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Nature of the Penetration Barrier in *Escherichia coli* K-12: Effect of Macromolecular Inhibition on Penetrability in Strains Containing the *envA* Gene

STAFFAN NORMARK AND BRITTA WESTLING

Department of Microbiology, University of Umeå, S-901 87 Umeå 6, Sweden

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The *envA* mutation in *Escherichia coli* K-12, which maps at 1.5 min, was previously shown to mediate sensitivity to gentian violet as well as to several antibiotics. Moreover, strains containing the *envA* gene were recently found to be lysed by lysozyme in the absence of ethylenediaminetetraacetate. It is here reported that the *envA* mutation mediates an increased uptake of gentian violet. The uptake of the dye was markedly affected by growth with different antibiotics interfering with macromolecular synthesis. Amino acid starvation of a strain containing *envA* with a stringent control of ribonucleic acid (RNA) synthesis resulted in a decreased uptake of gentian violet. However, no decrease in dye uptake was found during starvation in an *envA* transductant with a relaxed control of RNA synthesis. Inhibition of deoxyribonucleic acid (DNA) synthesis by nalidixic acid decreased the uptake of gentian violet of *envA* cells and, in addition, rendered the cells insensitive to the lytic action of lysozyme. Chloramphenicol treatment increased penetrability in wild-type and starved *envA* cells. In most instances, this effect of chloramphenicol was prevented by selectively interfering with DNA or RNA synthesis. A coordinate regulation of nucleic acid synthesis and penetrability is suggested.

Gentian violet (GV) and other basic dyes have a high affinity for nucleic acids (9, 25). Resistance and sensitivity to GV and other basic dyes have been attributed to a number of genes located close to the marker for lactose utilization (13, 14, 22). In a report by Nakamura (13), it was also concluded that resistant strains had a lower capacity to bind basic dyes. However, Kushner and Khan (10) found that proflavine-sensitive and -resistant strains bound the same amount of the dye and that binding of proflavine was a passive process. A release of bound dye caused the increased tolerance of the resistant cells.

It was previously found that the *envA* mutation increased sensitivity to GV as well as to several antibiotics and that it mediated an unspecific increase in permeability through the outer layers of the cell envelope (16). It is reported here that sensitivity to GV is correlated to an increased uptake of the dye. The uptake of GV was markedly decreased during amino acid starvation in *envA* strains with stringent but not with relaxed ribonucleic acid (RNA) control. Treatment with antibiotics affecting macromolecular synthesis markedly affected uptake of GV and the response to lysozyme in both wild-type and *envA* strains.

MATERIALS AND METHODS

Organisms. Strain D21 of *Escherichia coli* K-12 was described by Boman et al. (3). Strain D22 is an ethyl methane sulfonate-induced mutant from strain D21 (19). The altered gene *envA* at 1.5 min on the chromosomal map mediates chain formation and an increased penetrability to antibacterial agents (16). The isogenic transductant pair E64-113 (wild type) and E64-120 (*envA*) and the spontaneous revertant D22S1 were described by Normark (16). Strain CP791 is an *envA* transductant of the *rel* strain CP79 (5).

Media and growth conditions. The minimal medium used was medium E of Vogel and Bonner (24), supplemented with 0.2% glucose, 1 μg of thiamine per ml, and 50 μg of the L-epimer of the required amino acid per ml. The complete medium was the LB medium of Bertani (2) supplemented with medium E and 0.2% glucose. The Casamino Acids medium contained the basal minimal medium and 0.2% casein hydrolysate supplemented with L-tryptophan (50 μg/ml) and 0.2% glucose. The bacteria were always grown at 37 C. Growth was recorded by optical density readings using a Klett-Summerson colorimeter with a W66 filter.

Materials. Chloramphenicol was purchased from Erco, Stockholm, Sweden; GV was from Merck, Darmstadt, Germany; nalidixic acid was obtained from Winthrop Ltd., Surbiton on Thames, England; rifampin was obtained as a gift from I. Oeschger, New Haven, Conn.; lysozyme, three times crystallized, was obtained from Sigma Chemical Co., St. Louis, Mo.

Uptake of GV. Samples of 5 or 10 ml of the cell culture were immediately chilled and centrifuged. The pellet was resuspended in medium E supplemented with 1 μg of thiamine per ml and 0.2% glucose, containing GV (in most experiments 10 μg/ml). In all experiments the amount of GV per unit cell mass was kept constant. In most uptake experiments, the cell concentration was kept to 2×10^8 cells/ml, corresponding to 50 Klett units. The samples were incubated on a rotary shaker for 13 min at 37 C and then centrifuged. The amount of GV remaining in the supernatant fluid was measured with a Hitachi spectrophotometer at a wavelength of 590 nm.

RESULTS

Uptake of GV. It was previously shown that the EnvA phenotype decreased resistance to the basic dye GV (16). A further study of this effect showed that fast-growing log-phase cells of the *envA* strain D22 took up considerably more dye than the parental strain D21 (Fig. 1). This was true over a wide range of GV concentrations. Almost 90% of the added dye was taken up by strain D22 compared to 45% for the parent strain. A considerable difference in dye uptake was found irrespective of whether the strains were grown in LB, Casamino Acids or minimal medium.

Effect of amino acid starvation on uptake of GV. To follow the uptake of GV during tryptophan starvation, strains D21 and D22 were grown in Casamino Acids medium supplemented with tryptophan. At zero time the cultures were filtered, washed, and resuspended in prewarmed Casamino Acids medium without tryptophan. After 80 min of starvation, tryptophan was readded. Samples were taken at different time intervals, and ability to take up GV, optical density, and RNA content were followed. Figure 2 shows that for the *envA*-containing strain D22 there was a rapid decrease in the GV uptake about 10 min after the onset of amino acid starvation. After 35 min of starvation a plateau was reached. When tryptophan was readded to the culture there was an increasing uptake level for 35 min, at which time the normal high level of bound dye was reached. For the parent strain D21, starvation had little effect, and only a slight decrease in dye uptake was found during the starvation period. The RNA synthesis in both strains was inhibited during starvation, indicating a stringent control of RNA synthesis. After readdition of tryptophan, the RNA synthesis was

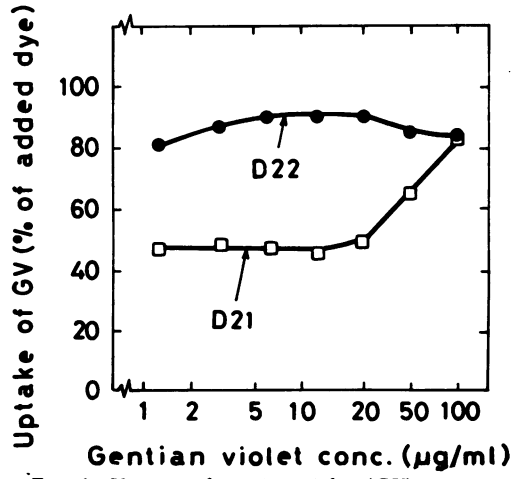

FIG. 1. *Uptake of gentian violet (GV) in strains D21 (wild type) and D22 (envA). The strains were grown in LB medium to a density of* 4×10^8 *cells/ml. Samples of 5 ml were chilled, centrifuged, and suspended in 5 ml of medium E containing 0.2% glucose and different concentrations of GV. After 13 min of incubation at 37 C, the dye remaining in the supernatant fluid after centrifugation was measured.*

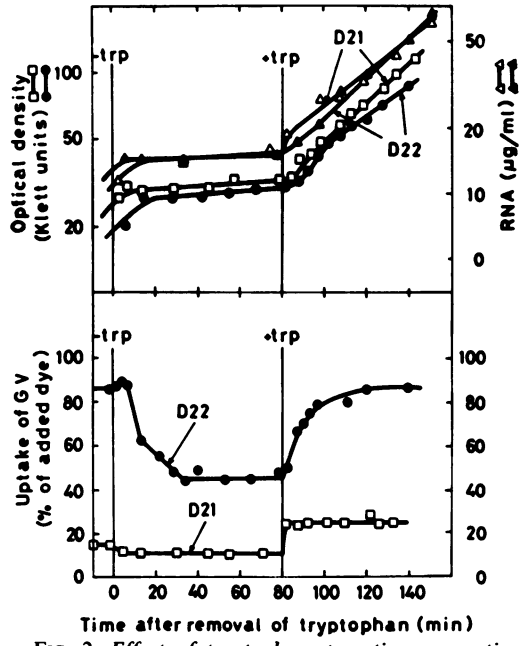

FIG. 2. *Effect of tryptophan starvation on gentian violet (GV) uptake, RNA content, and growth in strains D21 and D22. Both strains were grown to a density of* 10^8 *cells/ml in 150 ml of Casamino Acids medium supplemented with* L*-tryptophan (50 μg/ml). At zero time, tryptophan was withdrawn by filtration and extensive washing with Casamino Acids medium. The bacteria were then incubated in 150 ml of Casamino Acids medium for 80 min, when* L*-tryptophan was added to a final concentration of 50 μg/ml. Samples of 5 ml were withdrawn at various time intervals, and the ability of the cells to take up GV was followed. RNA content was measured on 1-ml samples precipitated with 5% trichloroacetic acid. After three extractions with 0.5* N *perchloric acid at 90 C for 20 min, RNA was determined by the orcinol method (20).*

slightly higher in the *envA* mutant D22. Immediately after the starvation both strains had similar growth rates, but after 20 min there was a decrease for the *envA* mutant D22.

To investigate whether the decreased dye uptake of amino acid-starved D22 cells could be related to the inhibition of RNA synthesis, the *envA* gene was transduced into strain CP79 with a relaxed control of RNA synthesis. Strains D22 and the *envA* transductant CP791 were grown in a minimal medium. The cultures were filtered, washed, and resuspended in prewarmed minimal medium lacking histidine. At zero time and after 30 min of histidine starvation, samples of the CP791 culture were taken out and treated with rifampin (20 μg/ml). Samples were taken at different time intervals, and the ability to take up GV was followed. Figure 3 shows that for strain D22 there was a rapid decrease in GV uptake during starvation, whereas no change was found for strain CP791. However, inhibition of RNA synthesis by rifampin in the latter strain resulted in a considerably decreased dye uptake. When rifampin was added at the onset of the starvation period, the uptake curves for strain D22 and rifampin-treated CP791 cells did coincide.

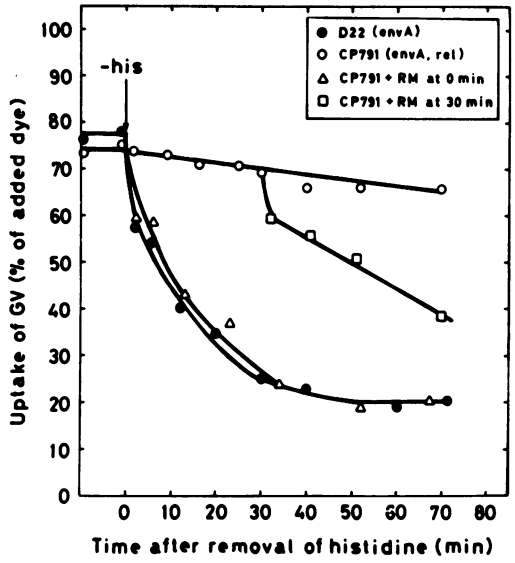

FIG. 3. *Effect of histidine starvation on gentian violet (GV) uptake in strains D22 (envA) and CP791 (envA, rel). Both strains were grown to 3 × 10⁸ cells/ml in 100 ml of minimal medium containing the L-epimer of proline, tryptophan, arginine, and histidine at 50 μg/ml. At zero time, histidine was withdrawn by filtration and washing with minimal medium lacking histidine. The bacteria were then incubated in the same minimal medium (100 ml) but without histidine. Samples of the starved CP791 culture were taken out at zero time and after 30 min of starvation, and were treated with rifampin (RM) at 20 μg/ml. Samples (5 ml) were withdrawn at various time intervals, and the ability of the cells to take up GV was followed.*

The effect on GV uptake of an uncoupling of RNA from protein synthesis was studied in starved cells of strain D22 (1). A culture of the *envA* mutant D22 was starved for tryptophan. After 52 min the culture was divided into four portions. One culture was kept as a control; to the others were added chloramphenicol (100 μg/ml), rifampin (20 μg/ml), and chloramphenicol and rifampin at the given concentrations. After 20 min of treatment with chloramphenicol, rifampin (20 μg/ml) was added to a sample of the chloramphenicol-treated culture. Figure 4 shows that uptake of GV was greatly affected by addition of chloramphenicol. For 40 min there was a gradual increase in the uptake of GV, until the cells showed the same uptake as nonstarved D22 cells. Addition of rifampin (20 μg/ml) to starved cells caused a slight decrease in dye up-

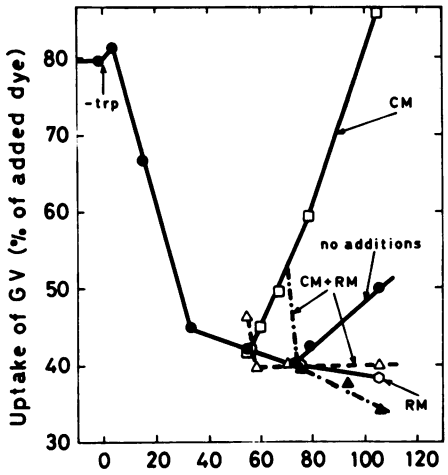

FIG. 4. *Effect of chloramphenicol (CM) and rifampin (RM) on the uptake of gentian violet (GV) in tryptophan-starved D22 cells. The envA mutant D22 was grown in 200 ml of Casamino Acids medium supplemented with L-tryptophan (50 μg/ml) to a density corresponding to 3 × 10⁸ cells/ml. Tryptophan was withdrawn by filtration and extensive washing. The bacteria were then incubated in 200 ml of Casamino Acids medium. After 52 min of starvation, the starved culture was divided into four portions. One was kept as control; chloramphenicol, rifampin, and chloramphenicol plus rifampin were added to the other respective portions. From the chloramphenicol-treated culture, a sample was taken out after 72 min of starvation, and rifampin was added. Samples (5 ml) were taken at different time intervals, and the ability of the cells to take up GV was followed. Symbols: no additions (●); 100 μg of CM per ml (□); 20 μg of RM per ml (○); 100 μg of CM and 20 μg of RM per ml (△); 100 μg of CM and 20 μg of RM per ml after 20 min (▲).*

take. When rifampin was added together with chloramphenicol, the effect of the latter antibiotic was completely blocked. When rifampin was added 20 min after chloramphenicol, there was a rapid decrease in dye uptake.

Modification of penetrability by antibacterial agents affecting macromolecular synthesis. Like all gram-negative bacteria, strain D21 is insensitive to the lytic action of lysozyme in the absence of ethylenediaminetetraacetate. Strain D22, however, was recently found to be lysozyme sensitive (18). To investigate whether inhibition of protein, RNA, or deoxyribonucleic acid (DNA) synthesis could modify the response towards lysozyme and GV uptake, exponentially growing LB cultures of D21 and D22 were treated with chloramphenicol, rifampin, or nalidixic acid. Incubation in the presence of the respective antibiotic was continued for an additional time of 60 min. The bacteria were then harvested by centrifugation and tested for their resistance to lysozyme (100 μg/ml) in tris(hydroxymethyl)aminomethane buffer (0.05 M, *p*H 7.4). Table 1 shows that chloramphenicol (100 μg/ml) induced sensitivity to lysozyme in strain D21 at the same time that it caused a marked uptake of GV. However, if RNA synthesis was inhibited by rifampin or DNA synthesis was inhibited by nalidixic acid, chloramphenicol could not induce lysozyme sensitivity. Rifampin alone caused no lysozyme sensitivity despite the fact that it caused an increased uptake of GV.

In the lysozyme-sensitive strain D22, lysis was prevented by 60 min of treatment with nalidixic acid. The combination of chloramphenicol and nalidixic acid as well as chloramphenicol and rifampin caused a lesser degree of lysis than chloramphenicol alone. Strain D22S1, an ampicillin-resistant revertant of D22, and an isogenic pair of transductants, E64-113 and E64-120, are included in Table 1, showing that both lysozyme sensitivity and an increased uptake of GV was correlated to the *envA* mutation. The transductants showed a similar response to lysozyme and GV uptake after antibiotic treatment. However, lysozyme resistance and a low GV uptake were induced in strain E64-120 (*envA*) after both rifampin and nalidixic acid treatment.

Chloramphenicol-treated cells of the parent strain D21 showed a considerably increased uptake of GV for at least 90 min after removal of the agent. For this reason one would expect such cells to show a decreased tolerance to different antibacterial agents. Since the *envA* mutant D22 showed a 100-fold decrease in tolerance to rifampin (16), it was suggested that in *E. coli* the normal tolerance to this antibiotic is due to difficulties in penetration into the cell. Strain D21 was therefore pretreated for 60 min in LB medium with chloramphenicol (100 μg/ml) and then spread on LA plates containing different concentrations of rifampin. Figure 5 shows that such pretreated D21 cells were highly sensitive to rifampin. A rifampin concentration of 0.05 μg/

TABLE 1. *Effect of inhibition of macromolecular synthesis on lysozyme sensitivity and uptake of gentian violet (GV)*

Strain	Treatment[a]	Lysozyme lysis[b] (%)	GV uptake[c] (% of added dye)
D21 (wild type)	No additions	6	19
D21 (wild type)	CM (100 μg/ml)	73	90
D21 (wild type)	RM (100 μg/ml)	0	70
D21 (wild type)	NAL (100 μg/ml)	2	15
D21 (wild type)	CM + RM (100 μg/ml)	2	89
D21 (wild type)	CM + NAL (100 μg/ml)	11	85
D22 (*envA*)	No additions	68	91
D22 (*envA*)	CM (100 μg/ml)	78	79
D22 (*envA*)	RM (100 μg/ml)	41	70
D22 (*envA*)	NAL (100 μg/ml)	13	37
D22 (*envA*)	CM + RM (100 μg/ml)	55	70
D22 (*envA*)	CM + NAL (100 μg/ml)	53	77
D22S1 (*envA*, *sup-200*)	No additions	0	28
E64-113 (wild type)	No additions	0	18
E64-120 (*envA*)	No additions	81	78

[a] Abbreviations: CM, chloramphenicol; RM, rifampin; NAL, nalidixic acid.

[b] Lysis in tris(hydroxymethyl)aminomethane buffer (0.05 M, *p*H 7.4) at room temperature was determined in a Klett-Summerson colorimeter after 60 min of lysozyme treatment at a concentration of 100 μg/ml.

[c] Uptake of GV (10 μg/ml) was determined on 5-ml samples as described in Materials and Methods.

ml caused 80% killing. This concentration had no effect on untreated cells.

DISCUSSION

Strains containing *envA* show the same increased sensitivity to GV as to other antibacterial agents (16). This sensitivity was correlated to an increased uptake of GV (Fig. 1). The uptake of the dye was found to be a rapid process and was completed within 2 min. The revertant, strain D22S1, showed an almost normal uptake, whereas two *envA* transductants were found to have a high dye uptake (Table 1 and Fig. 3). Other mutants showing an intermediate tolerance to GV were found to take up intermediate amounts of the dye (*unpublished data*). These results show that the *envA* mutation causes an increased uptake of GV which, in turn, gives an increased susceptibility to the dye. The lethal effect of GV is not known. However, GV, like other basic dyes, is known to bind to nucleic acids and to interfere with DNA and RNA synthesis (9, 25). This is in agreement with the finding that purified cell envelopes from strains D21 and D22 bind only a small percentage of the added dye.

The experiments reported here as well as those of the accompanying paper (17) suggest an intimate relationship between nucleic acid biosynthesis and penetration of drugs through the outer layers of the envelope. When DNA synthesis was inhibited by nalidixic acid (7), or when RNA as well as protein synthesis were inhibited by amino acid starvation (11), the *envA* mutant D22 showed a decreased uptake of GV (Table 1, Fig. 2–3). Furthermore, nalidixic acid induced tolerance to the lytic action of lysozyme (Table 1). It is therefore evident that in the *envA* mutant D22 the outer layers may regain their barrier function by an inhibition of DNA synthesis which allows protein and RNA synthesis to proceed. This effect of nalidixic acid was not caused by an induction of prophage λ, as a decreased dye uptake and lysozyme resistance were also obtained after nalidixic acid treatment in the nonlysogenic *envA* transductant E64-120.

During amino acid starvation, both strains D21 and D22 showed a stringent control of RNA synthesis (Fig. 2). When tryptophan was readded to starved cells of D22, RNA synthesis immediately started and cells rapidly increased the uptake of GV (Fig. 2). In separate experiments, it was also found that DNA synthesis did not start until about 40 min after addition of the amino acid. The *envA* transductant CP791 with a relaxed RNA control (5) showed no decrease in dye uptake during histidine starvation. However, when RNA synthesis was inhibited by rifampin (4, 23), a considerable decrease in dye uptake was found (Fig. 3). For these reasons, it is believed that in strain D22 inhibition of RNA synthesis during amino acid starvation is closely related to the decreased uptake of GV. This hypothesis was also supported by the following results.

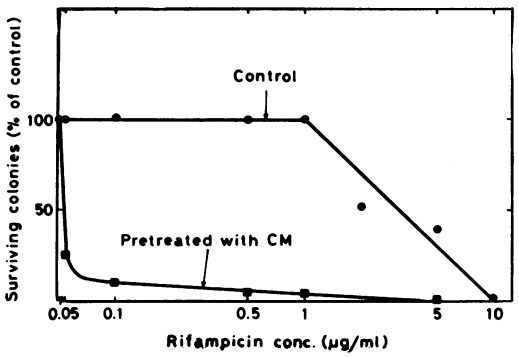

FIG. 5. *Effect of preincubation in the presence of chloramphenicol (CM) on the tolerance to rifampin (rifampicin). A log-phase culture of strain D21 was divided into two parts. One was kept as a control, and CM (100 μg/ml) was added to the other. After a 60-min treatment with CM, the two cultures were diluted and spread on LA plates containing different concentrations of rifampin. [For a discussion of this method see Nordström et al. (15).].*

When chloramphenicol was added to a starved culture of D22, there was a rapid increase in the uptake of GV (Fig. 4). This effect of chloramphenicol was completely blocked by inhibition of RNA synthesis by rifampin. Addition of rifampin after 20 min of chloramphenicol treatment rapidly decreased the uptake of GV. In starved cells of D22, chloramphenicol might therefore exert its effect on the uptake of dye by changing the concentration of some RNA precursors (e.g., the nucleoside triphosphates) rather than by increasing the RNA content. Starvation of strains with stringent RNA control causes an inhibition of lipid synthesis, whereas lipid synthesis continues during starvation in relaxed strains (21). Therefore the relationship between protein and lipid synthesis appears to be under the same control as is that between RNA and protein synthesis. In a similar way, chloramphenicol interferes with the coupling between protein and lipid synthesis during starvation in stringent mutants (21). The nature of the coupling mechanism is unclear, but it may operate through variations in the pool size of nucleotides (6, 8). Lipid changes are found in the cell envelope of strains containing *envA* (17). Thus it is possible that, during starvation of strain D22, inhibition of lipid biosynthesis results in a rearrangement of lipids in the outer layers of the cell

envelope, which subsequently will affect penetrability.

In the wild-type strain D21, chloramphenicol treatment resulted in an increased uptake of GV and an increased susceptibility to rifampin and lysozyme (Table 1 and Fig. 5). The chloramphenicol effect on lysozyme lysis could be inhibited by either nalidixic acid or rifampin (Table 1). The induced lysozyme sensitivity may therefore be caused by an uncoupling of RNA and DNA synthesis from protein synthesis. Similar conclusions were drawn by Matzura and Broda (12), who showed that during chloramphenicol treatment the residual RNA synthesis was sensitive to actinomycin D. Thus, in wild-type strains, an increased penetrability can be achieved when nucleic acid synthesis proceeds in the absence of protein synthesis.

Recent data have shown that strain D22, when grown at its maximal growth rate, contains more total RNA than its parent strain D21 (*unpublished data*). Moreover, after a starvation period the RNA synthesis was slightly higher in the *envA* mutant D22, despite the fact that it had a longer generation time than its parent strain D21 (Fig. 2). Whether this is a primary or a secondary effect of the *envA* mutation is not known. It is possible that, in the *envA* strain D22, an overproduction of RNA is related to the increased penetrability.

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