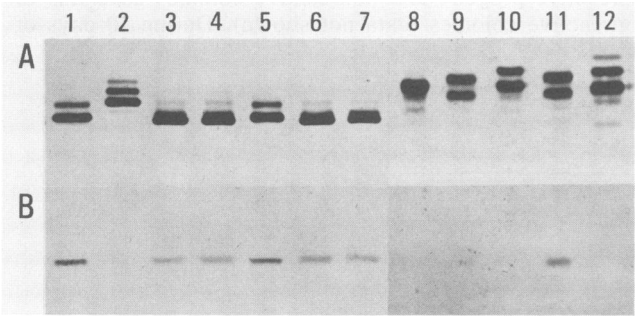

FIG. 2. SDS-PAGE and immunoblot analysis of proteinase K-treated lysates. The figure is a composite of two sets of duplicate gels. (A) Lysates were silver stained after SDS-PAGE. (B) Lysates were reacted with MAb 2-1-L8. The lanes represent lysates derived from the following strains: MUG116 (lane 1), DOV (lane 2), MUG121 (lane 3), MUG103 (lane 4), MUG102 (lane 5), MUG101 (lane 6), MUG100 (lane 7), MUG301 (lane 8), MUG302 (lane 9), MUG303 (lane 10), MUG304 (lane 11), and DOV (lane 12). Lanes 1 to 7 cannot be directly compared with lanes 8 to 12, since they are separate gels. LOSs of strain DOV (lanes 2 and 12) provide a reference for comparing the two parts of the composite.

TABLE 3. Susceptibility to killing by NHS

Strain	% NHS required[a]
DOV	2.5
MUG116	20
MUG100	10
MUG101	8.4
MUG102	80
MUG103	10
MUG121	40
MUG301	2.5
MUG302	0.62
MUG303	<0.62
MUG304	2.5

[a] Bactericidal endpoints were taken to be the lowest concentration of NHS that killed at least 50% of the test organisms.

DISCUSSION

This study was undertaken to determine the feasibility of using transformation to produce strains of *N. gonorrhoeae* with genetically altered LOS. Strains differing in three LOS-related phenotypes (SDS-PAGE profile, serum sensitivity, and the quantitative expression of the 3.6-kDa LOS that bears the L8 serotype antigen) were chosen as transformation donor and recipient. We were able to isolate a series of transformants that produced LOS of the same M_r as those of the donor, including the 3.6-kDa LOS that expressed the meningococcal L8 serotype antigen and was serum resistant. Thus, all three phenotypes could be transferred by transformation. Furthermore, it was possible to construct transformants that made combinations of donor and recipient LOS, or even new LOS molecules whose M_rs were different from those of either the donor or the recipient.

The genes that encode MAb 2-1-L8 reactivity transformed as a single marker, indicating linkage on the gonococcal chromosome. They did not cotransform with either of the tested antibiotic markers, as indicated by genetic linkage studies, the presence of sectored colonies after selection for antibiotic resistance, and the similarity of the LOSs made by strains that were transformed for different antibiotic resistance markers. The 1-order of magnitude increase obtained for the transformation of MAb 2-1-L8 reactivity, as compared with those observed for the antibiotic resistance genes, most probably occurs because transformation for antibiotic resistance is a selective process by which only cells that are fully expressing the marker will grow, whereas the immunological technique is a screening process that will detect any positive event. That the loci involved in LOS biosynthesis are unlinked to either the rifampin or the nalidixic acid resistance locus is further supported by the fact that we were able to obtain sectored colonies when we cotransformed MAb 2-1-L8 reactivity with the antibiotic resistance markers.

Although we used transformation to construct strains that produced both donor and recipient LOS components, strains

that reacted strongly with MAb 2-1-L8 by colony blot made only donor LOS and none of the parental LOS component (Fig. 2). Immunoblot analysis of SDS-PAGE gels revealed that only one LOS component, of M_r 3,600, reacted with MAb 2-1-L8 and that expression of this epitope is restricted to LOS of this M_r, regardless of whether this LOS is made by donor or transformed strains. The fact that only one immunoreactive component was observed in all the strains, even when multiple LOS bands were present, suggests that the gonococcus has a complex mechanism for controlling biosynthesis of the various components. Since some of the transformants were able to produce a 3.6-kDa LOS band in addition to several other LOSs not made by the donor strain, the enzymes responsible for producing a specific LOS must be compartmentalized in some manner to allow for the assembly of individual components. The production of new LOS molecules by transformed cells demonstrates that new genetic information has been introduced into the recipients.

Our data demonstrate that the genes involved in LOS biosynthesis are linked. Furthermore, they suggest that the gonococcus possesses an intricate control mechanism that allows it to synthesize a variety of LOS components. The mechanisms by which the transformed cells suppress synthesis of parental LOS, package the genetic elements that encode the enzymes necessary to produce a certain and complete LOS, and reorganize genetic elements to produce a new LOS remain completely unknown.

Although our studies were not designed to address the molecular basis of serum resistance, their results provide useful insights. Sensitivity to serum killing must represent a balance between expression of LOSs that promote serum lysis and those that suppress it. Expression of the 3.6-kDa LOS, which we previously correlated with ser^r, does not by itself ensure resistance. We are unable to judge whether it is the L8 epitope on the 3.6-kDa LOS that is associated with ser^r.

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