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# *Neisseria gonorrhoeae* Cell Envelope: Permeability to Hydrophobic Molecules

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Isogenic variants of antibiotic-resistant and -sensitive *Neisseria gonorrhoeae* were examined for differences in the inhibition of oxygen uptake by steroid hormones. Mutants designated as *env*, which possessed cell envelope mutations allowing phenotypic suppression of low-level antibiotic resistance, were more sensitive to steroid hormone inhibition of oxygen uptake than the wild-type parental strains. Possession of an *mtr* locus, which confers nonspecific resistance to multiple antibiotics, dyes, and detergents, was also associated with an increase in resistance to steroid hormone inhibition of oxygen uptake. The *penA2* locus, which confers an eightfold increase in resistance to penicillin, was not responsible for the increased resistance to steroid hormones. Phospholipids in the outer membrane of intact *env-2* cells were susceptible to digestion by phospholipase C, indicating exposure of phospholipid head groups on the outer surface. Cells of a wild-type and *mtr-2* strain were not susceptible to phospholipase C digestion unless they were pretreated with mixed exoglycosidases. This pretreatment also increased the sensitivity of *mtr-2* cells to progesterone inhibition of $O_2$ uptake. These data suggest that the permeability of the gonococcus to hydrophobic antibiotic and steroid molecules is mediated by the degree of phospholipid exposure on the outer membrane.

The genetics of low-level antibiotic resistance and hypersensitivity in *Neisseria gonorrhoeae* has been well described (12, 20, 21, 23). Loci designated as *penA*, *mtr*, and *penB* contribute additively to penicillin resistance, whereas *env* mutations suppress the phenotypic properties of *mtr* and *penB*. Mutations at *mtr* and *penB* confer nonspecific resistance to multiple antibiotics; *mtr* also confers resistance to hydrophobic dyes and detergents. Recently, the cell envelopes of antibiotic-resistant and -sensitive isogenic strains were analyzed to determine the biochemical changes which accompanied these mutations (7). Acquisition of *mtr-2* was accompanied by a sevenfold increase in the quantity of a 52,000-molecular-weight outer membrane protein and an increase in the extent of peptidoglycan cross-linking. This latter change was also found to accompany the introduction of *penA2*. The introduction of *penB2* by genetic transformation into a wild-type strain resulted in an increase in the molecular weight of the principal outer membrane protein due to the cotransformation of *nmp-1* (3).

We have previously described the effects of steroid hormones on several strains of *N. gonorrhoeae*, which, unlike other gram-negative bacteria, were inhibited by steroids (11, 14, 16). The degree of inhibition was dependent upon the molecular configuration of the steroid, as well as growth medium composition, pH, and the carbon and energy source (11). Steroid hormones, detergents, and many dyes and antibiotics are hydrophobic molecules which can penetrate membranes by dissolving into the phospholipid bilayer (18). Gram-negative bacteria possess both an inner (cytoplasmic) and outer membrane (19). On the basis of several lines of evidence, Nikaido and Nakae (19) have suggested that the outer membrane in the *Enterobacteriaceae* is an asymmetric structure that serves as a barrier to hydrophobic molecules. The lipid portion of lipopolysaccharide is found only on the outer surface of the outer membrane, whereas phospholipids are found only on the inner surface. Kamio and Nikaido (8) found that deep-rough mutants of *Salmonella typhimurium* are much more permeable to hydrophobic molecules and have outer membranes containing phospholipid bilayer regions. Therefore, we decided to examine antibiotic-sensitive and -resistant strains of *N. gonorrhoeae* to determine whether known alterations in the cell envelope would affect the degree of steroid inhibition or the presence and exposure of phospholipids on the cell surface.

## MATERIALS AND METHODS

**Organisms and growth conditions.** The isogenic strains of *N. gonorrhoeae* used in these experiments were kindly provided by P. F. Sparling (University of North Carolina, Chapel Hill) and are listed in Table 1. The origin, genotype, and sensitivities of these strains to antibiotics, dyes, and detergents have previously been described (7, 21, 23). Cells of colony type 4, which were nonpiliated and transparent ($P^-$ Tr), were used in all experiments. Bacteria for the steroid inhibition studies were grown in a complex medium (pH 7.2) containing glucose, as previously described (11).

**Steroid inhibition of oxygen uptake.** Oxygen uptake of gonococci from exponential-phase cultures was monitored with an oxygen electrode as previously described (11). Steroids were added to a final concentration of 40 μg per $5 \times 10^7$ cells. Inhibition was reported as the percentage of the original rate of $O_2$ uptake 10 min after the addition of steroids.

**Chemicals and radioisotopes.** Phospholipase C from *Bacillus cereus* was obtained from Boehringer Mannheim Corp., San Francisco, Calif. Alkaline phosphatase, type VII, L-α-phosphatidyl choline, type III-E, L-α-phosphatidyl-DL-glycerol, grade 1, and L-α-phosphatidyl ethanolamine, type III, were obtained from Sigma Chemical Co., St. Louis, Mo. Mixed exoglycosidases from *Turbo cornutus* were obtained from Miles Laboratories, Elkhart, Ind. and contained α- and β-mannosidase, α- and β-glucosidase, α- and β-galactosidase, α-L-fucosidase, β-xylosidase, α- and β-*N*-acetylglucosaminidase, and α- and β-*N*-acetylgalactosaminidase. Dipotassium [$^{32}$P]phosphate (specific activity 500 mCi/mmol) was obtained from New England Nuclear Corp., Boston, Mass.

**Preparation and assay of phospholipase C.** Phospholipase C (EC 3.1.4.3) was partially purified by gel filtration through a column (1.6 by 82 cm) of Sephadex G-75 in 50 mM Tris-hydrochloride buffer (pH 7.6) containing 5 mM $CaCl_2$ and 50% glycerol (24). Two milligrams of enzyme (obtained as a suspension in 3.2 M $(NH_4)_2SO_4$ solution) were dialyzed overnight in the cold against 600 volumes of buffer, and again for 1 h against 200 volumes, before being applied to the column and eluted at a flow rate of 7 ml/h. Fractions (2 ml) were assayed for phospholipase C activity by the method of Krug et al. (10), in which alkaline phosphatase cleaves inorganic phosphate from phosphoryl choline. The reaction mixture contained (micromoles): Tris-hydrochloride (pH 7.3), 4.6; $CaCl_2$, 0.36; $MgCl_2$, 1.01; and $(NH_4)_2SO_4$, 0.36; together with 35% (vol/vol) glycerol, 0.04% (vol/vol) Triton X-100, 0.3 U of alkaline phosphatase, and phospholipase C. Phosphatidyl choline (0.2 μmol) was added to bring the final volume to 100 μl. The substrate solution was prepared by evaporating the carrier solvent under a stream of $N_2$ and suspending the residue, with sonication, into a sample of a solubilizing solution composed of 10 ml of 0.1 M Tris-hydrochloride buffer (pH 7.5); 0.8 ml of a 5% (vol/vol) Triton X-100 solution; and 0.1 ml of a 0.1 M $CaCl_2$ solution (5). Samples were incubated at 37°C, and inorganic phosphate was determined as described (10) by comparison with a standard curve. Protein concentration was measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.), using bovine gamma globulin as standard. The specific activity of the final pooled fractions was 3.65 μmol of inorganic phosphate liberated per min per mg of protein at 37°C with phosphatidyl choline as substrate. The enzyme was equally active with the major gonococcal phospholipids, phosphatidyl ethanolamine and phosphatidyl glycerol (15).

**Phospholipase C treatment of $^{32}$P-labeled gonococci.** A modification of the procedure of Kamio and Nikaido (8) was used to label cells. Gonococci were grown in complex liquid medium (pH 7.2) (14) containing one-tenth (3.0 mM) the normal amount of phosphate and buffered with 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). Liquid cultures were inoculated with a cell suspension obtained from growth on solid agar medium incubated overnight at 37°C with 4% $CO_2$ and were incubated at 37°C with shaking. Turbidity was measured at 540 nm with a Klett-Summerson colorimeter. When cultures began growing exponentially, ca. 0.25 mCi of dipotassium [$^{32}$P]phosphate was added to each of two separate 300-ml Klett flasks containing 10 ml of culture. After 1.5 generations, cultures were centrifuged at $3,400 \times g$ for 10 min, and the cell pellets were combined and suspended in 34 ml of fresh medium containing 30 mM phosphate. The label was chased by incubation for at least 30 min, until cells reached a Klett reading of 100. Cultures were then centrifuged, and the cell pellet was washed once in 10 mM HEPES buffer (pH 7.2) containing 10 mM $MgCl_2$. Cells were recentrifuged and suspended in prewarmed (37°C) HEPES buffer containing $Mg^{2+}$ by blending in a Vortex mixer in the presence of three 3-mm-diameter glass beads. Prewarmed phospholipase C (18 μg of protein per 3 mg of cell dry weight) or an equal volume of column buffer was added to a final volume of 1.5 ml. Cell suspensions were incubated at 37°C with shaking. Samples (200 μl) were removed at 0, 2, 5, 10, and 15 min into an equal volume of ice-cold 10% trichloroacetic acid and incubated for 30 min at 0°C. After centrifugation, the trichloroacetic acid-precipitable pellet was extracted with 1.5 ml of 70% ethanol for 30 min at 45°C, and the

TABLE 1. *Strains of N. gonorrhoeae used*[a]

| Strain | Genotype | Origin |
|---|---|---|
| FA18 | Wild-type | A. Reyn |
| FA32 | *env-3 str-10 tet-3 chl-3 mtr-3 penA3 penB3* | UV mutant of FA18 |
| FA5 | *env*$^+$ *str-1 tet-1 chl-1 mtr-1 penA1 penB1* | D. Kellogg |
| FA52 | Same as FA5, but *env-1* | UV mutant of FA5 |
| FA19 | Wild-type | A. Reyn |
| FA47 | *env-2 str-7 penA2* | UV mutant of FA19 |
| FA48 | Same as FA47, but *env*$^+$ *tet-2 chl-2 mtr-2 penB2* | UV mutant of FA47 |
| FA102 | *penA2* | FA48 × FA19 |
| FA136 | *penA2 mtr-2* | FA48 × FA102 |
| FA140 | *penA2 mtr-2 penB2* | FA48 × FA136 |
| FA171 | *mtr-2* | FA48 × FA19 |
| BR87 | Same as FA140, but *str-7 env-2* | FA47 × FA140 |

[a] From references 7 and 21.

radioactivity in the ethanol extract was determined as previously described (17).

**Determination of outer membrane phospholipid-to-protein ratios.** Outer membranes from mid-exponential-phase cells grown in the medium used for $^{32}P$ labeling were prepared by treatment with sodium *N*-lauroylsarcosine (Sigma) as previously described (7), except that cells were broken in a Braun homogenizer (Bronwill Scientific, Inc., Rochester, N.Y.).

Protein concentrations were measured by the Bio-Rad protein assay. Lipids were extracted by a modification (Howard Jenkin, personal communication) of the method of Bligh and Dyer (2), and portions of the extract were ashed and analyzed for total phosphate (1).

## RESULTS

**Steroid inhibition of $O_2$ uptake.** The effects of steroid hormones on $O_2$ uptake by isogenic strains of *N. gonorrhoeae* are shown in Table 2. The wild-type strains FA18 and FA19 were sensitive to all steroids tested. The *env-3* mutant FA32 was more sensitive to β-estradiol diacetate than was the parental strain FA18. The *env-2* mutant FA47 was more sensitive to β-estradiol diacetate and progesterone than was the parental strain FA19. The *env-1* mutant FA52 was as sensitive to β-estradiol diacetate as were the other *env* mutants and was more sensitive to β-estradiol diacetate and progesterone than was its parental strain FA5. Strain FA5, which possesses *mtr-1*, *penA1*, and *penB1* loci, was much more resistant to β-estradiol diacetate and progesterone than were the wild-type strains and *env* mutants.

To assess the role of the *mtr*, *penA*, and *penB* loci in gonococcal resistance to steroid inhibition of $O_2$ uptake, we examined several isogenic variants of strain FA19 possessing various combinations of the *mtr*, *penA*, and *penB* loci. Strain FA102 (*penA2*) was not resistant, but was about as sensitive to steroids as the wild-type parental strain FA19 (Table 2). Strain FA136 (*penA2 mtr-2*) was highly resistant to steroid hormone inhibition of $O_2$ uptake. Strains FA48 (*mtr-2 penB2*) and FA140 (*penA2 mtr-2 penB2*) were also highly resistant to steroid inhibition of $O_2$ uptake. However, strain FA140 was not as resistant to progesterone as were the other variants which possessed loci conferring antibiotic resistance. Strain FA171 (*mtr-2*) was also resistant to steroid inhibition of $O_2$ uptake. The recombinant strain BR87, which is a cross between an *env* and an *mtr* strain, is phenotypically $Env^-$ and was as sensitive to steroids as the other *env* mutants.

TABLE 2. *Effects of steroid hormones on isogenic variants of N. gonorrhoeae*

| Strain | $O_2$ uptake[a] (% of original) with: | | |
|---|---|---|---|
| | β-Estradiol diacetate | Progesterone | Testosterone acetate |
| FA18 | 5–61[b] | 33 | 19 |
| FA32 | 2–16 | 28 | 25 |
| FA5 | 73–106 | 89 | 29 |
| FA52 | 3–17 | 29 | 23 |
| FA19 | 16–64 | 40 | 18 |
| FA47 | 4–29 | 24 | 11 |
| FA48 | 78–111 | 94 | 58 |
| FA102 | 9–61 | 49 | 29 |
| FA136 | 70–108 | 88 | 72 |
| FA140 | 63–96 | 75 | 42 |
| FA171 | 66–103 | 80 | 70 |
| BR87 | 5–23 | 37 | 18 |

[a] Results represent the average of at least two determinations and are expressed as the percentage of the linear rate of $O_2$ uptake 10 min after the addition of 40 μg of steroid per $5 \times 10^7$ cells.

[b] Inhibition by β-estradiol diacetate was biphasic.

All the *mtr*-containing strains derived from the parental wild-type strain FA19 exhibited increased resistance to steroids. Resistance to steroid inhibition of $O_2$ uptake, conferred by *mtr*, was probably not due to differences in cytoplasmic membrane enzyme systems. The oxidation of L-lactate by membrane preparations from both the wild-type FA19 and the *mtr-2* mutant FA171 was equivalent and was similarly inhibited by progesterone (data not shown). Therefore, it was likely that differences in steroid susceptibility were due to permeability changes in the outer membrane.

**Treatment of intact cells with phospholipase C.** The susceptibility of deep-rough mutants of *S. typhimurium* to hydrophobic molecules is associated with the presence of phospholipids in the outer layer of the outer membrane (8). To determine whether phospholipid head group exposure on the outer surface of the gonococcal outer membrane was correlated with steroid inhibition, we exposed intact cells of three isogenic variants to phospholipase C and observed the release of $^{32}P$-labeled material. Phospholipase C-treated strain BR87 (*env-2*) cells lost ethanol-extractable, $^{32}P$-labeled material with time (Fig. 1), suggesting that phospholipids were exposed on the outer surface of the outer membrane. The amount of ethanol-extractable, $^{32}P$-labeled material from cells of strains FA171 (*mtr-2*) and FA19 (wild type) increased with time, both with and without phospholipase C treatment (Fig. 1). This increase suggested a sizable intracellular pool of $^{32}P$, as well as de novo phospholipid synthesis. Preincubation of these cells at 37°C for 30 min was sufficient to deplete the pool of $^{32}P$-labeled phospholipid precursors. However, even after this

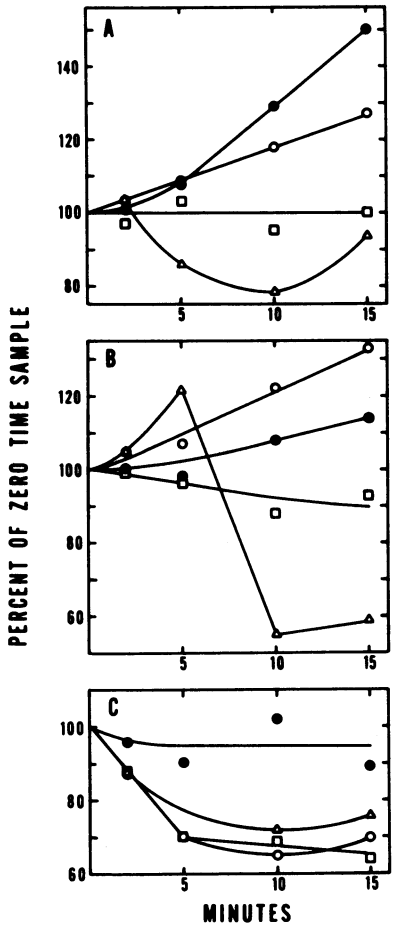


FIG. 1. *Digestion of phospholipids by treatment of intact gonococci with phospholipase C. (A) Strain FA171 (mtr-2); (B) strain FA19 (wild type); (C) strain BR87 (env-2). $^{32}$P-labeled cells (3 mg dry wt) were suspended in HEPES buffer containing $Mg^{2+}$ at 37°C (see the text). In some experiments, cell suspensions were preincubated with mixed exoglycosidases (10 mg/ml) for 30 min before phospholipase C was added. Either phospholipase C buffer or 18 μg of phospholipase C was added to a final volume of 1.5 ml. Cell suspensions were incubated at 37°C, and samples were added to an equal volume of cold 10% trichloroacetic acid at 0, 2, 5, 10, and 15 min, mixed, and allowed to stand for 30 min. After centrifugation, the pellet was extracted with 70% ethanol for 30 min at 45°C, and the radioactivity in the ethanol extract was determined. Symbols: ●, buffer control; ○, phospholipase C; □, preincubated for 30 min at 37°C before phospholipase C addition; △, preincubated with mixed exoglycosidases for 30 min at 37°C before phospholipase C addition.*

preincubation, there was still no degradation of phospholipids by phospholipase C. When cells of strain BR87 were similarly preincubated (Fig. 1), no additional increase in the release of $^{32}$P-labeled material was noted.

To insure that autolysis did not expose cytoplasmic membrane phospholipids to phospholipase C, magnesium was added for cell stability. Little or no phospholipase C degradation of the phospholipids of strains FA171 and FA19 occurred in buffer containing 10 mM $Mg^{2+}$, even after preincubation for 30 min (Fig. 1). However, when cells of strain FA19 were suspended in buffer without $Mg^{2+}$, 38% of the phospholipids were hydrolyzed within 15 min of phospholipase C addition (data not shown). Furthermore, additional studies have shown that the presence of $Mg^{2+}$ prevented the release of intracellular adenine-labeled material (pool and nucleic acid) and stabilized the outer membrane as indicated by marked inhibition of the release of the periplasmic enzyme, beta-lactamase (manuscript in preparation).

**Effect of treatment with mixed exoglycosidases.** Preincubation of cell suspensions of strains FA171 and FA19 with mixed exoglycosidases rendered them sensitive to the action of phospholipase C (Fig. 1), suggesting that glycosylated components in the outer membrane were masking the phospholipid head groups. No additional release of phospholipids was seen when cells of strain BR87 were preincubated under the same conditions. Mixed exoglycosidases (10 mg/ml) had no detectable phospholipase C activity against phosphatidyl choline, nor was protease activity detected against the general protease substrate Hide Powder Azure (Calbiochem-Behring Corp., La Jolla, Calif.). The addition of mixed exoglycosidases to growing cells of strain FA171 rendered them sensitive to progesterone inhibition of $O_2$ uptake (Table 3). Respiration was not affected by mixed exoglycosidases alone. The sensitivity to progesterone increased with the amount and length of preincubation with enzymes.

Throughout these studies, replicate experiments exhibited similar increases or decreases in labeled phospholipids. Although the magnitude of the response varied with each experiment, the trend of the responses remained the same. Phospholipids from strains FA171 and FA19 were never degraded by phospholipase C unless cells were pretreated with mixed exoglycosidases, whereas phospholipids from strain BR87 were always degraded. The data in Fig. 1 were obtained from single batches of cells simultaneously treated under four different conditions. Therefore, the relationships between the conditions were internally controlled and valid. Slight variations in the resuspension of cells or in the length of incubation of cells before the addition

TABLE 3. *Effect of mixed exoglycosidase treatment on progesterone sensitivity of N. gonorrhoeae strain FA171 (mtr-2)*

| Treatment with mixed exoglycosidases | Rate of $O_2$ consumption (μl/min) | |
|---|---|---|
| | Before progesterone[a] | After progesterone[b] |
| None | 1.22 | 0.99 |
| 25 μg/ml (no preincubation) | 1.32 | 1.07 |
| 50 μg/ml for 20 min | 1.20 | 0.90 |
| 100 μg/ml for 30 min | 1.35 | 0.84 |

[a] Gonococci were grown and diluted in a complex liquid medium to ca. $5 \times 10^7$ cells/ml, and 4 ml was added to the cuvette chambers of an oxygen monitor. Cells were treated with mixed exoglycosidases as indicated, and $O_2$ uptake was measured.

[b] Progesterone was added to a final concentration of 40 μg per $5 \times 10^7$ cells. Values are the linear rate 10 min after addition.

of phospholipase C may have contributed to the differences seen between replicate experiments. An important factor in obtaining reproducible results was the degree of aeration of growing cells. Cells of strains FA171 and FA19 grown in 20 instead of 10 ml of medium did not exhibit the continued incorporation of $^{32}P$ into phospholipids after resuspension. These cells were also less sensitive to phospholipase C degradation of outer membrane phospholipids after pretreatment with mixed exoglycosidases. These differences are currently being investigated.

**Determination of outer membrane phospholipid-to-protein ratios.** Outer membrane preparations were analyzed to determine the relative amounts of lipid phosphorus and protein among strains FA171, FA19, and BR87. The results in Table 4 show that outer membranes from the *env* strain BR87 possessed a significantly greater phospholipid-to-protein ratio than did either the wild-type or *mtr* strain.

TABLE 4. *Phospholipid-to-protein ratios in outer membranes of N. gonorrhoeae*

| Strain | μg of phospholipid per mg of protein |
|---|---|
| FA19 (wild-type) | 133 |
| FA171 (*mtr*) | 146 |
| BR87 (*env*) | 190 |

## DISCUSSION

The acquisition of *env* loci, which results in an increased sensitivity to various hydrophobic molecules (21), also results in an increased sensitivity to steroid hormones. The presence of the *penA2* locus, which specifically increases penicillin resistance (23), does not confer resistance to steroid inhibition of $O_2$ uptake. The acquisition of *mtr* loci has been found to result in a two- to fourfold increase in penicillin resistance, and even higher increases in resistance to other antibiotics, dyes, and detergents (23). The results of the present study indicate that the acquisition of *mtr-2* alone is sufficient for resistance to steroid inhibition of $O_2$ uptake. The role of *penB* could not be directly evaluated, since the presence of *mtr* is required for its expression (23).

The common denominator among all the steroid-resistant strains tested in this study was the possession of *mtr* loci. Therefore, membrane alterations due to the expression of *mtr* may be responsible for increased resistance to steroids. Acquisition of *mtr-2* has been shown to be accompanied by a sevenfold increase in the amount of a 52,000-molecular-weight outer membrane protein and an increase in the extent of peptidoglycan cross-linking (7). The role which the 52,000-molecular-weight outer membrane protein may play in decreasing gonococcal envelope permeability has been previously discussed with regard to antibiotic sensitivity (7). Results from our laboratory have shown that the presence of lipophilic groups on steroid molecules increases their ability to inhibit gonococcal $O_2$ uptake (11). These, as well as other observations (14), suggest that penetration of the outer membrane is of major importance for steroid inhibition of cytoplasmic membrane functions. Our results on resistance to steroid inhibition support the hypothesis that decreased envelope permeability may be a consequence of *mtr* acquisition (6, 7). Erythromycin, rifampin, fusidic acid, crystal violet, acridine orange, deoxycholate, and Triton X-100, which have been used to challenge these strains (6, 7, 23), are, like steroid hormones, hydrophobic molecules which are thought to penetrate membranes by dissolving into the phospholipid bilayer (18). Possession of *mtr* has been found generally to confer a greater magnitude of resistance to these substances than it does to penicillin, tetracycline, or chloramphenicol (23), which apparently utilize the hydrophilic pathway and diffuse through aqueous membrane pores (18). Therefore, it seems that *mtr*-mediated resistance is primarily to hydrophobic molecules.

An important difference separating the *env-2* strain from the wild-type and *mtr-2* strains is the degree to which phospholipid head groups are exposed in the outer membrane. Phospholipids of the *env-2* mutant BR87 were degraded by phospholipase C and therefore appeared to be exposed, whereas those of the wild-type FA19

and the *mtr-2* mutant FA171 were resistant to phospholipase C and appeared to be arranged differently. The difference between strains is not due to differences in autolytic capacity, since both BR87 and FA19 autolyzed to a similar extent when suspended in HEPES buffer (data not shown) but did not autolyze in the presence of $Mg^{2+}$. The apparent difference is that the wild-type and *mtr-2* variant possess glycosylated substances, which mask the phospholipid head groups and which can be removed by treatment with mixed exoglycosidases. Therefore, the loss of a glycosylated component of the outer membrane may be a specific consequence of *env-2* acquisition. The nature of this component is presently under investigation. The differences seen after treatment with mixed exoglycosidases are also not due to cell lysis, since phospholipid degradation of strain BR87 was the same, with or without pretreatment. Mixed exoglycosidase treatment increased the sensitivity of strain FA171 to progesterone inhibition without affecting the initial rate of $O_2$ uptake, which would have been expected had cells lysed. Furthermore, 98% of adenine-labeled material remained cell associated when cells of strain FA19 were incubated with mixed exoglycosidases for 30 min under experimental conditions (data not shown).

The wild-type strain FA19 and the *mtr-2* strain FA171 both possessed glycosylated substances and were resistant to phospholipase C. However, the wild-type strain was more sensitive to steroids. Phospholipids of both strains may be inaccessible to phospholipase C due to masking, but the wild-type phospholipids may be more accessible to the much smaller steroid molecules due to differences in the arrangement or the amount of the glycosylated component. Alternatively, the presence of increased amounts of the 52,000-molecular-weight outer membrane protein (conferred by *mtr-2*) may alter the outer membrane so that the lipid phase is less accessible to hydrophobic molecules. Hydrophilic molecules, such as benzylpenicillin, may be unable to penetrate the outer envelope due to other alterations caused by the 52,000-molecular-weight protein, or they may be unable to penetrate the more heavily cross-linked peptidoglycan, which is also conferred by *mtr* (7).

In the *Enterobacteriaceae*, deep-rough mutants produce defective lipopolysaccharide and subsequently fail to incorporate many outer membrane proteins (19). This causes a compensatory increase in the phospholipid content of the outer membrane, resulting in the formation of bilayer regions which render the cell sensitive to penetration by hydrophobic molecules. Such may not be the case for these isogenic strains of *N. gonorrhoeae*, which have a similar lipopolysaccharide composition (7). However, differences in the amount of lipopolysaccharide per cell have not yet been ruled out.

No significant differences in either the total extractable lipid phosphorus or the qualitative phospholipid composition have been noted among these gonococcal strains (7), suggesting that the difference in susceptibility to phospholipase C was not due to increased synthesis of outer membrane phospholipids as was that of deep-rough mutants of *S. typhimurium* (19, 22). However, we found the outer membrane of the *env* strain BR87 to have a higher phospholipid-to-protein ratio than either the wild-type FA19 or *mtr* strain FA171. Presumably, the difference was not due to lesser amounts of protein in the outer membranes of strain BR87, since polyacrylamide gel electrophoresis patterns appear to be quantitatively similar to those of FA19 (7). It is also unlikely that the difference is due to coextraction of phosphate in lipopolysaccharide (9, 22) from strain BR87, since the phosphate content of the lipopolysaccharide from these gonococcal strains is equivalent (7). The increase in the phospholipid-to-protein ratio may be due to increased amounts of phospholipid in the outer membrane of strain BR87. However, the percentage increase over the wild-type and *mtr* strain is far less than that observed for deep-rough mutants of *Escherichia coli* and *S. typhimurium* (9, 22), which reveal phospholipid-to-protein ratios two- to threefold greater than their wild-type parents. Therefore, the increased sensitivity of gonococcal strain BR87 to hydrophobic molecules seems not to be due to gross increases in the amount of outer membrane phospholipid, as is the case for *E. coli* and *S. typhimurium*. A more comprehensive analysis of gonococcal outer membrane components now seems to be in order.

It might be argued that treatment of gonococci with mixed exoglycosidases produces an altered lipopolysaccharide which cannot interact properly with outer membrane components. A release of proteins and compensatory increase in phospholipids, previously described for the *Enterobacteriaceae* (9, 19, 22), could account for our observations of increased degradation of phospholipids and cannot be ruled out at this time. However, this possibility is unlikely. Although the gonococcal strains tested have a similar lipopolysaccharide composition, mixed exoglycosidase treatment caused no additional degradation of strain BR87 phospholipids by phospholipase C, suggesting that lipopolysaccharide was not the primary substrate.

Wild-type enterics possessing outer mem-

branes of low permeability to hydrophobic molecules may have a selective advantage when exposed to intestinal bile salts and long-chain fatty acids. However, a mucosal surface pathogen like the gonococcus, which can exist at a number of sites within its human host, may be more flexible with respect to the structure of the outer membrane. Approximately 15% of gonococcal clinical isolates from North Carolina contained *env* mutations (4). Most of these isolates also possessed phenotypically suppressed *mtr* mutations. The occurrence of these outer envelope mutants suggests that, under certain conditions, there may be a selective advantage for strains possessing this phenotype.

In summary, our results suggest that the gonococcal outer membrane contains phospholipid bilayer regions capable of interacting with hydrophobic molecules such as steroids (11), fatty acids (13), and antibiotics (23). Masking of the phospholipid bilayer may result in resistance to these hydrophobic molecules. The degree of exposure of phospholipid head groups may be controlled by the amount of an outer membrane protein or by the presence of glycosylated components, which serve to regulate outer membrane permeability by hydrophobic molecules.

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