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Cytoplasmic Membrane Proteins of Spectinomycin-Susceptible and -Resistant Strains of *Neisseria gonorrhoeae*

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Cytoplasmic membranes were isolated and examined from two spectinomycin-susceptible and three spectinomycin-resistant clinical strains of *Neisseria gonorrhoeae*. A laboratory-derived spectinomycin-resistant mutant, obtained by serial passage on gradually increasing concentrations of the antibiotic, and a susceptible revertant, spontaneously arising from one of the resistant clinical strains, were also studied. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis revealed that a major protein, comprising about 7% of total cytoplasmic membrane protein (molecular weight 24,000), was absent in the three clinically isolated spectinomycin-resistant strains. In a revertant, this protein reappeared. During treatment of one of the susceptible strains with spectinomycin, the protein disappeared. However, this correlation was not maintained in the laboratory-derived spectinomycin-resistant mutant. This mutant was of comparable resistance to the clinical isolates, but the 24,000-molecular-weight protein was present in normal quantities. In addition, spectinomycin resistance in clinical isolates was variable compared with stable resistance exhibited by the laboratory-derived mutant. These findings suggested that differences in laboratory-derived versus clinical spectinomycin resistance may be due to different types of resistance mutations.

The biochemical composition of the gonococcal outer cell membrane has been the subject of much investigation. Identification of antigens for vaccine production has been a major stimulus. Much interest has also focused on those components of the outer membrane that affect host-microorganism interaction (pili) and resistance to antibiotics (23). By contrast, relatively little is known about the cytoplasmic membrane.

There is evidence to suggest that the components of the cytoplasmic membrane may also be important in antibiotic resistance and as possible antigen sources for vaccine production. Potentially antigenic components of membrane may be masked in the intact organism (24). Autolysins are enzymes that lead to degradation of peptidoglycan, releasing antigens into the surrounding environment as well as exposing others present on the cytoplasmic membrane. L-forms and wall-defective variants can arise (i) due to the highly autolytic nature of the gonococcus, (ii) from lysozyme found in leukocytes, and (iii) by antibiotic induction during the course of therapy (19). These examples illustrate important in vivo exposure of cytoplasmic membrane constituents in *Neisseria gonorrhoeae*.

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This investigation was undertaken to characterize and compare the cytoplasmic membrane of spectinomycin-susceptible and -resistant strains of *N. gonorrhoeae*. Membranes were analyzed by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (SDS-PAGE).

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MATERIALS AND METHODS

Bacteria and media. The spectinomycin-susceptible (Spc^s) strain F-29 was supplied by Clyde Thornsberry, Center for Disease Control, Atlanta, Ga., and strain 8-PHD was recovered from a clinic patient at the Peoria Health Department, Peoria, Ill. Three post-treatment strains resistant to spectinomycin (Spc^r) were examined. Strain 736430 was recovered in Georgia (November 1976) and reported on by Thornsberry et al. (27). Strain 18676 was recovered by A. Reyn in Denmark (1973) from a two-time spectinomycin therapy failure (21). The third strain, 1428-PHD, was recovered from another patient seen at the Peoria Health Department (August 1977). This patient had a history of three successive spectinomycin therapy failures.

A laboratory-derived spectinomycin-resistant mutant from parent strain F-29, designated $F\text{-}29^r$, was

obtained by serial passage on media containing increasing levels of spectinomycin. Growth from a 16-h plate containing GC agar base with 1% supplement C and 1% hemoglobin (GC-C, Difco Laboratories, Detroit, Mich.) was removed with a sterile cotton swab and uniformly spread onto the surface of a GC-C plate containing 4 μg of spectinomycin per ml. After 24 to 48 h of incubation, all discrete colonies were removed, suspended into 0.5 ml of tryptic soy broth (TSB; Difco), and blended in a Vortex mixer. The suspension was uniformly spread onto a GC-C plate containing 6 μg of spectinomycin per ml. This procedure was repeated with media containing progressively higher levels of spectinomycin, at 2-μg/ml increments, until growth was achieved at a concentration of 50 μg/ml. Subsequent serial passage was carried out at 50-μg/ml increments until a level of 500 μg of spectinomycin per ml was achieved.

A spontaneously occurring Spc^s mutant, from parent strain 736430, was obtained by replica plating (13) and designated 736430^s. A 16-h culture grown on GC-C was removed with a sterile cotton swab and suspended in TSB to a density of 40 to 50% light transmission (wave length, 530 nm; 10^7 organisms/ml). Appropriate dilutions were made in TSB, and 0.1 ml was uniformly spread onto GC-C plates with incubation for 24 to 48 h at 36°C in a 6% CO_2 incubator. The isolated colonies on master plates (≤300) were replica plated onto GC-C plates containing 20 μg of spectinomycin per ml. Colonies not growing on antibiotic-containing medium after 48 h of incubation were picked from the master plate and reidentified as *N. gonorrhoeae*; then susceptibility was determined. Those organisms with susceptibility to <20 μg of spectinomycin per ml were considered susceptible (30).

All strains of colony type 4 were subcultured for 14 to 16 h at 35°C in a 6% CO_2 incubator on GC-C. For membrane preparation, approximately 5×10^{10} bacteria were suspended in a 500-ml flask containing 250 ml of TSB with a final concentration of 10 mM $MgSO_4$ and 10 mM $NaHCO_3$. The culture was gently agitated with a 50-mm stirring bar and incubated at 35°C for 18 h.

Chemicals and equipment. Lysozyme, pancreatic RNase, and pancreatic DNase were obtained from Sigma Chemical Co., St. Louis, Mo. Other materials used in gel electrophoresis (SDS, acrylamide, etc.) were obtained from Bio-Rad Laboratories, Richmond, Calif. Molecular weights were estimated by using phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme as standards. The Bio-Rad slab gel electrophoresis unit with model 500 power supply was used. Records of gel electrophoresis were made with a Photovolt thin-layer chromatography densitometer with a slit width of 0.1 mm. Proteins were quantitated by determining total area of each band from gel scans.

Preparation of membranes for gel electrophoresis. With only a few modifications, the procedure of Johnston and Gotschlich (10) was followed. Cells were harvested by centrifugation at 10,000 × *g* for 15 min at 0°C and washed once in cold 10 mM Tris-hydrochloride (Tris) buffer, pH 7.8. The average wet packed cell weight per 250 ml of culture was 1.5 g after 16 h of incubation. The cells were resuspended in cold 0.6 M sucrose in 10 mM Tris buffer, pH 7.8, to which was immediately added lysozyme to a final concentration of 100 μg/ml (dissolved in 100 mM Tris, pH 7.8) and 2 mM EDTA in 10 mM Tris, pH 7.4. After 1 h of incubation at 30°C with gentle agitation, the spheroplasts were lysed by adding four times the volume of cold 5 mM $MgCl_2$ in Tris, pH 7.0. Spheroplast formation was monitored by Gram staining, and wet mounts were observed by phase microscopy, and confirmed by thin-sectioned preparations examined by electron microscopy. DNase (1,400 U) and RNase (1,500 U) were added to the lysed suspension, which was incubated at 37°C for 30 min. The membranes were harvested at 23,000 × *g* for 20 min and washed with 5 mM $MgCl_2$ (in 10 mM Tris), followed by recentrifugation. The pellet containing membranes was suspended in 25 mM EDTA in 10 mM Tris-EDTA (pH 7.0) and dialyzed overnight against the same buffer. Sodium azide was added to the dialysis buffer to prevent microbial growth.

Isopycnic buoyant density gradient centrifugation. Sucrose density gradient centrifugation was carried out in a Beckman L5-40 ultracentrifuge, using an SW50.1 rotor. Step gradients were prepared by layering 1.5 ml each of 60, 40, and 20% (wt/wt) sucrose solutions. All sucrose solutions contained 5 mM EDTA (pH 7.0). A 0.5-ml sample of membrane was layered on the top of the gradient, and centrifugation was carried out at 32,000 rpm for 4 h at 0°C. Tube contents were fractionated by puncturing the bottom of the centrifuge tube and collecting drops, with the refractive index determined by use of a Bausch & Lomb ABBE-3L refractometer.

Electron microscopy. Membrane fractions were prepared for electron microscopy as previously described (9). Specimens were sectioned with a Reichert OMV3 microtome and examined on a JOEL model JEM 100 C electron microscope.

Enzyme measurements. Enzyme assays were carried out by the method described by Osborn et al. (20). Specific activities of succinic dehydrogenase (EC 1.3.99.1) and D-lactate dehydrogenase (EC 1.1.1.28) were expressed as micromoles of substrate converted per minute per milligram of protein.

Solubilization and gel electrophoresis of membrane proteins. SDS-PAGE was performed by the method of Laemmli (12). The cytoplasmic membrane fraction was dissolved in 2.3% SDS in 0.0625 M Tris (pH 6.8) containing 5% 2-mercaptoethanol. The final concentration was 10 to 25 mg of protein per ml, determined by the method of Lowry et al. (14). The suspension was heated to 100°C for 5 min or 37°C for 2 h. The sample was then diluted to 7 to 18 mg of protein per ml in 0.0625 M Tris (pH 6.8), and glycerol was added to a concentration of 15% (vol/vol) along with 0.001% bromophenol blue. A portion of 15 to 25 μg of protein was applied to each well of a slab gel (9 by 14 cm). A 1.0% SDS–10% acrylamide–0.27% methylenebisacrylamide gel was used throughout. The electrophoresis buffer used was 0.025 M Tris containing 0.192 M glycine and 0.1% SDS (final pH 8.4). Electrophoresis was run for 4 h at 30 mA/gel. The gels were fixed and stained overnight in 0.05% Coomassie blue–50% methyl alcohol–10% acetic acid and destained in a solution of 10% methyl alcohol–acetic acid.

Antibiotics and susceptibility test methods. Spectinomycin sulfate (Trobicin) was supplied as a sterile dry powder in 100-mg vials through the courtesy of R. T. Pfeifer, The Upjohn Co., Kalamazoo, Mich. Commercial preparations of penicillin G (Pfizer) and tetracycline hydrochloride (Pfizer Inc., New York) were used. The β-lactamase-resistant cephalosporin cefuroxime was a gift from C. H. O'Calloghan, Glaxo Laboratories Ltd., London, England.

Cultures for inoculum were grown for 16 h on chocolate agar with 1% supplement C and 1% hemoglobin. A bacterial suspension (10^7 organism/ml) was prepared in Mueller-Hinton broth with 1% supplement C (MHB-C) by agitation on a Vortex shaker. Twofold drug dilutions were made in MHB-C in 0.5-ml portions to which 0.05 ml of a bacterial suspension was added (5×10^5 organisms). The tubes were incubated in a 6% CO_2 environment, and results were read at 48 h. The first drug dilution in which no growth was visible was designated the minimal inhibitory concentration (MIC) (14). The tube contents were subcultured by plating 0.05 ml on a chocolate agar plate containing 1% supplement C. The drug dilution from which four or fewer colonies grew was recorded as the minimal bactericidal concentration (MBC).

An agar dilution method was also used in susceptibility testing with GC-C as the basal medium. Strains to be tested were grown for 16 h on chocolate agar with 1% supplement C and diluted in MHB-C. Plates containing appropriate concentrations of an antibiotic were spot inoculated with 5×10^5 organisms. The lowest concentration inhibiting all visible growth or allowing growth of no more than one colony constituted the MIC.

RESULTS

Antibiotic susceptibility profiles. The MICs recorded are shown in Table 1. The MBCs for all antibiotics, except spectinomycin, were either the same or twofold higher than the MIC. For those strains susceptible to spectinomycin, the MBC was twofold higher than the MIC. In spectinomycin-resistant strains, the MBC often could not be established. Our MIC findings obtained by the broth dilution method compared favorably with those found by the agar dilution method, and correlated within one dilution. It was observed that clinical spectinomycin-resistant strains did not give consistent MICs with either the broth or agar dilution method. For strain 736430, variations were noted, ranging from an MIC of 39 to 2,500 μg/ml (data not shown). This day-to-day phenomenon was also observed with strains 18676 (125 to 1,000 μg/ml) and 1428-PHD (7.8 to 62.5). No MIC variability was noted in the laboratory-derived Spc^r mutant $F\text{-}29^r$.

Analysis of cytoplasmic membrane proteins by gel electrophoresis. The membrane preparations exhibited characteristics typical of cytoplasmic membranes as evidenced by chemical analysis, sucrose density gradient centrifugation, and electron microscopy. The cytoplasmic membrane demonstrated a single band of turbidity with a buoyant density of 1.141 g/cm (2). Succinic and D-lactate dehydrogenase were localized in this membrane fraction (20) and were increased six- to eightfold over the total unfractionated cell envelope. Specific activities for these two enzymes in the crude envelope were 0.68 ± 0.4 and 0.57 ± 0.6. For the cytoplasmic membrane, the activities were 4.76 ± 0.65 and 3.99 ± 0.57. Electron microscopy of cytoplasmic membrane fractions revealed membrane-enclosed (9-nm thick) spherical structures that have been reported to appear as single or double units (9). These findings suggested that our preparations were free of outer membrane contamination.

TABLE 1. *MICs of clinical strains of N. gonorrhoeae to various antibiotics*

Strain	MIC (μg/ml)[a]			
	Pen	Spc	Tet	Cef
F-29	0.022	3.12	0.39	0.19
$F\text{-}29^r$ (laboratory derived)	0.022	>500	0.39	0.19
736430	0.078	39–2,500	0.78	0.78
736430^s (spontaneous revertant)	0.078	12.5	0.78	0.78
18676	0.011	125–1,000	0.19	0.19
1428-PHD	0.125	7.8–62	0.78	0.39
8-PHD	0.125	6.25	0.78	0.39

[a] MICs to penicillin (Pen), spectinomycin (Spc), tetracycline (Tet), and cefuroxime (Cef).

Our experiments with cytoplasmic membrane preparations revealed a multibanded electrophoretic pattern of 21 to 25 proteins. They were reproducibly observed in at least four preparations from each strain. Figure 1 shows the gel electrophoresis pattern of cytoplasmic membrane protein from Spc^s strain F-29 and Spc^r strain 736430. One major protein (no. 25) was present only in the Spc^s strain F-29 (Fig. 1 and 2A, C). Spc^s strain 8-PHD (Fig. 3B) also demonstrated this protein (no. 25), which was consistently absent from the three clinical Spc^r strains 736430, 18676, and 1428-PHD (Fig. 2C and 3A, C). Protein no. 25 had a molecular weight of 24,000 (Fig. 1) and accounted for 6 to 7% of the total cytoplasmic membrane protein.

The three consistently predominant proteins in each strain, independent of spectinomycin susceptibility, had molecular weights of 59,000 (protein no. 14), 34,000 (no. 23), and 29,000 (no. 24) (Fig. 1). No one protein, however, was the major constituent of the cytoplasmic membrane, as has been observed in the outer membrane of *N. gonorrhoeae* (10, 11). Six proteins (no. 1

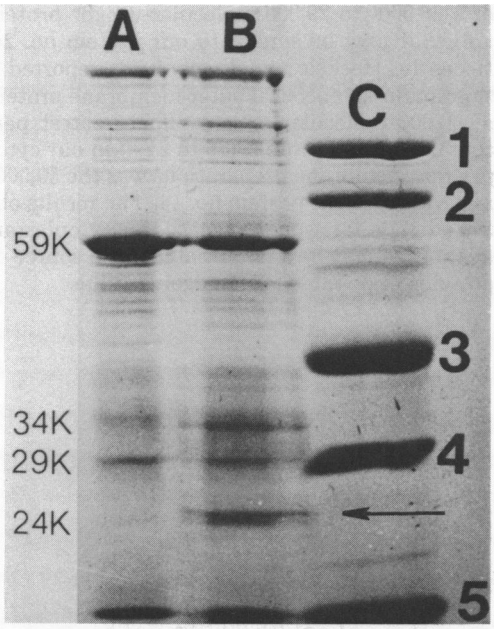

FIG. 1. *SDS-PAGE of isolated cytoplasmic membranes of (A) 736430 Spc^r, (B) F-29 Spc^s, and (C) molecular weight standards of: (1) phosphorylase B, 94,000; (2) bovine serum albumin, 68,000; (3) ovalbumin, 45,000; (4) carbon anhydrase, 30,000; and (5) lysozyme, 14,300. Arrow indicates major protein difference.*

through 6) had apparent molecular weights greater than 94,000.

Differences in the presence or absence of protein bands other than no. 25 were observed among the five strains studied (Table 2). These differences did not correlate with susceptibility or resistance to spectinomycin. Differences in proteins were not due to changes from repeated subcultures, as frozen stock cultures served as the reservoir for each strain. Possible variation introduced by serial subculture (10 times) was tested, but no significant differences were noted. The geographically diverse strains F-29 and 736430 and PHD-8 and 18676 demonstrated remarkable similarities in protein profiles, with differences being reflected only in protein no. 25. None of the patterns was modified when samples were heated with SDS at 100°C for 5 min or 37°C for 2 h.

Effect of spectinomycin on cytoplasmic membrane protein composition. At mid-log-phase growth, 2.0 μg of spectinomycin per ml was added to Spc^s strain F-29. Cells were harvested after 4 h, at which time there was complete cessation of growth (monitored turbidimetrically). The cells obtained from spectinomycin-containing broth medium were harvested, and purified cytoplasmic membranes were prepared. The profile of cytoplasmic membrane proteins revealed the significant disappearance of band no. 25, with other bands remaining essentially unaltered (Fig. 2B).

Cytoplasmic membrane proteins of Spc^s and Spc^r isogenic variants. By replica-plating technique, a naturally occurring Spc^s mutant (736430^s) was obtained from parent strain 736430. No other changes in susceptibility to antibiotics were noted (Table 1). The electrophoretic profile of this Spc^s variant revealed the presence of a protein at a location similar to the migration position of protein no. 25 (Fig. 2D). The molecular weight of this protein ranged from 24,000 to 25,000. The protein was not modified by heating with SDS at 100°C for 5 min or 37°C for 2 h.

The laboratory-derived Spc^r mutant (F-29^r) from Spc^s strain F-29 demonstrated a spectinomycin MIC of >500 μg/ml. Susceptibility to other antibiotics remained unchanged (Table 1). SDS-PAGE revealed a cytoplasmic membrane protein profile indistinguishable from that of the Spc^s parent F-29. These results would suggest that laboratory-derived versus clinically isolated spectinomycin resistance may differ.

DISCUSSION

The major outer membrane proteins of *N. meningitidis* and *N. gonorrhoeae* have been reported to demonstrate a wide range of strain-specific variation as revealed by SDS-PAGE (4, 7). The results of this study indicate similar findings for cytoplasmic membrane proteins in *N. gonorrhoeae*. Previously, only 13 proteins were reported in gonococcal plasma membranes (10, 22).

Spectinomycin susceptibility in clinical isolates was associated with the presence of a specific protein (no. 25) having a molecular weight of 24,000. Although a number of proteins in the outer membrane of *Neisseria* species exhibit differing molecular weights on SDS-PAGE depending upon the temperature of solubilization (5, 7, 28), no such modification occurred with protein no. 25. All three clinical strains with spectinomycin resistance lacked this protein.

Several predominant proteins in the cytoplasmic membrane, e.g., no. 14, 23, and 24, displayed mobility on SDS-PAGE similar to that reported for proteins in the outer membrane. Serological specificity in *N. gonorrhoeae* has been identified in part with a principal outer membrane protein having a molecular weight range of 32,000 to 39,000 (11). Our cytoplasmic membrane preparations demonstrated a predominant protein (no. 23), accounting for 5 to 17% of total protein and having a molecular weight of 34,000. Association with leukocytes depends on the presence

of a 28,000- to 29,000-molecular-weight protein (26) that may be similar to our protein no. 24. Recently, Heckels and Everson (8) reported a third major gonococcal outer membrane protein of 60,000 molecular weight. The greatest percentage of total protein (10 to 21%) in our cytoplasmic membrane preparations was the 59,000-molecular-weight protein no. 14. The results obtained thus far would suggest the cytoplasmic membrane may be a potentially useful source of proteins of possible immunogenic value.

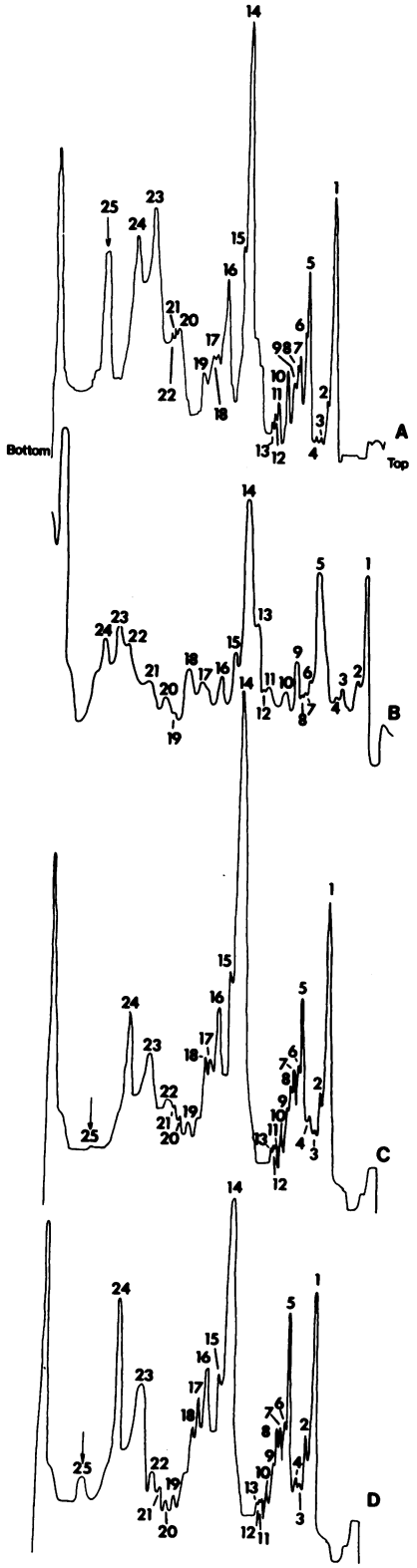

FIG. 2. *Scan of polyacrylamide gels of cytoplasmic membrane proteins from (A) F-29 Spc^s, (B) F-29^r, a laboratory-derived Spc^r mutant from parent F-29, (C) 736430 Spc^r, and (D) 736430^s, a spontaneously arising Spc^s variant of parent 736430. Arrow indicates major protein difference.*

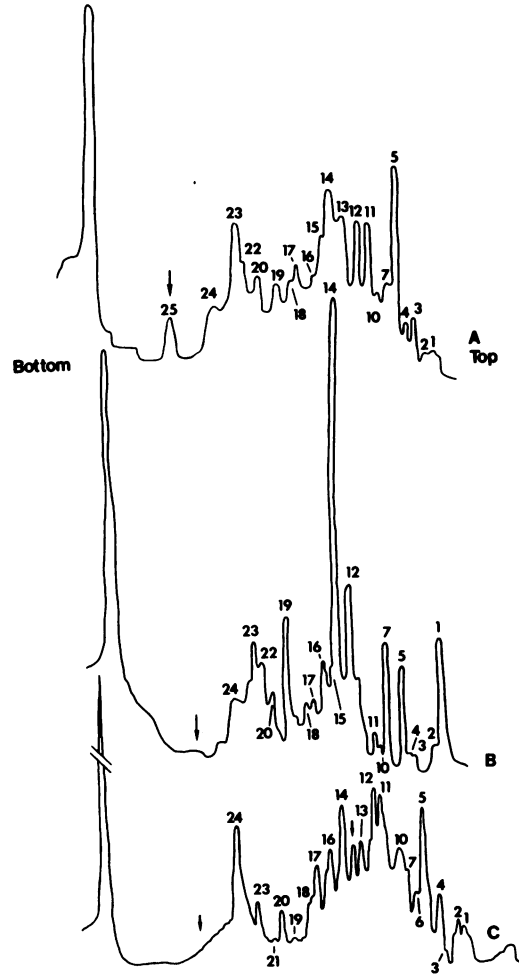

FIG. 3. *Polyacrylamide gel scans of strains (A) 18676 Spc^r, (B) 8-PHD, Spc^s, and (C) 1428-PHD Spc^r. Arrow indicates major protein difference.*

TABLE 2. *Variation of cytoplasmic membrane proteins in strains of N. gonorrhoeae*

Strain and phenotype	Protein absent (mol wt)
F-29 Spc^s	None
736430 Spc^r	25 (24,000)
18676 Spc^r	6 (>94,000); 8 and 9 (92,000–94,000); 21 (37,000) and 25 (24,000)
1428-PHD Spc^r	8 and 9 (92,000–94,000); 15 (58,000); 22 (36,000); plus additional band at 63,000
8-PHD Spc^s	6 (>94,000); 8 and 9 (92,000–94,000); 21 (37,000)

The results of this study provide, for the first time in *N. gonorrhoeae*, direct evidence of altered cytoplasmic membrane composition in antibiotic-susceptible versus -resistant strains. The findings of this paper are similar to those for *Escherichia coli* noted by Mizuno et al. (18). Two types of spectinomycin-resistant mutants were reported by these authors which differed in cytoplasmic membrane protein composition and phenotypic properties.

In the present paper, significant differences were noted between naturally occurring clinical spectinomycin resistance and resistance found in a laboratory-derived mutant. The laboratory-derived Spc^r mutant and Spc^s strains of *N. gonorrhoeae* exhibited cytoplasmic membrane profiles that were indistinguishable. Both contained a protein with a molecular weight of 24,000. When cells were grown in the presence of 2 μg of spectinomycin per ml, this protein (no. 25) was lost from the cytoplasmic membrane of Spc^s strain F-29 but not from the resistant mutant. Ribosomes have been shown to be resistant to antibiotic action in other laboratory-derived Spc^r mutants. This suggested that the action of spectinomycin was on the ribosomes and not directly on the cytoplasmic membrane (18).

Spectinomycin resistance may be genitically complex (multiple loci), or there may be phenotypic interactions between several genes. Evidence for the latter has been demonstrated in *N. gonorrhoeae* and *E. coli*. Sarubbi et al. (25) observed that recombinants of various mutant loci for spectinomycin resistance and tetracycline resistance brought about changes in the level of tetracycline resistance observed. In *E. coli*, there are data suggesting close interaction between ribosomal subunits in which the phenotype of mutations in one subunit is modified by mutations in the other (3). There are indications for interplay between specific proteins which can suppress streptomycin dependence and partially suppress spectinomycin resistance (1, 6).

More in-depth genetic studies are warranted to determine the basis of the variability observed in clinical spectinomycin resistance. If such gene interplay exists in *N. gonorrhoeae* and is highly unstable, the therapeutic and epidemiological significance of clinical spectinomycin resistance may gain new importance.

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