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Altered Crystal Violet Permeability and Lytic Behavior in Antibiotic-Resistant and -Sensitive Mutants of *Neisseria gonorrhoeae*

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Wild-type, antibiotic-resistant and hypersensitive isogenic strains of *Neisseria gonorrhoeae* were studied for uptake of crystal violet, rates of autolysis, and response to lysozyme. Total uptake of crystal violet was similar in all strains at 0 C but varied significantly at 37 C. Mutation at the nonspecific resistance locus *ery* resulted in relative impermeability to crystal violet at 37 C, as compared to wild type. The penetration barrier to crystal violet at 37 C was overcome by addition of 5 mM ethylenediaminetetraacetic acid. Mutation at *ery* also resulted in reduced rates of autolysis and reduced sensitivity to high concentrations of lysozyme under conditions of divalent cation (Mg^{2+}) depletion. In contrast, mutation at the nonspecific drug hypersensitivity locus *env* resulted in increased uptake of crystal violet at 37 C, due to increased binding of dye to crude envelope as well as increased penetration into cytoplasm. The *env* mutants were also more rapidly autolytic and more sensitive to lysozyme than wild type in the absence of Mg^{2+}. These results suggest that the cell envelopes of *ery* mutants are more stable and less permeable and those of *env* mutants are less stable and more permeable than wild-type strains.

Sparling et al. (29) have presented evidence that low-level resistance of *Neisseria gonorrhoeae* to many antibiotics, detergents, and dyes resulted from mutation at a locus designated *ery*. Nonspecific low-level resistance to certain antibiotics, principally penicillin and tetracycline, also occurred after mutation at *penB* (29). Other mutations of the gonococcus have been shown to result in loss of low-level resistance to multiple drugs (26). These mutations, which resulted in extraordinary sensitivity (greater than wild type) to antibiotics, dyes, and detergents occurred, at a locus designated *env* (26). The *ery*, *penB*, and *env* mutations were found both in laboratory-induced mutants and in naturally occurring (clinical) isolates (26, 29).

There is considerable evidence in other gram-negative bacteria that changes in the structure and function of the cell envelope, particularly in the outer membrane, may alter sensitivity to antibiotics (4, 9-11, 15, 18-23, 27, 28, 30, 31), dyes (2, 4, 9, 10, 13, 21, 23), and detergents (2, 4, 8-10, 14, 19, 21, 23). We report here initial efforts to determine whether similar mechanisms might be the basis of the pleiotropic effects of certain gonococcal mutations, particularly *ery* (nonspecific resistance) and *env* (nonspecific hypersensitivity).

MATERIALS AND METHODS

Bacterial strains. Three isogenic sets of *N. gonorrhoeae* were used in these experiments. The origin, genotype, and antibiotic sensitivities of these strains have been described (26, 29). Colony type 4 cells were used in all experiments.

Media and growth. Cells were grown in a medium consisting of 1 g of KH_2PO_4, 4 g of K_2HPO_4, 0.5 g of NaCl, and 15 g of proteose peptone no. 3 (Difco) per liter to which 0.6% glucose and 1% supplement C (Difco) were added prior to use. Growth was at 37 C in 125- to 300-ml side arm flasks which were agitated in a water bath shaker. Culture size varied from 10 to 30 ml, depending on the number of samples needed. The inocula were broth suspensions of cells taken from GC base agar (Difco) plates which had been incubated in a 37 C, 4% CO_2 incubator for 18 h. An inoculum sufficient to yield an initial reading of 15 to 20 Klett units on a Klett-Summerson colorimeter (no. 540 filter) was used. Under these conditions growth appeared normal in the absence of added CO_2, with mass doubling times of 60 to 80 min.

Crystal violet uptake. Permeability of *N. gonorrhoeae* to crystal violet was determined by methods similar to those described by Gustafsson et al. (13). Aliquots (3.8 ml) of mid-exponential-phase cultures (Klett reading of 70, no. 540 filter) were maintained at 0 or 37 C for 5 min. Crystal violet (0.2 ml) was added to a final concentration of 5 μg/ml. Unless otherwise stated, cells and crystal violet were incubated at 0 or 37 C for an additional 10 min before the

cells were pelleted by centrifugation (12,000 × *g*, 10 min, 2 C). The amount of dye remaining in the supernatant fluid was determined by measuring absorbance at 590 nm on a Beckman Acta CIII spectrophotometer.

The time course of dye absorption was determined by adding crystal violet (5 μg/ml) to a cell suspension maintained at 25 C. At intervals, aliquots (1 ml) of the cell-dye mixture were transferred to chilled tubes (−5 C) to rapidly chill the cells to prevent further dye absorption. The cells were then centrifuged at 12,000 × *g* at 2 C for 10 min. The amount of dye remaining in the supernatant was determined.

The temperature dependence of dye absorption was determined by incubating aliquots of cells grown at 37 C at the desired temperature for 10 min prior to adding dye. Uptake was allowed to continue for 10 min at the same temperature. Otherwise the procedure was as described above.

Location of crystal violet in cells. After a standard determination of dye uptake, the cell pellet was resuspended in 10 ml of ice-cold 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.8. This cell suspension was passed twice through a chilled French pressure cell at 16,000 lb/in^2. The disrupted cells were centrifuged for 30 min at 48,000 × *g* at 4 C to pellet crude cell envelope. The amount of crystal violet in the supernatant (cytoplasm-containing fraction) was determined by measuring absorption at 590 nm. Any dye not accounted for in the supernatant of the initial standard dye uptake determination, or in the cytoplasmic fraction from disrupted cells, was assumed to be bound to the envelope.

Binding of crystal violet to ribosomes. Mid-exponential-phase cells were harvested by centrifugation at 20 C and were resuspended in ice-cold 10 mM tris(hydroxymethyl)aminomethane buffer (pH 7.8) containing 10 mM Mg^{2+}. Cells were broken by two passes through a French pressure cell at 16,000 lb/in^2 pressure at 4 C. Cellular debris was removed by centrifuging the suspension at 48,000 × *g* for 45 min at 4 C. The resulting supernatant was then centrifuged at 50,000 rpm in a Ti65 rotor (Beckman) for 2 h at 4 C to pellet the ribosomes. The pellet was resuspended in tris(hydroxymethyl)aminomethane-Mg^{2+} buffer as above and dialyzed against the same buffer for 12 h at 4 C. The concentration of ribosomes was determined by measuring the absorbance of the solution at 260 nm (1.0 A_{260} unit = 60 μg of ribosomes per ml). Crystal violet (2 μg/ml final concentration) was then added to 5-ml aliquots of the ribosome suspension (0.3 mg of ribosomes per ml). The mixture was incubated at 37 C for 10 min and then centrifuged at 50,000 rpm in a Ti65 rotor for 2 h at 4 C. The amount of dye remaining in the supernatant was determined as desribed above.

Rates of autolysis. Cells grown to a Klett reading of 70 or 100 as described were harvested by centrifuging at 10,000 × *g* for 10 min at 2 C. The cells were then suspended in ice-cold 50 mM KPO_4 buffer, pH 7.0, with and without 3 mM Mg^{2+}. Aliquots of the cell suspension were warmed to room temperature (22 to 25 C), and the change in turbidity at 600 nm was measured in a Beckman Acta CIII recording spectrophotometer. In some experiments lysozyme (1 mg/ml) was added to the cell suspension, and the change in turbidity was monitored.

Electron microscopy. Mid-exponential-phase cultures were prepared for electron microscopy by a modified Ryter-Kellenberger procedure (25) by adding one-tenth volume of fixative (5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 6.9) to the cultures, which were then harvested by centrifugation. The pellets were suspended in fixative for 1 h at room temperature. After washing and enrobing the cells in agar they were postfixed for 16 h in veronal acetate-buffered osmium tetroxide. They were washed, stained for 2 h in uranyl acetate, subjected to acetone dehydration, and infiltrated and embedded in Vestopal W. Thin sections were cut on a Reichert OM-2 ultramicrotome, poststained with lead citrate and uranyl acetate, and examined using an AEI electron microscope operated at 60 kV.

Chemicals. Lysozyme was from Schwarz Bioresearch (activity, 1,000 U/mg). Crystal violet was from Eastman. All other materials were of the highest purity available.

RESULTS

Uptake of crystal violet. We wished to ascertain whether mutations which nonspecifically increased or decreased resistance of the gonococcus to antibiotics and other antibacterial agents did so by altering an outer penetration barrier. Since recent studies of envelope mutants of *Escherichia coli* (13) and *Salmonella minnesota* (1) showed that uptake of the basic dye crystal (gentian) violet correlated well with changes in antibiotic sensitivity and envelope structure, it seemed reasonable to use similar methods to study our mutants.

The amount of crystal violet removed from broth suspension by cells at 0 and 37 C was measured for various wild-type, antibiotic-resistant and hypersensitive strains. All strains removed approximately equal quantities of dye at 0 C. This value ranged from 15 to 25% of the total dye in solution. At 37 C, however, the amount of dye absorbed by the strains varied. In general, the naturally occurring and ultraviolet-induced multiply drug resistant (MDR) mutants removed little or no dye in excess of that absorbed at 0 C (i.e., 15 to 25%), whereas wild-type-sensitive strains absorbed approximately 35 to 45% of the dye available and several antibiotic-hypersensitive (*env*) mutants removed 50 to 75% of the dye from solution. These results were calculated as the difference in percentage of dye removed at 37 and 0 C (ΔP = percentage of dye removed at 37 C − percentage of dye removed at 0 C) and are shown in Fig. 1. The ΔP values are averages of 5 to 10 experiments for each strain. Results were similar with con-

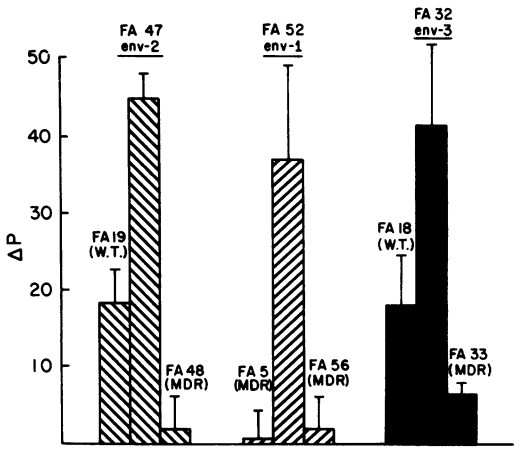

FIG. 1. *Crystal violet uptake in three sets of strains of N. gonorrhoeae. ΔP = percentage of uptake of total dye at 37 C − percentage of uptake at 0 C. W.T., Wild type; env are drug-hypersensitive mutants (26). MDR are all env⁺ and carry mutations at ery in addition to many others. FA47 and FA48 were constructed by mutagenesis from FA19. FA32 and FA33 were constructed by mutagenesis from FA18. FA52 and FA56 were mutants from clinical isolate FA5 (26, 29). Values are means, with one standard deviation, from five to ten experiments.*

centrations of dye from 1 to 7 μg/ml (not shown).

Reproducibility of results depended on the use of fresh, mid-exponential-phase cells. In preliminary experiments, uptake of dye was variable and was often relatively greater when later exponential- or stationary-phase cells were used.

The temperature dependence of dye absorption was compared for wild-type strain FA19, its *env-2* mutant FA47, and an MDR derivative of FA47, FA48 (Fig. 2). Basal dye absorption was observed below 10 C for all strains. Above 10 C absorption of dye by strains FA19 and FA47 increased to a maximum, which occurred at 18 to 25 C for FA19 and 25 to 37 C for FA47. There was little increase in dye uptake in FA48 at temperatures up to 37 C.

The rates at which strains FA19 and FA47 absorbed crystal violet at 25 C were very rapid and almost identical for the first minute (Fig. 3). Absorption of dye was essentially complete for FA19 at this point but continued for several more minutes for the hypersensitive *env-2* mutant FA47.

To determine whether the additional dye removed at 37 C was due to increased binding to cell envelope or increased permeability into the cytoplasm or both, cells were fractionated into crude cytoplasmic and envelope fractions, and the amount of dye in each fraction was determined. The results for FA19 and its derivative

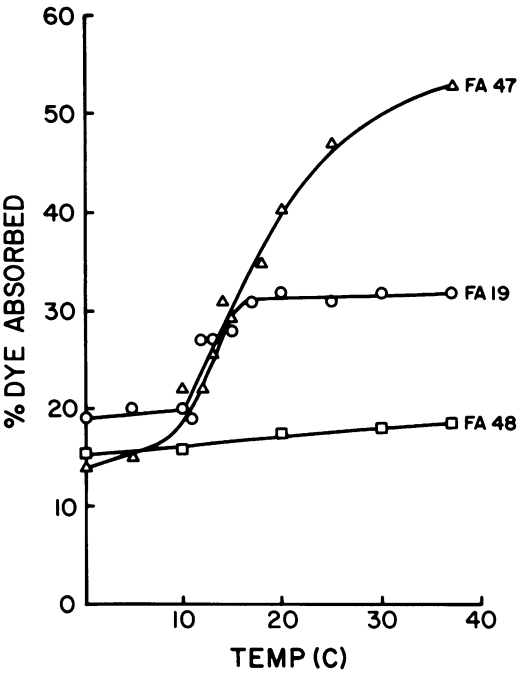

FIG. 2. *Crystal violet uptake (percentage of total dye added) as a function of temperature in wild-type strain FA19, env-2 mutant FA47, and MDR mutant FA48.*

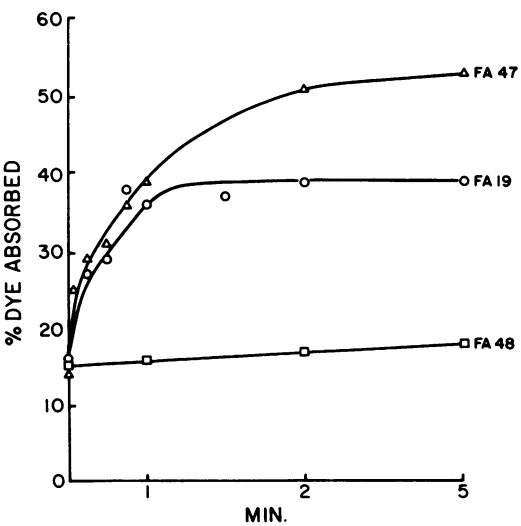

FIG. 3. *Kinetics of crystal violet uptake (percentage of total dye added) at 25 C in wild-type strain FA19, env-2 mutant FA47, and MDR mutant FA48.*

TABLE 1. *Distribution of crystal violet in cell envelope and cytoplasm after a standard uptake experiment*

Strain[b]	Temp (C)	Crystal violet uptake (μg/ml)[a]		
		Whole cells (%)[c]	Cytoplasm	Cell envelope
FA19	0	1.2 (24.0)	0.6	0.6
	37	2.1 (41.4)	1.0	1.1
FA47 (*env-2*)	0	1.0 (20.0)	0.7	0.3
	37	3.7 (73.0)	2.0	1.7
FA48 (MDR)	0	1.0 (18.0)	0.4	0.6
	37	1.1 (22.0)	0.4	0.7

[a] After a standard dye uptake experiment, cells containing crystal violet were fractionated, and dye concentration in fractions was determined as described in Materials and Methods. Uptake by whole cells = initial dye concentration − residual dye concentration in supernatant.

[b] FA19 is wild type. FA47 is the *penA2 str-7 env-2* mutant of FA19. FA48 is an MDR polygenic mutant of FA47, which is *envA*$^+$ (26).

[c] Figures in parentheses are percentage of total dye added taken up by whole cells.

strains FA47 and FA48 are shown in Table 1. It appeared that both increased binding to cell envelope and permeability into the cytoplasm were responsible for the increased amount of dye absorbed at 37 C by strains FA19 and FA47. Results for wild-type strain FA18, its *env-3* mutant FA32, and a multiply resistant derivative of FA32 (FA33) were very similar to those for FA19, FA47, and FA48, respectively (not shown).

Ribosomal binding of crystal violet. Crystal violet is a cation and binds avidly to negatively charged particles such as ribosomes (13). Therefore, differences in absorption of crystal violet by whole cells could reflect a difference in the amount of dye bound and held by cytoplasmic ribosomes rather than differences in permeability of the cell envelope. To exclude this possibility, we attempted to measure amounts of crystal violet bound to ribosomes after a standard whole-cell uptake experiment by using 0.1 M $CaCl_2$ to release crystal violet from ribosomes (13). Release of crystal violet from ribosomes by this method was variable and always incomplete, and no meaningful conclusions were possible. Ribosomes were therefore prepared from cells not previously exposed to crystal violet, and the amount of dye removed from solution by ribosomes was measured. Ribosomes from strains FA19, FA47, and FA48 bound 3.2, 3.4, and 3.3 μg of dye per mg of ribosomes, respectively. This indicated that the observed difference in dye permeability was not due to differential binding by ribosomes.

Effect of EDTA on crystal violet absorption. The addition of 5 mM ethylenediaminetetraacetate (EDTA) to a cell suspension 1 min before the addition of crystal violet increased the amount of dye absorbed by all strains tested at 0 C (Table 2). At 37 C the addition of EDTA increased the amount of dye absorbed by strains FA48 and FA5 about twofold but did not increase the amount of dye absorbed by strains FA19 and FA47 over that normally observed in the absence of EDTA. The relatively greatest effect of EDTA, therefore, was on antibiotic-resistant strains FA48 and FA5, which were as permeable to crystal violet as wild-type strain FA19 under these conditions.

Uptake of crystal violet by transformed strains. Isogenic transformants of FA19 containing various antibiotic resistance markers from FA48 (29) were tested for their ability to absorb crystal violet. The results (Table 3) indicated that introduction of the antibiotic resistance markers *penA2*, *str-7*, *chl-2*, and *tet-2* did not significantly alter dye absorption. However, when the *ery-2* marker was added to strains carrying other markers (strains FA136 and FA140) or no other markers (strain FA171), uptake of crystal violet was sharply reduced. Thus, *ery-2* (and *ery-1* from FA5, not shown) was responsible for the decreased uptake of crystal violet exhibited by the polygenic, MDR strains FA48 and FA5.

Autolysis in phosphate buffer. We observed that certain strains tended to autolyze when suspended in phosphate buffer after centrifugation. This tendency to autolyze was compared for various strains suspended in phosphate buffer at pH 7 at room temperature (Fig. 4A). Most rapid autolysis was observed in the

TABLE 2. *Effect of EDTA on crystal violet uptake*

Strain[a]	Temp (C)	Crystal violet uptake (% total dye added)	
		−EDTA	+5 mM EDTA
FA19	0	17	29
	37	44	43
FA47 (*env-2*)	0	17	31
	37	58	52
FA48 (MDR)	0	19	28
	37	25	45
FA5 (MDR)	0	14	37
	37	18	48

[a] FA19 is the wild-type parent of drug-hypersensitive strain FA47 and MDR strain FA48. FA5 is unrelated but phenotypically similar to strain FA48.

TABLE 3. *Penetration barrier to crystal violet due to ery-2*

Strain[a]	Genotype	Crystal violet uptake (% total dye added)		
		0 C[b]	37 C[b]	ΔP[c]
FA19	Wild-type parent	24	43	19 ± 4
FA102	*penA2*	29	53	24 ± 2
FA128	*tet-2, chl-2, str-7*	19	37	18 ± 2
FA136	*penA2, ery-2*	26	30	4 ± 3
FA140	*penA2, ery-2, penB2*	24	31	7 ± 3
FA171	*ery-2*	20	23	3 ± 5

[a] Strains were constructed by transformation of FA19 with limiting concentrations (0.01 μg/ml) of deoxyribonucleic acid from MDR strain FA48 (29).

[b] Average of two to ten experiments.

[c] ΔP = percentage of dye uptake at 37 C − percentage of dye uptake at 0 C.

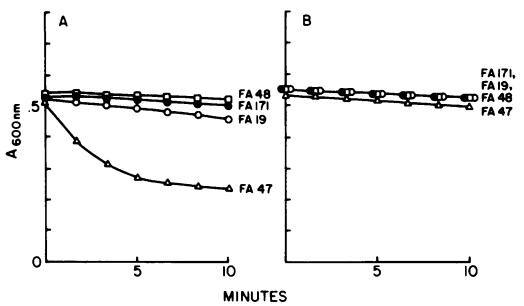

FIG. 4. *Autolysis of wild-type strain FA19, env-2 mutant FA47, MDR mutant FA48, and ery-2 transformant FA171 in pH 7.0 phosphate buffer (A) without added Mg^{2+} and (B) with 3 mM Mg^{2+} added.*

hypersensitive *env-2* mutant FA47, whereas FA19 autolyzed at a slower rate and FA48 showed little if any loss of turbidity over a 10-min period. The *ery-2* transformant from FA48 × FA19 (FA171) was similar to FA48 in this respect. If Mg^{2+} (3 mM) was added to the suspending buffer, autolysis was prevented for strains FA47 and FA19 (Fig. 4B).

When a high concentration of lysozyme (1 mg/ml) was added to the cell suspensions in phosphate buffer without Mg^{2+}, there was a rapid increase in turbidity (Fig. 5A) and a simultaneous macroscopic clumping of strain FA47. Strains FA48 and FA171 showed little or no increase in turbidity or clumping, whereas FA19 was intermediate between FA47 and FA48. The addition of Mg^{2+} largely prevented the lysozyme-induced turbidity increase in strains FA47 and FA19 (Fig. 5B).

Electron microscopy. Thin-section transmission electron micrographs of exponential-phase cells of FA19, FA47, and FA48 revealed typical gram-negative trilaminar membranes, but no differences in the morphological features of the double-track inner or outer membranes or the single-track electron-dense middle (peptidoglycan) layer (not shown). No differences were noted in other morphological features, including cell division, by either electron or phase microscopy.

DISCUSSION

Our results demonstrate that isogenic strains of gonococci varying in their antibiotic resistance differ in characteristics that can be expected to be associated with alterations of the cell envelope. MDR strains, or transformants carrying only the single marker *ery*, were relatively impermeable to crystal violet, resistant to autolysis under conditions of divalent cation (Mg^{2+}) depletion, and resistant to aggregation by high concentrations of lysozyme, as compared to their wild-type parents. The *ery* transformants were also more resistant to diverse drugs, including several surface-active detergents (29). Addition of EDTA, which is known to damage the outer membrane of other gram-negative bacteria (3, 16–18) by chelating divalent cations, with subsequent loss of the normal penetration barrier to agents such as actinomycin D (16, 17), resulted in loss of the penetration barrier of the MDR gonococci to crystal violet (Table 2).

In contrast, gonococci carrying various *env* mutations (FA32, FA47, FA52) were more permeable to crystal violet, more rapidly autolytic in phosphate buffer, and more sensitive to lysozyme than their corresponding *env*$^+$ parents. They also were more sensitive to a wide variety of drugs and surface-active agents (26) than wild-type strains. The sensitivity of *env* mutants to lysozyme, as well as their rapid

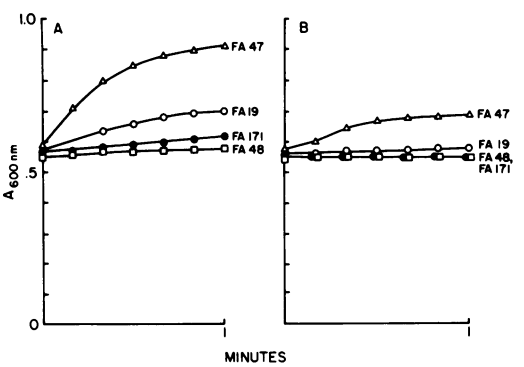

FIG. 5. *Cell clumping due to lysozyme (1 mg/ml) in pH 7.0 phosphate buffer (A) without added Mg^{2+} and (B) with 3 mM Mg^{2+} added.*

autolysis in phosphate buffer, was dramatically reduced by addition of 3 mM Mg^{2+} to the medium (Fig. 4 and 5). These observations are all consistent with alteration of the envelope by *env*, resulting in decreased stability under adverse conditions and increased permeability. Mutation at *ery*, which produces effects opposite to these, presumably results in increased stability and decreased permeability of the envelope.

There is convincing evidence that the outer membrane of *E. coli*, *Salmonella*, and other gram-negative bacteria normally acts as a permeability (penetration) barrier (4, 11, 18). This barrier function may be increased, resulting in drug resistance, by mutations that affect the structure of the lipopolysaccharide (LPS) in the outer membrane (4, 20, 22, 23, 27, 28). The outer membrane barrier may also be decreased, resulting in drug hypersensitivity, by a variety of mutations affecting LPS (4, 8, 27, 28, 30, 31), protein (33), or both protein and LPS (2, 14). Other mutations to increased sensitivity have been associated with tolerance to colicins (10, 21).

In two of the best-studied examples, outer membrane mutants of *E. coli* (13) and *S. minnesota* (1) with increased sensitivity to antibiotics were also more permeable to crystal violet. In *Salmonella*, the extent of alteration of LPS was directly correlated with the total amount of crystal violet absorbed (1). In *E. coli*, alterations of LPS structure were best correlated with initial rates of crystal violet uptake, although total uptake differed in several mutants, including one with no clear alteration of LPS (13). In our studies, initial rates of crystal violet uptake were extremely rapid and not appreciably different between wild type and *env-2* mutant FA47; both were markedly different from multiply resistant strain FA48 (Fig. 3). Measurement of total uptake clearly differentiated the various mutants, however (Fig. 1 and 2).

As noted previously in *E. coli* by Gustafsson et al. (13), all strains of gonococci absorbed approximately 20% of available crystal violet at 0 C. This presumably represents nonspecific ionic binding to the negatively charged cell surface and implies that surface charge is not markedly different in the various mutants. The sudden increases in crystal violet absorption noted at about 10 to 12 C in wild-type strain FA19 and *env-2* mutant FA47 (Fig. 2) are consistent with a temperature-induced phase transition of a lipid membrane, although other explanations are possible.

The changes we have observed could conceivably be due to changes in the middle (peptidoglycan) layer of the gram-negative envelope, either due to altered peptidoglycan structure or altered autolytic activity. Certain drug-hypersensitive mutants of *E. coli* apparently have increased autolytic activity (13, 15), and partial inhibition of peptidoglycan synthesis by low concentrations of ampicillin or lysozyme resulted in increased sensitivity of several gram-negative bacilli to other antibiotics and detergents as well (5). The evidence for a barrier function of peptidoglycan is less equivocal in gram-positive bacteria (6). Since the concentration of Mg^{2+} may have an effect on the stability of peptidoglycan structure (24) and activity of certain autolytic enzymes (12) as well as integrity of the outer membrane (3, 16–18), the effects of EDTA and Mg^{2+} which we observed cannot be taken to indicate the site of the primary biochemical lesion in either the *ery* or *env* mutants. Similarly, the increased rates of autolysis observed in *env* mutants do not necessarily indicate that this is the primary defect; increased rates of autolysis could be secondary to changes in outer membrane due to interdependence of structure and function between outer membrane and peptidoglycan.

Clinical observations strongly suggest that only antibiotic-sensitive gonococci are capable of causing systemic infection in man (32). Penicillin-resistant envelope mutants of salmonella are more sensitive to host antibody (22), and detergent, lysozyme, and EDTA-resistant mutants of shigella are less invasive (7). One or more of the putative envelope loci (*ery*, *env*) which we have described in the gonococcus may be associated with decreased virulence, although this and several other questions require further study.

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