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Outer Penetration Barrier of *Escherichia coli* K-12: Kinetics of the Uptake of Gentian Violet by Wild Type and Envelope Mutants

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Wild-type strains of *Escherichia coli* K-12 adsorb gentian violet to the cell surface, but the dye is not transported into the cytoplasm. However, when some mutants that have an altered outer membrane are exposed to gentian violet, the dye is also found in the ribosomal fraction. The transport into the cytoplasm is inhibited at 0 C and requires that the concentration of gentian violet exceeds a threshold value. The initial rate of uptake as well as the amount of gentian violet found in the cytoplasm increases with the concentration of the dye in the medium. The rate of transport of the dye into the cytoplasm is much lower for stationary mutant cells than for exponentially growing cells. The rate of uptake into the cytoplasm increases with increasing deficiency of carbohydrate in the lipopolysaccharide (carbohydrate content *lpsB* > *lpsA* > *galU*). However, other components are also responsible for the barrier since an *envA* mutant which is not altered in the lipopolysaccharide carbohydrates show an extremely rapid uptake of the dye. The rate of uptake for the *envA* mutant was the highest found and the same as that of spheroplasts. Growth in the presence of agents affecting the murein sacculus, e.g., lysozyme and sublethal concentrations of penicillin, increased the rate of uptake of gentian violet. Brief treatments with tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetic acid drastically impaired the barrier function. Inhibition of protein synthesis by chloramphenicol also opened the barrier to gentian violet. In conclusion, the outer part of the bacterial envelope is a penetration barrier for gentian violet and probably also for other substances. The lipopolysaccharide, the murein and also other components are important for the function of this barrier. Resistance to gentian violet was found to be inversely correlated to the rate of penetration of the dye into the cytoplasm.

Gram-negative bacteria are generally more tolerant to antibacterial agents than are gram-positive bacteria. This tolerance has been attributed to a penetration barrier outside the cytoplasmic membrane (8, 23). The existence of a barrier has been revealed by various chemical treatments of the cell and by the isolation of mutants. Lieve et al. (11, 12) reported that treatment with ethylenediaminetetraacetic acid (EDTA) makes the cells sensitive to actinomycin D and also releases part of the envelope lipids and lipopolysaccharide. This response suggested that the lipopolysaccharides constitute the main penetration barrier in *Escherichia coli*. Mutants affected in the carbohydrate composition of the lipopolysaccharide show a changed sensitivity to antibacterial agents (6, 14, 21, 22, 25). This finding also points to the importance of the lipopolysaccharide for the barrier function. However, antibiotic-supersensitive mutants with apparently normal lipopolysaccharide have been isolated (15, 25). Additional barrier components have therefore been proposed (30). It has been suggested that murein participates directly or indirectly for the barrier function (3, 10, 19).

The barrier function has been studied in various ways: by measuring resistance levels of different envelope mutants (2, 5, 6, 16, 21, 22, 28) and under different physiological conditions (2, 3, 4, 15, 17) by measuring the crypticity of cellular enzymes such as β-lactamases (3, 8, 18, 23) and by direct measurements of the uptake of antibacterial agents (7, 9, 11, 13, 15, 17). With the exception of the latter these approaches are indirect.

Most antibacterial agents affect the cell envelope directly or indirectly. To study the normal barrier function it is necessary to study the initial rate of uptake. To do so the rate of uptake must be measured during fractions of a cell generation. We have found that gentian violet is a suitable substance for such studies as it can be measured spectrophotometrically at low concentrations even in broth media, and a mutant isolated in this laboratory (*envA*) has been shown to have a markedly increased uptake of this dye (15, 17).

MATERIALS AND METHODS

Bacterial strains. All strains used were derivatives of *E. coli* K-12. Their characters are described in Table 1.

Media and growth conditions. The basal medium was medium E of Vogel and Bonner (27). The Casamino Acids medium contained the basal medium, thiamine (1 μg/ml), 0.2% Casamino hydrolysate and was supplemented with L-tryptophan (50 μg/ml) and 0.2% glucose. The acetate medium consisted of the basal medium, thiamine (1 μg/ml), 0.2% acetate and was supplemented with L-histidine (50 μg/ml), L-proline (50 μg/ml), and L-tryptophan (50 μg/ml). The bacteria were grown on a rotary shaker at 37 C. Growth was recorded by optical density readings by use of a Klett-Summerson colorimeter with a W66 filter or a Zeiss PMQII spectrophotometer at a wavelength of 420 nm. A reading of 50 Klett units corresponded to 2×10^8 cells/ml or to a dry weight of 40 μg/ml.

Materials. D-Ampicillin and chloramphenicol were gifts from AB Astra, Södertälje, Sweden, and Erco, Stockholm, Sweden, respectively; gentian violet, sodium azide, and EDTA were from Merck, Darmstadt, Germany; lysozyme was obtained from Sigma Chemical Co., St. Louis, Mo.

Measurement of the total uptake of gentian violet. The method for measuring the total uptake of gentian violet has previously been described by Normark and Westling (17) but was modified in the following way. The culture was grown to the desired density, in most experiments 50 Klett units. Gentian violet was added directly to the growth flask to the final concentration chosen. The flask was incubated on a rotary shaker at 37 C or, in some cases, 0 C. After 15 and 30 min, samples were taken and centrifuged at 0 C. The amount of gentian violet remaining in the supernatant fluid was measured by use of a Unicam SP700 spectrophotometer at a wavenumber of 16.95 kc/cm (= 590 nm). With one exception, the uptakes after 15 and 30 min were the same. The values for uptake are not adjusted for the possible uptake during the centrifugation.

Kinetic studies of the uptake of gentian violet. The cells were grown to decided Klett reading. At zero time, gentian violet was added. In most experiments the final dye concentration was 10 μg/ml. At intervals samples were transferred to ice-cold tubes which were kept at 0 C. At this temperature no further uptake of gentian violet was observed. The accuracy of the estimation of sampling time was ±1 second. The samples were centrifuged at 0 C. The dye remaining in the supernatant fluid was measured in a Unicam SP700 at a wavenumber of 16.95 kc/cm (= 590 nm).

Determination of resistance to gentian violet. The bacteria were grown in Casamino Acids medium to a cell density of about 2×10^8 cells/ml. A number of prewarmed (37 C) tubes containing Casamino Acids medium and different concentrations of gentian violet (0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5, and 7.5 μg/ml) were inoculated with about 10^4 cells per ml. Resistance was defined as the highest concentration of gentian violet at which a turbid culture was obtained after incubation overnight.

Separation of dye-containing cell material from cells pretreated with gentian violet. A 30-ml culture was grown at 37 C to 50 Klett units and divided into three portions. Two portions (A and B) were chilled to 0 C. The third (C) was kept at 37 C. Gentian violet (10 μg/ml) was added to portions A and C. Portion B was not treated with gentian violet but was fractionated in the same way as A and C, and the respective fractions were used as blanks in the spectrophotometric determinations of gentian violet. Gentian violet was measured at 590 nm in a Beckman Acta CII spectrophotometer. After 15 min of incubation at 0 and 37 C, respectively, the cultures were centrifuged at 0 C for 15 min at 4,500 rpm in a Wifug desk-top centrifuge. The dye remaining in the supernatant fluid was mea-

TABLE 1. *Escherichia coli K-12 strains used and their relevant characters*[a]

Strain	Origin	Envelope genotype	Carbohydrate per mg of LPS (μg)[b]				Other relevant markers
			Rha	Gal	Glu	Hep	
D21	Boman et al. (1)	wt	14	29	78	95	*proA, trp, his*
D22	Normark et al. (16)	*envA*	11	27	71	70	*proA, trp, his*
D21e8	Eriksson-Grennberg et al. (6)	*galU*	0	0	8	41	*proA, trp, his*
D21e7	Eriksson-Grennberg et al. (6)	*lpsA*[c]	0	1	46	47	*proA, trp, his*
D21e19	Eriksson-Grennberg et al. (6)	*lpsB*[c]	2	1	63	90	*proA, trp, his*

[a] Abbreviations: gal, galactose; glu, glucose; hep, heptose; his, histidine; lps, lipopolysaccharide; pro, prolin; rha, rhamnose; trp, tryptophan; wt, wild type.

[b] Data taken from Grennberg et al. (6), and Normark (15).

[c] These loci have been designated *rfa* on the recent *E. coli* linkage map (26).

sured. The pellets were resuspended in 10 ml of ice-cold 0.01 M tris(hydroxymethyl)-aminomethane(Tris)-hydrochloride, pH 7.8. According to the results of Normark (M.D. thesis, Univ. of Umeå, Sweden, 1971), this prevents a release of gentian violet molecules. The cells were disrupted twice in a cooled Aminco French press. The disrupted cells were centrifuged for 30 min at 48,000 × g in a Sorvall RC-2B centrifuge in order to spin down cell wall and debris (envelope fraction). The pellets were resuspended in 10 ml of the above-mentioned buffer supplemented with $CaCl_2$ (0.1 M). This treatment will cause release of 60% of the bound dye (S. Normark, M.D. thesis, Univ. of Umeå, Sweden, 1971). The suspension was recentrifuged and the pellet was resuspended and again centrifuged. The released dye in the two supernatant fluids was measured. $MgCl_2$ (0.01 M) was added to the supernatant fluids from the first Sorvall centrifugation, and the ribosomes were removed by centrifugation for 90 min at 250,000 × g in a Spinco ultracentrifuge. The pellets were resuspended in 10 ml of Tris buffer containing $CaCl_2$ (0.1 M), and the dye remaining in the pellet (ribosomal fraction) and supernatant fluid (cytoplasmic fraction) was measured.

RESULTS

Kinetics of gentian violet uptake. The uptake of gentian violet was followed in strain D21 and in a number of envelope mutants of this strain (Fig. 1). At 0 C all strains instantaneously took up 20% of the 10 μg of gentian violet added per ml, after which no further uptake was recorded. At 37 C the parent strain D21 showed the same uptake as at 0 C. The envelope mutants also showed the same instantaneous uptake at 37 C as at 0 C. However, at the higher temperature the mutants exhibited a continued gradual uptake of dye until a plateau value was reached. The final total uptake was approximately the same for strains D21e7 (*lpsA*), D21e8 (*galU*), and D21e19 (*lpsB*), whereas a higher total uptake was found for strain D22 (*envA*). The rate of uptake differed markedly among the envelope mutants.

During these conditions there was no change in viable count of the wild-type strain D21, whereas at least 90% of the *envA* cells were rapidly killed within 5 min. The other three strains showed a slower and less pronounced killing.

The results depicted in Fig. 1 demonstrate that the uptake of gentian violet can be divided into at least two processes. The instantaneous uptake that occurs even at 0 C has been referred to as type I, whereas the gradual uptake recorded only at higher temperatures is denoted type II.

The location of the gentian violet taken up by the wild-type strain D21 and the envA mutant D22 was then studied (Table 2). The wild-type strain at both 0 C and 37 C and the *envA* strain at 0 C took up the same amount of dye. All the gentian violet was located in the envelope fraction. Gentian violet uptake of type I is therefore due to binding to the cell envelope. The *envA* strain at 37 C showed a much higher

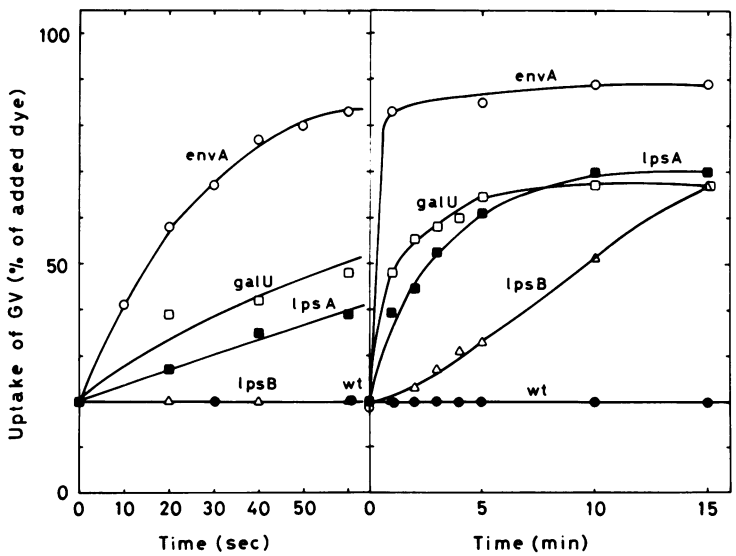

FIG. 1. *Uptake of gentian violet by strain D21 (wild type) and its envelope mutants D22 (envA), D21e8 (galU), D21e7 (lpsA), and D21e19 (lpsB). The bacteria were grown exponentially in Casamino Acids medium to a cell density corresponding to 50 Klett units (2 × 10^8 cells/ml). At zero time, 10 μg of gentian violet was added per ml of culture. The uptake of gentian violet was measured as described in Materials and Methods.*

TABLE 2. *Separation of dye-containing cell material from gentian violet pretreated envA and wild-type cells*[a]

Fraction	Uptake of gentian violet					
	envA, 0 C		*envA*, 37 C		Wild type, 0 and 37 C	
	μg/ml	% of total	μg/ml	% of total	μg/ml	% of total
Intact cells	2.2	100	8.7	100	2.2	100
Envelope	1.9	87	2.9	33	2.1	95
Ribosomal	0.0	0	4.7	54	0.0	0
Cytoplasmic	0.1	3	0.1	1	0.0	0

[a] Strains D21 (wild type) and D22 (*envA*) were grown in Casamino Acids medium to a cell density corresponding to 50 Klett units. One sample of the respective culture was chilled to 0 C. Gentian violet (10 μg/ml) was added and incubation at 0 and 37 C, respectively, was continued for 15 min. The uptake of dye was measured (intact cells). The separation of cell material and further dye measurements were carried out as described in Materials and Methods.

total uptake than the wild type. The main part of the increased amount of gentian violet taken up was recovered in the ribosomal fraction. Thus gentian violet uptake of type II represents transport of dye into the cytoplasm.

Figure 2 shows the uptake of gentian violet at different cell concentrations. Uptake by the wild-type strain was a linear function of the total cell mass. In strains exhibiting type II uptake (*envA* and *galU*), the total uptake of dye at 37 C increased with cell concentration until a plateau value was reached.

We then measured the total uptake of gentian violet at different concentrations of the dye (Fig. 3). The uptake was linearly dependent on the concentration of gentian violet for the strains tested.

Properties of type I and type II uptake. At 37 C, strain D22 (*envA*) exhibited both types of uptake. At 0 C, however, uptake of type II was abolished. As shown in Fig. 4, uptake of type II for strain D22 could also be reduced by sodium azide. Potassium cyanide had the same effect. Furthermore, the type II uptake was much slower when the *envA* cells were grown in minimal-acetate medium than it was in Casamino Acids medium. Stationary phase *envA* cells showed a decreased rate of uptake and a lower final plateau value than log-phase cells.

In the parent strain D21, no gentian violet uptake of type II was recorded at 37 C. However, after treatment of the cells with agents known to affect the cell envelope (EDTA, lysozyme, or lysozyme-EDTA) strain D21 was also capable of type II uptake. The spheroplasts obtained by treatment with lysozyme-EDTA showed the same kinetics of gentian violet uptake as did the *envA* mutant D22.

It has earlier been reported that sublethal

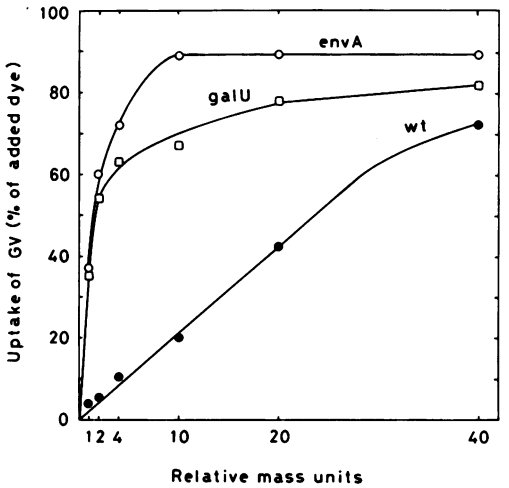

FIG. 2. *Uptake of gentian violet as a function of cell concentration in strains D21 (wild type), D21e8 (galU), and D22 (envA). The bacteria were grown in Casamino Acids medium to a cell density corresponding to 200 Klett units (= 40 relative mass units). Serial dilutions were made with prewarmed (37 C) Casamino Acids medium. Gentian violet (10 μg/ml) was added. The uptake of the dye was measured after 15 min of incubation at 37 C.*

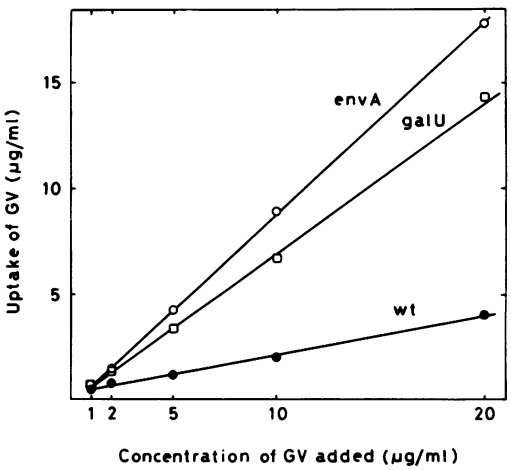

FIG. 3. *Uptake of gentian violet as a function of gentian violet concentration in strains D21 (wild type), D21e8 (galU), and D22 (envA). The experimental conditions were as in Fig. 1. At zero time, different concentrations of gentian violet were added to the culture. The uptake of dye was measured after 15 min of incubation at 37 C.*

concentrations of ampicillin (3) and chloramphenicol (17) affect the penetration properties of the cell envelope. Figure 5 shows that, after treatment with D-ampicillin (10 μg/ml) or chloramphenicol (100 μg/ml), strain D21 was capable of type II uptake of gentian violet.

It should be noted that, in all experiments reported in Fig. 4 and 5, the instantaneous uptake of gentian violet (type I) was the same (20%).

Initial rate of uptake of gentian violet (type II). Strain D21e8 (*galU*) was chosen for these studies as it exhibited a moderate rate of type II uptake. The initial rate of uptake by strain D21e8 was measured at different concentrations of gentian violet (Fig. 6). The original uptake data obtained for one concentration of dye as well as the calculation of the corresponding initial rate is inserted in Fig. 6. The rate of uptake was very slow for low concentrations of gentian violet. At concentrations above a threshold value there was a linear correlation between rate of uptake and concentration of gentian violet.

Resistance to gentian violet. The resistance of the different strains to gentian violet was tested (Fig. 7).

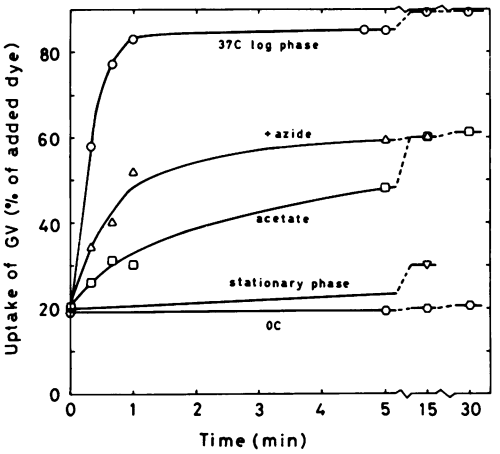

FIG. 4. *Uptake of gentian violet in the envA strain D22 under different growth conditions. The bacteria were grown in Casamino Acids medium to a cell density corresponding to 50 Klett units or overnight and then diluted to 50 Klett units. One culture was grown to 50 Klett units in minimal acetate medium (acetate). One sample of the cells harvested in the exponential growth phase in Casamino Acids medium was chilled to 0 C. To a second sample sodium azide (1 mM) was added 5 min before zero time (azide). Gentian violet (10 μg/ml) was added at zero time, and the uptake of the dye was assayed at intervals.*

DISCUSSION

Kinetics of gentian violet uptake. The uptake of gentian violet consists of at least two processes, in this paper referred to as type I and type II. Type I was instantaneous, independent of energy metabolism, and was the same for all mutants tested as well as for the parent strain (Fig. 1). The dye taken up by this process is solely located in the envelope of the bacteria (Table 2). We think that this uptake represents a binding to the surface layers of the bacteria.

Type II uptake of gentian violet is affected by agents known to block energy metabolism (Fig. 4). It represents a transport into the cytoplasm, where the main part of the dye is bound to the ribosomal fraction (Table 2).

The parent strain D21 showed no uptake of type II. However, spheroplasts (lysozyme-EDTA) of strain D21 showed the same rapid uptake of gentian violet as did the *envA* mutant D22. The inability of intact D21 cells to display type II uptake can thus be attributed to a penetration barrier in the cell envelope. It is known that EDTA treatment partially removes the lipopolysaccharide from *E. coli* (11, 12) and increases its sensitivity to a number of agents. EDTA did not affect the surface binding but made the cells of strain D21 able to express uptake of type II, although at a slightly lower rate than that of spheroplasts (Fig. 5).

The results discussed above indicate that the cell envelope is a barrier preventing the penetration of gentian violet into the cells. The mutants D21e19 (*lpsB*), D21e7 (*lpsA*), and D21e8 (*galU*) have an increasing deficiency of carbohydrates in their lipopolysaccharide (see Table 1). Figure 1 shows that the rate of type II uptake increases with the carbohydrate deficiency. It is therefore clear that the lipopolysaccharide is of importance for the normal barrier function in *E. coli*. Indirect evidence for such a function has previously been obtained using sodium cholate (6).

The *envA* mutant D22 shows an extremely rapid uptake of gentian violet. However, the carbohydrate composition of the lipopolysaccharide is not altered in this mutant (Table 1). Moreover, the amount of phenol-extractable LPS from purified outer membranes of wild type and *envA* strains did not differ (30). Therefore, other components in the cell envelope must also be of importance for barrier function. When outer membranes from *envA* and wild-type strains were compared, a relative decrease in the amount of phosphatidylglycerol was observed in the former (30). We do not know whether this difference is the sole explanation for the drastic increase of type II uptake. The possibility that the murein sacculus also acts as a penetration

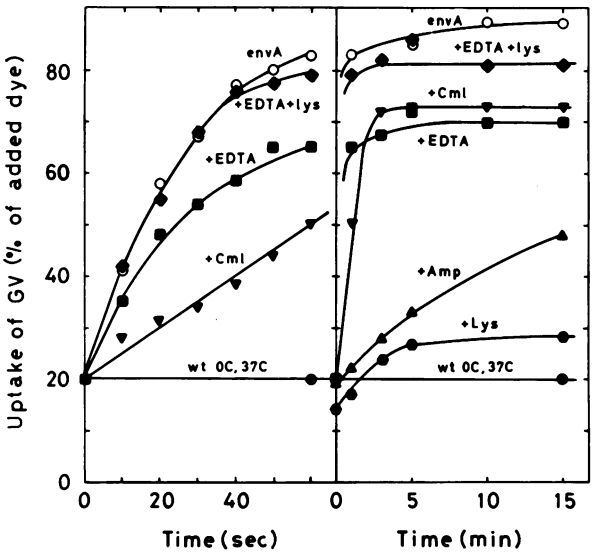

FIG. 5. *Uptake of gentian violet in the wild-type D21 treated in various ways. Strain D21 (wild type) was grown in Casamino Acids medium. At a cell density corresponding to 25 Klett units, D-ampicillin (Amp) (10 μg/ml) or lysozyme (lys) (100 μg/ml) was added to two samples. Incubation continued until a cell density corresponding to 50 Klett units was reached (zero time in figure). Chloramphenicol (cml) (100 μg/ml) was added to a third sample. A sample was chilled to 0 C. Part of the untreated culture was centrifuged, resuspended in K-phosphate buffer (0.01 M, pH 7.4) and treated with 0.01 M EDTA for 5 min, centrifuged, and resuspended in growth medium. Finally, a sample harvested at 50 Klett units was resuspended in Tris buffer (0.03 M, pH 8.0) containing 30% (wt/vol) of sucrose. Lysozyme (400 μg/ml) and EDTA (0.01 M) were added, and incubation at 37 C was continued for 10 min. The spheroplasts were harvested by centrifugation and resuspended in 37 C Casamino Acids medium containing sucrose (30% [wt/vol]). As a control the envA strain D22 was grown in Casamino Acids medium to 50 Klett units. Gentian violet was added (10 μg/ml), and the uptake of the dye was measured at intervals.*

barrier has been discussed in some recent papers (3, 10, 19). The recent observation that *envA* cells seem to have an elevated *N*-acetylmuramyl L-alanine amidase activity (B. Beck, personal communication) and an increased turnover of diaminopimelic acid (S. Normark, unpublished data) may therefore be relevant when discussing the nature of the EnvA phenotype. When D21 (wild type) cells were grown in the presence of lysozyme and ampicillin, type II uptake was induced (Fig. 5). This could reflect the participation directly or indirectly of the murein in the penetration barrier. Proof that murein contributes to the barrier must await further analyses of the composition and biosynthesis of murein in antibiotic-supersensitive mutants.

Gentian violet uptake of type II was also obtained when the parent strain D21 was treated with chloramphenicol (Fig. 5). This suggests that the outer membrane is affected by chloramphenicol. This is consistent with the finding of Rothfield and Pearlman-Kothlencz (20) that outer membrane fragments are excreted into the medium when *E. coli* or *Salmonella typhimurium* are treated with chloramphenicol or starved for a required amino acid. It has also been reported that treatment of *E. coli* with chloramphenicol renders the cells sensitive to lysozyme (17) and also causes a leakage of periplasmic β-lactamase into the medium (A. Lundbäck, Ph.D. thesis, Univ. of Umeå, Sweden, 1973). Chloramphenicol treatment also greatly increases the cholate sensitivity of *Pseudomonas aeruginosa* (unpublished data).

In conclusion, we have shown that the cell envelope constitutes a barrier to the penetration of gentian violet into the cytoplasmic membrane for further transport into the interior of the cell. The lipopolysaccharide is an important part of the normal barrier function, but other constituents of the outer membrane, e.g. phospholipids, are also important. The murein sacculus itself may be a component of the barrier or may indirectly affect the integrity of the outer membrane.

Cell envelopes of gram-positive and gram-negative bacteria show similar binding of crystal violet (29). The uptake data presented in

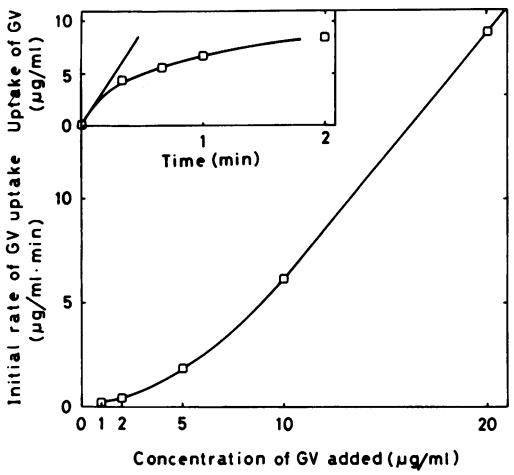

FIG. 6. *Initial rate of gentian violet uptake in the galU strain D21e8. Experimental conditions were as in Fig. 1. At zero time different amounts of gentian violet were added. The uptake of dye was measured at intervals. The initial rate was estimated as shown in the insert.*

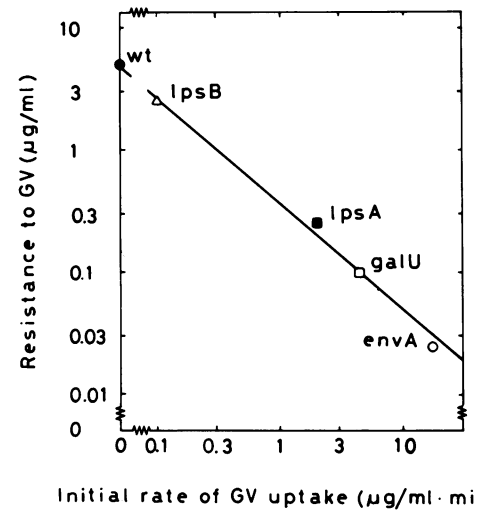

FIG. 7. *Resistance to gentian violet as a function of initial rate of gentian violet uptake in strain D21 (wild type) and its envelope mutants D22 (envA), D21e8 (galU), D21e7 (lpsA), and D21e19 (lpsB). The resistance to gentian violet was measured as described in Materials and Methods. The initial rate of uptake of the dye was determined as described in Fig. 6; 10 μg of gentian violet was added per ml of culture.*

this paper may have some relevance for an explanation of how the Gram reaction discriminates between gram-positive and gram-negative bacteria.

Model for the uptake of gentian violet. We propose that the uptake of gentian violet proceeds by the following scheme (see Fig. 8). (i) First there is a strong binding of gentian violet, presumably to negative groups on the surface of the bacteria. The extent of this binding can be estimated from the lower curve in Fig. 8, which can be extrapolated to 0.35 μg of gentian violet per ml. This value corresponds to about 2×10^8 molecules of gentian violet per bacterium. (ii) When these sites are saturated, more gentian violet molecules bind to the surface, but these molecules are less tightly bound to the cells. The type I uptake is the same in the parent strain and in the envelope mutants. Hence, both the firmly and the loosely bound molecules are located outside the penetration barrier. (iii) In the envelope mutants, gentian violet molecules are allowed to pass the barrier. We believe that this passage is simple diffusion and that it results in an increase in gentian violet molecules at the outside of the cytoplasmic membrane. (iv) The gentian violet molecules present at the outside of the cytoplasmic membrane are then transported through the membrane by an unknown process. (v) In the cytoplasm the majority of the gentian violet molecules bind to the ribosomes. This binding leads to a reduction of the concentration of gentian violet in solution in the cytoplasm and explains why a flux of molecules into the cells results that gives a 1,000-fold enrichment (strain D22 at 10 μg of gentian violet per ml, Fig. 1).

Sensitivity to gentian violet. The most likely explanation for the toxicity of gentian violet is that the ribosomes to which the dye is bound functions less efficiently in protein synthesis. The envelope mutants studied in this work all exhibited virtually the same total uptake of gentian violet (Fig. 1). Therefore,

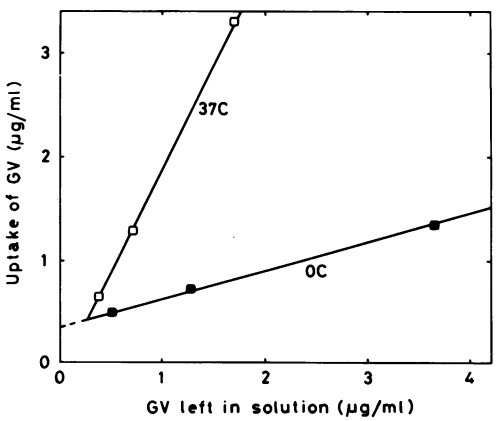

FIG. 8. *Total uptake of gentian violet by the galU strain D21e8 at 37 and 0 C. Experimental conditions were as in Fig. 3.*

these plateau values cannot explain the great differences in resistance levels shown by the mutants. However, the mutants differ greatly in the rate of gentian violet uptake (type II) (Fig. 1). The resistance levels have been plotted against the initial rate of type II uptake (Fig. 7). This figure shows that the more rapid the uptake of gentian violet the less is the resistance level. We conclude that the resistance level is determined by the ratio between rate of volume increase and rate of gentian violet uptake.

We hope that the scheme outlined above for the uptake of gentian violet will also prove to apply to other antibacterial agents against which there is a penetration barrier.

ACKNOWLEDGMENTS

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