JOURNAL OF BACTERIOLOGY, Apr. 1971, p. 1-13
Vol. 106, No. 1

Colicin Tolerance Induced by Ampicillin or Mutation to Ampicillin Resistance in a Strain of *Escherichia coli* K-12

LARS G. BURMAN AND KURT NORDSTRÖM

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

Received for publication 28 December 1970

A mutant (G11e1) of *Escherichia coli* selected as being resistant to ampicillin and showing signs of an envelope defect was also found to be tolerant to colicins E2 and E3. The colicin tolerance of G11e1 could be partially repressed by Mg^{2+} ions. Transition from tolerance to sensitivity and vice versa by shifting the concentration of Mg^{2+} in the growth medium required several generations. This indicated that synthesis of new envelope material was needed for transition. Previous physiological results have indicated a change in the envelope lipopolysaccharide (LPS) of G11e1. However, chemical analyses revealed no differences in carbohydrate composition between LPS from G11e1 and its parent strain G11a1. Genetic experiments showed that the mutation in G11e1 is located at about 20 min on the *E. coli* K-12 chromosome. The mutation was dominant over wild type in partial diploids with the mutation located on the episome. Because colicin tolerance was the most striking phenotypic effect as a result of mutation in the actual locus, this gene will be named *tolD* until the exact gene product is known. Spheroplasts formed from G11a1 and G11e1 by ethylenediaminetetraacetate-lysozyme treatment did not adsorb colicin E2; however, penicillin spheroplasts of G11a1 and G11e1 were tolerant to colicin E2. Thus, colicin tolerance can be induced biochemically. It is suggested that colicin tolerance often is a secondary consequence of a change in the cell envelope.

The killing of bacteria by colicins and by abortive phage infection shows certain similarities (32). Irreversible adsorption of colicins or phages to receptor sites on the bacterial surface initiates a lethal process. These receptors are assumed to be specific and limited in number and can be lost by mutation. Such a mutant bacterium is referred to as resistant to the actual colicin or phage. Sometimes receptors are common to groups of phages or colicins and even shared by a colicin and a phage (15). Such conclusions are mainly based on cross-resistance patterns. T-phage receptors have been subject to certain studies of their composition and localization in the *Escherichia coli* cell envelope (1, 53), but knowledge about the postulated colicin receptors is poor.

Adsorption of a colicin molecule to the surface of a sensitive bacterium leads to drastic intracellular changes and death of the cell (39). However, the colicin molecule remains outside the cytoplasmic membrane during this process, accessible to inactivation by trypsin (41). This conclusion was supported by experiments with radioactive colicin (33). In cell-free systems, the colicin molecules are harmless (27, 38).

A model has been proposed according to which the colicin molecule, while remaining at its receptor site, affects the intracellular target indirectly through a specific signal system in which the cytoplasmic membrane would play an important role (39). To elucidate such a system, Nomura (38) and Clowes (9) had independently selected bacterial mutants to which colicins were adsorbed without being able to transmit their lethal message. Such "colicin tolerant" (*tol*) mutants have now been isolated by several workers (10, 21, 36, 42, 48). It has been proposed that *tol* mutants might not only give information about a colicin transmission system but also enable a systematic genetic study of the cytoplasmic membrane (36).

By using mainly colicins E1, E2, E3, and K as selective agents, many classes of *tol* mutants with different tolerance patterns have been found in *E. coli* K-12. The corresponding mutations have been shown to occur in distinct loci on the *E. coli*

chromosome, always different from that of the receptor shared by E colicins and phage BF23 (26, 40). Most *tol* mutants belong to one of three multiple tolerant classes mapping near *gal* at 17 min on the *E. coli* K-12 chromosome map. However, the complexity of this picture has been steadily increasing. Depending on the strain and the colicin used for mutant selection, the frequency of occurrence of a particular mutant type varies greatly. By using only colicin E2 for selection, at least eight different *tol* mutant classes have been obtained (42). Furthermore, mutation in one gene may cause tolerance to several colicins with completely different mechanisms of action.

During isolation of ampicillin-resistant mutants of *E. coli* K-12 (Hfr Cavalli) in this laboratory, several classes of mutants have been found. Mutants of class I are mutated in gene *ampA* at 82 min on the *E. coli* genetic map (13) and synthesize 10 times more of the cell-bound penicillinase than is normally found in most *E. coli* K-12 wild-type strains (29). Mutations of class II are defined by their doubling of penicillinase-mediated ampicillin resistance on plates (44) without increasing the penicillinase activity of the cells. Mutants of class II are changed in the properties of the cell envelope (43). The physiology of a class II mutant G11e1 (*ampA*, *tolD; see below*) and its class I parent strain G11a1 (*ampA*) has been studied in more detail (43). In G11e1 the colonies are mucoid, the cell envelope is fragile, and part of the periplasmic penicillinase leaks out. Adsorption of phage T4 is slower in G11e1 than in the parent strain G11a1. G11e1 also shows high sensitivity to sodium cholate and ethylenediaminetetraacetate (EDTA) and changed resistance to dyes and many antibiotics in addition to penicillins, probably as a result of changed penetrability by these substances through the cell envelope. R-factor-mediated antibiotic resistance is influenced by the class II mutation in the same manner as is chromosomal resistance.

Moreover, G11e1 is colicin tolerant, and in this paper the comparison between strains G11e1 and G11a1 is continued by experiments with colicins of the E group. We show that the class II mutation in G11e1 has occurred in a new gene denoted *tolD* until the gene product is known. The *tolD* gene is mapped and found to be located close to 20 min on the *E. coli* K-12 chromosome. Finally, we report that colicin tolerance can be induced by biochemical treatment of the bacterial envelope, and the basis for colicin tolerance is discussed.

MATERIALS AND METHODS

Bacteria, media, and growth conditions. The *E. coli* K-12 strains used are listed in Table 1. G11a1 is a spontaneous mutant of G11, and G11e1 is derived from G11a1 (14). Single cells of strains G11, G11a1, and G11e1 are resistant to 2, 15, and 25 μg of D-ampicillin per ml, respectively. G11a11 is a spontaneous colicin E2-resistant mutant isolated from G11a1 (*see below*). The F^- strains RE149, MC100, MS3, and MS31 were made lysogenic for phage λ. Samples of *E. coli* W3110 colicinogenic for colicin E1, E2, or E3 were obtained from D. Helinski. Phage BF23 was obtained from M. Nomura. The ribonucleic acid phage MS2 described by Davis et al. (12) was from the stock collection of this department but originated from R. Lavallé.

The minimal medium used was medium E described by Vogel and Bonner (52). It was supplemented with 0.2% glucose, 1 μg of thiamine per ml and 25 μg of the L-enantiomorphs of the required amino acids per ml. When selecting for Gal^+ recombinants, glucose was exchanged by 0.2% galactose. The complete medium used was LB of Bertani (4) supplemented with medium E and 0.2% glucose. It was solidified with 1.5% agar (LA plates). LA plates usually contained 2.5×10^{-3} M $CaCl_2$. In the spheroplast experiments LB-S was used, consisting of LB supplemented with medium E, glucose, 20% sucrose, 8×10^{-3} M $MgSO_4$, and 3×10^{-3} M $CaCl_2$. Soft agar (SA) consisted of Difco nutrient broth (1.3%), 0.6% agar, and 2.5 mM $CaCl_2$. In the spheroplast experiments, SA was supplemented with 20% sucrose and 8×10^{-3} M $MgSO_4$, giving SA-S.

Unless otherwise stated, the experiments were performed at 37 C. The bacteria were cultivated on a rotary shaker (100 rev/min), and optical density of the cultures was measured with a Klett-Summerson colorimeter with filter W66. A Klett reading of 100 units corresponds to 4×10^8 cells/ml growing exponentially in LB medium.

Materials. D-Ampicillin was kindly provided by AB Astra, Södertälje, Sweden. Sodium cholate and EDTA were obtained from Kebo, Stockholm, Sweden. Trypsin was from Mann Research Laboratories, New York, N.Y. Lysozyme and deoxyribonuclease were obtained from Sigma Chemical Co., St. Louis, Mo. Nylon microculture containers used in replica plating were obtained from Elesa, Milan, Italy.

Preparation of colicins. The procedure described by Nagel de Zwaig and Luria (36) was used to prepare colicins. The colicin solutions were stored at −20 C. The colicin titers obtained were about 10^{12} killing units per ml. One killing unit was defined as the amount of colicin that kills one bacterium. Titration of colicin solutions was performed as follows. Sensitive cells (4×10^8 per ml) were incubated in the presence of colicin at 37 C for 10 min. The mixture was diluted 100 times in ice-cold LB medium to inhibit further adsorption. Appropriate dilutions were spread on LA plates for viable counts. Survival (ratio between viable count after and before colicin treatment) was assumed to be equivalent to the zero term in the Poisson distribution, i.e., e^{-m}, where m is the multiplicity (number of killing units of colicin per bacterium).

Determination of colicin adsorption. Adsorption of colicin was measured in the following way. The culture to be tested was grown in LB medium to about 4×10^8 cells per ml. Colicin was added, and incubation was continued for 10 min at 37 C. The cultures were then centrifuged, and the supernatant was assayed for the

TABLE 1. *Escherichia coli K-12 strains used*

Strain	Origin	Sex	Ampicillin resistance class	Auxotrophic markers[a]	Galactose marker	Response to str[a]	λ Lysogenic	Other important characteristics[a]
G11	Stent and Brenner (50)	Hfr[b]	Wild type	*ilv, metB*	+	s	+	
G11a1	Eriksson-Grennberg et al. (14)	Hfr[b]	Class I	*ilv, metB*	+	s	+	
G11e1	Eriksson-Grennberg et al. (14)	Hfr[b]	Class II	*ilv, metB*	+	s	+	
G11a11	This paper	Hfr[b]	Class I	*ilv, metB*	+	s	+	Colicin E2-r[c]
HfrH	W. Hayes (20)	Hfr[b]	Wild type		+	s	−	
KL16	B. Low (30)	Hfr[b]	Wild type		+	s	−	
KL16-99	B. Low (30)	Hfr[b]	Wild type		+	s	−	*recA*
D1	RC711 of Meynell and Datta (35)	F⁻	Wild type	*his, proB, trp*	+	r	+	
D11	Boman et al. (6)	F⁻	Wild type	*his, proB, trp*	+	r	+	
D114	This paper	F⁻	Wild type	*proB, trp*	+	r	+	*recA*[d]
D21	Boman et al. (6)	F⁻	Class I	*his, proB, trp*	+	r	+	
D214	Normark (45)	F⁻	Class I	*proB, trp*	+	r	+	*recA*
D21-C19	This paper, cross 1, Table 7	F⁻	Class II	*proB, trp*	+	r	+	
MC100	Markovitz and Baker (34)	F⁻	Wild type	*leu, pro, purE, trp*	*gal*	r	+[e]	
MS3	Reeve and Doherty (47)	F⁻	Wild type	*pyrD*	*gal*	r	+[e]	
MS31	This paper	F⁻	Wild type	*pyrD, trp*	*gal*	r	+[e]	
RE149	Reeve and Doherty (47)	F⁻	Wild type	*bio*	*gal*	r	+[e]	

[a] Abbreviations: *bio*, biotin; *gal*, galactose; *his*, histidine; *ilv*, isoleucine-valine; *leu*, leucine; *met*, methionine; *pro*, proline; *pur*, purine; *pyr*, pyrimidine; *r*, resistance; *s*, sensitivity; *str*, streptomycin; *trp*, tryptophan. The capital letter after some gene symbols refers to the *E. coli* K-12 genetic map of Taylor (51).

[b] Injection direction and origin: G11 and its derivatives, counter-clockwise, 16 min; HfrH, clockwise, 88 min; KL16 and KL16-19, counterclockwise, 56 min (see Fig. 5).

[c] Spontaneous mutant resistant to colicin E2, isolated from G11a1.

[d] His⁺-recombinant from cross KL16-99 × D11 which has received the *recA* gene.

[e] These strains were lysogenized with phage λ during the progress of this work.

presence of colicin by adding a sensitive indicator strain as described above for titration of colicins.

Isolation of colicin-resistant strains. Cells of G11a1 were harvested in the exponential growth phase and spread on LA plates together with about 10^9 killing units of colicin E2. Colonies appearing after overnight incubation were picked and restreaked on plates with colicin on them. These purified clones were then grown in LB medium and spotted onto an LA plate spread with about 10^7 BF23 phage particles. Colicin E2-resistant clones are resistant to this phage (40). As a final test, adsorption of colicin (*see* Table 3) to the cells was measured. One of the selected clones, G11a11, was not killed by and did not adsorb colicin E2 (*see* Table 3).

Selection of auxotrophs. Selection of auxotrophs was made by the penicillin-recycling method (31). In this way, the tryptophan auxotroph MS31 was isolated from MS3.

Preparation of recA clones. *RecA* strains (30) were prepared by crossing Hfr KL16-99 (*recA*) with a *his*⁻ F⁻ strain and selecting for His⁺ recombinants. A number of recombinants were restreaked on the selective medium and grown in microculture containers (*see below*). LA plates were inoculated with the recombinants by replication and ultraviolet-irradiated for different times. About 10 to 20% of the recombinants tested were considerably more ultraviolet-sensitive than the parent strain and were regarded as *recA* recombinants. They were tested in mating experiments with G11a1 used as donor and gave about 10^{-7} Pro⁺ colonies per input donor. *ProB* is a gene which is transferred early by G11 strains.

Mating experiments. Matings by the gradient method (19) were performed in the following way. The strains were grown logarithmically in LB medium, and about 5×10^7 donor cells per ml were mixed with 5×10^8 recipient cells per ml. After incubation for 5 min at 37 C for conjugated pair formation, the mating mixture was diluted 100 times into prewarmed LB medium, and incubation was continued on a slow rotary shaker for another 3 hr to allow complete chromosome transfer and formation of recombinants from the zygotes before plating on selective medium. When selecting biotin prototrophs, the procedure of Reeve and Doherty (47) was followed to deplete the bacteria of biotin.

Characterization of recombinants and mutants. The recombinants and mutants were tested by replication from microculture containers (Bertani, *personal communication*). These are made of nylon and are autoclavable. They consist of 25 square wells and fit into 9-cm petri dishes. A 0.5-ml amount of medium (usually LB) was added to each well. The wells were then individually inoculated from colonies by means of sterile tooth picks. After incubation, one drop of each culture was

transferred onto the desired plate by a replicator carrying 25 steel needles (3-mm ϕ). Usually overnight microcultures were diluted before replication on plates by two serial transfers to microculture containers with 0.5 ml of 0.9% NaCl in each well. By this procedure, drops containing virtually no LB and about 50 cells were finally replicated. When measuring ampicillin resistance, the resistance levels obtained in this test were slightly higher than those obtained in single-cell tests.

Test for maleness. About 10^8 bacteria were seeded in soft agar, and MS2 was spotted on the agar surface. Plaques were recorded after 4 to 6 hr of incubation at 42 C.

Extraction and analysis of lipopolysaccharide (LPS) of bacterial envelopes. The bacteria were grown in 12 liters of LB in 20-liter bottles with aeration and stirring. They were chilled at the end of log phase (about 10^9 cells/ml) by pouring the culture over ice at −20 C and harvested by centrifugation in a continuous-flow centrifuge. The cells were suspended in 0.05 M potassium phosphate buffer (*p*H 7.2) containing 10 μg of deoxyribonuclease per ml and 5 mM $MgCl_2$. They were broken with 0.17- to 0.18-mm ϕ glass beads (B. Braun, Melsungen, Germany) in a Sorvall Omnimixer, and the envelopes were spun down at 5 C and washed three times with cold water by centrifugation (23,000 × *g*, 20 min). LPS was extracted from envelopes by the phenol-water method (54) or the EDTA method (28), dialyzed, and lyophilized. Hydrolysis of LPS and analysis of sugars by gas-liquid chromatography was done by the method of Holme et al. (25) with the following modification (T. Holme, *personal communication*). Excess sulfuric acid used for hydrolysis was neutralized with $BaCO_3$, the precipitate was filtered off, and sodium borohydride was added. Excess of this compound was destroyed by acetic acid. Methanol was added, and the methyl borate formed was evaporated to dryness. Residual water was removed by two washes in ethanol and toluene (1:1, v/v). The samples were injected into a Perkin-Elmer model 900 gas chromatograph with a 3% ECNSS-M glass column. The column temperature used was 200 C.

N-acetylglucosamine was determined by the Morgan-Elson procedure as described by Ghuysen et al. (16).

Phosphate was determined by the method of Ames and Dubin (2).

RESULTS

Killing by colicin E2. The class II mutant G11e1 and its parent strain G11a1 were tested for their response to colicins E1, E2, and E3. Figure 1 shows that G11e1 survived treatment with colicin E2 and colicin E3 whereas the parent strain G11a1 was highly sensitive to these colicins. Both strains were sensitive to colicin E1. Colicin E2 was used for the remainder of the experiments described in this paper. The kinetics of killing by colicin E2 is shown in Fig. 2a. Killing of G11a1 was rapid, and 10 min was selected as the standard time for incubation with colicin.

We found that G11e1 is sensitive to phage BF23. The same has been shown for the colicin-tolerant (*tol*) mutants described by Nomura (38) and Clowes (9). These mutants adsorb colicins without being killed. The following experiment was performed to show that G11e1 cells can still adsorb colicin E2. Cultures of G11a1, G11e1, and G11e11 (4×10^8 cells/ml) were treated with different concentrations of colicin E2. After 10 min of incubation at 37 C, the cultures were centrifuged and the supernatants were assayed for colicin with G11a1 as the test organism. At a multiplicity of up to 15 killing units of colicin per bacterium, the supernatant from G11a1 and G11e1 incubation mixtures had no significant killing effect on the test organism (*see* experiment 1, Table 3). The supernatant of the colicin-resistant strain G11a11 still contained the amount of colicin that was added initially. Thus, G11e1 cells adsorb colicin E2 to the same extent as G11a1 cells, whereas G11a11 do not adsorb any colicin molecules.

Holland (22) has reported that a majority of *tol* mutations are temperature conditional. However, G11e1 was tolerant to colicin E2 at all temperatures tested (24 to 42 C).

Survival of tolerant strains in the presence of colicin is often less than 100% (cf. Fig. 1 and 2; references 22 and 36). This could be because the cells are particularly susceptible to the killing action of colicin at one specific stage in the division cycle. However, the experiment described in Fig. 2b does not favor such a conclusion, since consecutive addition of colicin did not increase killing of G11e1.

Reversal of colicin tolerance by Mg^{2+} ions. In a previous paper (43), we reported that the phenotype of the class II mutant G11e1 could be partially reversed by divalent cations. Table 2 shows that this also applies to colicin tolerance. Increasing concentration of Mg^{2+} ions in the pregrowth medium and during colicin treatment gave rise to a gradually increasing sensitivity of strain G11e1 to colicin E2. However, even at very high concentrations of these ions, G11e1 was not as sensitive as G11a1. Figure 3 shows the kinetics of transition from tolerance to sensitivity and vice versa. The cells were grown for several generations, and the response to colicin E2 was measured after each doubling. Several generations were required to obtain the degree of sensitivity that was typical for the medium used after the shift. Transition from tolerance to sensitivity was somewhat slower than transition from sensitivity to tolerance. The medium shifts had no effect on the response of strain G11a1 to colicin E2.

Phenotypic colicin tolerance. By treating cells in various ways, we tried to study which part of the cell envelope was involved in adsorption of colicin E2. Since some of these treatments

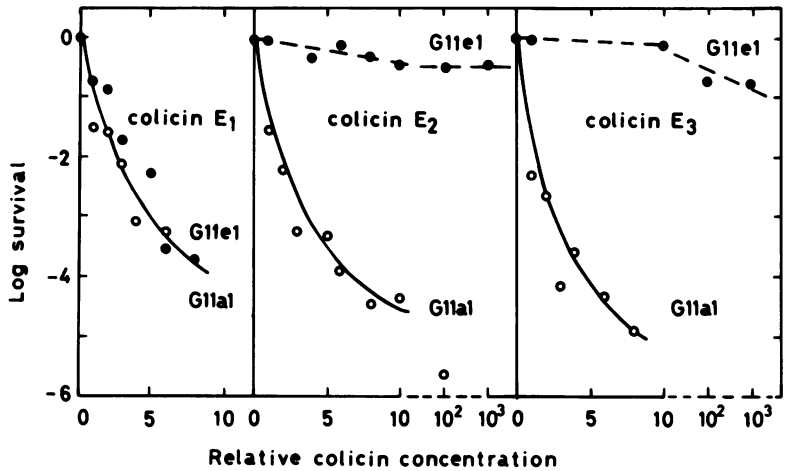

FIG. 1. *Killing of G11a1 and G11e1 by colicins E1, E2, and E3. Mixtures of 0.4 ml of log-phase bacteria in LB medium (4 × 10⁸ cells per ml) and 0.1 ml of different colicin dilutions were incubated at 37 C. After 10 min, the mixtures were diluted in ice-cold LB, and viable counts were made. The concentrations given on the abscissa axis are relative; 1,000 refers to the solutions of colicins obtained as described in Materials and Methods.*

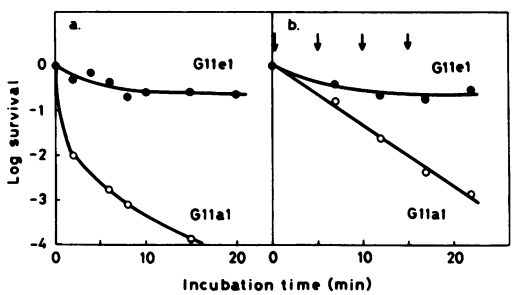

FIG. 2. *Kinetics of killing by colicin E2. Log-phase cells were grown in LB to a cell density of 4 × 10⁸/ml. (a) At zero time, 3.2 × 10⁹ killing units of colicin E2 were added per ml, and incubation was continued at 37 C. At intervals, samples were taken and diluted 100 times in ice-cold LB medium; viable count was made. (b) At the times indicated by vertical arrows, 8 × 10⁸ killing units of colicin E2 were added per ml. Incubation was continued at 37 C, and viable count was followed.*

yielded cells that were perfectly viable, it was also possible to distinguish between tolerance and sensitivity of cells that were able to adsorb colicins. The experiments are reported in Table 3 and can be summarized as follows. Colicin was added at a multiplicity of about 5 killing units per cell. This amount of colicin was completely adsorbed by both G11a1 and G11e1 cells (experiment 1). No attempt was made to titrate the number of receptors per bacterium or to investigate whether there was any difference between the two strains in this respect. By treatment with EDTA, about 50% of the LPS content is removed from the cells (28), but this treatment had no effect on the tolerance

TABLE 2. *Effect of Mg^{2+} ions on colicin tolerance of Escherichia coli G11e1*

Concn of Mg^{2+} (M)	Survival[a]	
	G11e1	G11a1
0.000	0.59	0.0075
0.003	0.34	
0.01	0.17	
0.03	0.02	0.0041
0.10	0.02	0.0036

[a] Log-phase cells (4 × 10⁸ per ml) were treated with colicin E2 at a multiplicity of 5 for 10 min at 37 C. Survival is defined as the ratio of viable counts after and before colicin treatment. The Mg^{2+} concentrations indicated were used during both pregrowth and colicin treatment of the cells.

of G11e1 or the sensitivity of G11a1 to colicin E2 (experiment 2). No change in response to colicin E2 was observed when the EDTA-treated cells were treated with trypsin, which is known to cleave the bonds between the lipoprotein and the murein sacculus (reference 7; experiment 3). Preparation of spheroplasts by treatment of the cells with EDTA and lysozyme in the presence of 20% sucrose resulted in an almost complete loss of ability to adsorb colicin (experiment 4). The cells so treated were no longer viable. Spheroplasts can also be prepared by growth in the presence of ampicillin. Experiment 5 in Table 3 shows that ampicillin spheroplasts of both G11a1 and G11e1 can adsorb colicin E2. Since these spheroplasts revert to normal rods when ampicillin is removed, the effect of colicin E2 on survival

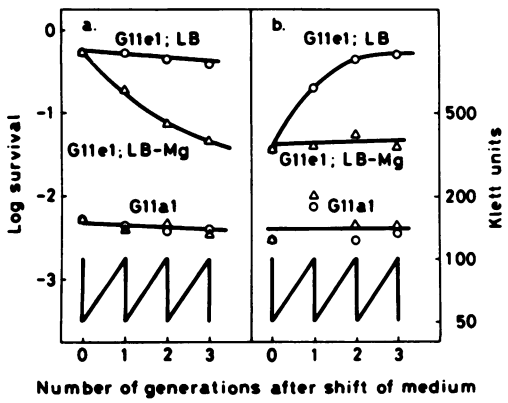

FIG. 3. *Effect on response to colicin E2 by shift of medium from LB to LB containing 0.05 M Mg^{2+} ions and vice versa. The cells of G11a1 or G11e1 were grown in the pregrowth medium (a, LB; b, LB-Mg) to a cell density corresponding to 100 Klett units (equivalent to 4×10^8 cells/ml), harvested by centrifugation, and resuspended in fresh prewarmed medium (○, LB; △, LB-Mg) giving 50 Klett units. Incubation was continued; when the Klett reading reached 100 units, the culture was again diluted to 50 Klett units and incubation was continued. Each time 100 Klett units was reached, a sample was taken for test of response to colicin E2, as in Fig. 1, using a multiplicity of 5 killing units of colicin per bacterium.*

could be tested. Ampicillin spheroplasts of G11a1 as well as of G11e1 were tolerant to colicin E2. Thus, colicin tolerance can be induced biochemically.

In an experiment similar to those of Table 3, cell suspensions and spheroplast suspensions were centrifuged, and the supernatants were tested for presence of colicin receptors with G11a1 used as test organism. In no case was there any measurable spontaneous or induced release of colicin receptors into the medium.

Experiments with LPS. In previous experiments (43), G11e1 cells showed leakage of a periplasmic enzyme (penicillinase) during normal growth and slow adsorption of phage T4. This suggests a disturbance in the cell envelope LPS which is known to take part in T4 adsorption (53, 55) and which is supposed to be an important part of the "outer membrane" of gram-negative cells. However, a chemical analysis of the polysaccharide part and determination of total *N*-acetylglucosamine and phosphate showed no significant difference between G11a1 and G11e1 (Table 4).

LPS from strains G11a1 and G11e1 was tested for inactivation of phage T4. However, in no case did EDTA- or phenol-extracted LPS preparations show any inactivation of phage T4 up to the highest concentration of LPS tested (0.5 mg/ml).

We also tested to see whether incubation of G11a1 cells with phenol-extracted LPS from G11e1 could influence the sensitivity to colicin E2 of these cells. The experiment reported in Table 5 indicates that this treatment rescues the cells from the killing action of colicin E2. Preincubation of colicin E2 with LPS alone had no inhibitory effect on colicin E2.

Revertants and mutants. G11e1 was originally isolated on rich medium plates containing ampicillin (14). In Table 6 are summarized some phenotypic properties of 86 independent, spontaneous, ampicillin-resistant (class II) mutants of G11a1. Two properties, response to cholate and to colicin E2, were studied and all four possible phenotypes were obtained. In the same table we report the result of a study of 29 cholate-resistant clones isolated from G11e1. They were tested for two phenotypic properties, level of ampicillin resistance (class I or class II) and response to colicin E2. Clones were also found showing each of the four possible phenotypes. Thus, there is no strict correlation between these envelope effects.

Mapping of the mutation giving the class II phenotype in G11e1. Preliminary experiments indicated that the mutation giving the class II phenotype in G11e1 was located in the *pro-trp* region of the *E. coli* K-12 chromosome.

We tried to locate the mutation that gives the class II phenotype in G11e1 by crosses. It was not possible to use interrupted-mating experiments, partly because a long time is required for phenotypic expression of this mutation (cf. the shift experiments described above and in Fig. 3), partly because the mutation (*tolD* in Fig. 5) is injected as a very late marker by G11e1. We therefore used a number of females as recipients and selected for the transfer of prototrophic markers or galactose fermentation. Recombinants were picked and tested for cotransfer of other markers including response to cholate. The class II phenotype was also checked by testing the recombinants on LA plates containing different amounts of D-ampicillin. We used the gradient method for the mating experiments (19). This method allows recombinants to be formed from the zygotes before spreading on selective media and it also allows time for phenotypic expression.

The results of the crosses are summarized in Table 7. In cross 1, D21 was used as recipient and, as is apparent from the table, the mutation that gives the class II phenotype is located between *trp* and the end of the donor chromosome (at about 16 min). Cross 2, in which RE149 was used as recipient, shows that *bio* is located between *gal* and the studied gene. There was low linkage in this region (cf., *gal-bio*). However, this

TABLE 3. *Effect of colicin E2 on cells and spheroplasts*

Expt no.	Cell material[a]	Treatment	Survival[b]		
			G11a1	G11e1	G11a11
1	Whole cells	None	1.0	1.0	1.0
		Colicin E2[c]	0.003	0.4	1.0
		Supernatant from B[d]			
		G11a1	0.9		
		G11e1	1.0		
		G11a11	0.003		
2	EDTA treated cells[e]	None	1.0	1.0	1.0
		Colicin E2[c]	0.003	0.4	1.0
		Supernatant from B[d]			
		G11a1	0.9		
		G11e1	0.8		
		G11a11	0.002		
3	Cells treated with EDTA and trypsin[f]	None	1.0	1.0	
		Colicin E2[c]	0.003	0.4	
		Supernatant from B[d]			
		G11a1	1.0		
		G11e1	0.9		
4	EDTA-lysozyme spheroplasts[g]	None	0	0	
		Colicin E2[c]	0	0	
		Supernatant from B[d]			
		G11a1	0.003		
		G11e1	0.003		
5	Ampicillin spheroplasts[h]	None	0.27	0.27	
		Colicin E2[c]	0.07	0.10	
		Supernatant from B[d]			
		G11a1	1.0		
		G11e1	1.0		

[a] Cells were grown in LB-S to a cell density of 10^8 cells/ml. This suspension was designated "whole cells." This material was then treated in various ways (see footnotes *e* through *h*).

[b] Viable count was made by diluting the samples in LB-S and adding 0.1 ml of an appropriate dilution to SA-S which was then poured onto LA-plates and incubated overnight. The values given are relative values, and the top line is defined as viable count of 1.0.

[c] Samples (1 ml) were mixed with 0.1 ml of colicin E2 (about 10^{10} killing units per ml) and incubated at 37 C for 10 min. Viable counts were made as described above.

[d] Cells or spheroplasts were spun down after treatment with colicin E2, and the supernatant was assayed for presence of colicin with G11a1 used as test organism at a concentration of 10^8 cells/ml.

[e] Cells were suspended in 0.9% NaCl containing 0.12 M tris(hydroxymethyl)aminomethane-hydrochloride buffer and 0.01 M EDTA. After 4 min at 37 C, $MgCl_2$ was added to a final concentration of 0.05 M. The cells were then spun down, suspended in LB, and tested for killing by and adsorption of colicin E2.

[f] Cells were treated with EDTA and $MgCl_2$ as described in footnote *e*. They were then washed twice in 0.9% NaCl, and 50 μg of trypsin per ml was added. After 30 min at 37 C, the cells were centrifuged and washed twice in 0.9% NaCl. Finally, they were resuspended in LB and tested for killing by and adsorption of colicin E2.

[g] EDTA-lysozyme spheroplasts were prepared by harvesting the cells, chilling to 0 C, and centrifugation at a cell density of 4×10^8 cells per ml. After washing, the cells were suspended in 0.05 M potassium phosphate buffer (*p*H 7.2) containing 20% sucrose; 1 mM EDTA and 20 μg of lysozyme per ml were added, and spheroplasting was allowed to continue for 30 min at 37 C.

[h] Spheroplasts were made from bacteria growing in LB-S by adding 500 μg of D-ampicillin per ml at a density of 10^8 cells/ml and incubating at 37 C for 60 min with aeration. The efficiency of spheroplast formation was checked by phase-contrast microscopy and tested by diluting samples in 0.15 M NaCl and spreading an appropriate dilution on LA plates. For both strains, survival after 60 min of ampicillin treatment was 0.004.

TABLE 4. *Chemical analysis of phenol-water extracted lipopolysaccharide (LPS)*

Strain	Compound[a] (μg/mg of LPS)					
	Rha	NAG	Gal	Glu	Hep	P
G11a1	12	23	25	82	59	121
G11e1	13	24	26	83	56	134

[a] Abbreviations: Rha, rhamnose; NAG, *N*-acetylglucosamine; Gal, galactose; Glu, glucose; Hep, heptose; P, phosphate.

TABLE 5. *Rescuing from colicin E2 killing by addition of LPS*[a]

Incubation mixture			Colonies per plate
G11a1 cells	LPS (μg/ml)	Colicin E2 (killing units/ml)	
+	0	0	252
+	400	0	264
+	0	10^9	1
+	400	10^9	83
+	40	10^9	10
+	4	10^9	0

[a] G11a1 cells were grown in LB medium to a cell density of 3×10^8 per ml. After 10^5 dilution in 37 C LB medium, LPS from G11e1 was added. After about 2 min, colicin E2 was added, and the total mixture was incubated for 30 min at 37 C. A sample (0.1 ml) was transferred to 2.5 ml of melted SA which was then poured onto LA plates and incubated overnight.

was not due to the presence of the class II mutation, since cross 3 shows that the same linkage was obtained with G11a1 as donor. A position for the class II mutation between *gal* and *trp* is also indicated by cross 4 in which MC100 was used as recipient. Finally, MS31 was used as recipient in cross 5. The result shows that the studied mutation is located between *pyrD* and *gal*.

All these data limit the region in which the mutation can be located to 18 to 21 min on the *E. coli* K-12 chromosome. However, as these crosses involved the very last region that is transferred by G11e1, very low yields of recombinants were obtained. We therefore selected one His^+ recombinant from cross 1 and used this strain (D21-C19) in crosses 6 and 7. D21-C19 is *trp*, *pro*, *cho*-s and F^- (determined by using phage MS2). Thus, by using wild-type (cholate-resistant) donors, we studied the transfer of cholate resistance as an early marker. We selected two Hfr strains with opposite direction of transfer. Both crosses indicated a position between *proB* and *trp*.

The results of crosses 1, 5, and 6 can be plotted by the gradient method. The result is shown in Fig. 4. Cross 1 gives a slightly less reliable result than the other two since a rather long extrapolation has to be made here. Therefore, the most probable map position for the mutation that gives the class II phenotype in G11e1 is 20 min (*tolD* in Fig. 5).

Dominance studies. Secondary F′ clones from

TABLE 6. *Phenotypes of 86 mutants of the class II type and of 29 cholate-resistant revertants from one class II mutant*

Phenotype of class II mutants of G11a1[a]			Phenotype of cholate-resistant revertants of G11e1[b]		
Strains	Response to cholate[c]	Growth in presence of colicin E2[d]	Strains	Ampicillin resistance[e]	Growth in presence of colicin E2[d]
G11a1	r	−	G11a1	Class I	−
G11e1	s	+	G11e1	Class II	+
20 Mutants	r	−	2 Revertants	Class I	−
5 Mutants	r	+	4 Revertants	Class I	+
39 Mutants	s	−	7 Revertants	Class II	−
22 Mutants	s	+	16 Revertants	Class II	+

[a] Mutants were isolated by cultivating log-phase cells of G11a1 in LB medium and spreading on LA plates containing 35 μg of D-ampicillin per ml. No mutagenic treatment was used. Colonies appeared with a frequency of about 10^{-5}.

[b] G11e1 is one of the mutants obtained in an experiment similar to that in footnote *a* (14). Log-phase cells of G11e1 growing in LB medium were spread on LA plates containing 40 mg of sodium cholate per ml. No mutagenic treatment was used. Colonies appeared with a frequency of about 10^{-5}.

[c] Tested by replica plating on LA plates containing 40 mg of sodium cholate per ml. G11e1 is resistant to only 5 mg/ml.

[d] Tested by replica plating on LA plates onto which about 10^9 killing units of colicin E2 had been spread.

[e] Tested by replica plating on LA plates containing D-ampicillin. The class I types (e.g., G11a1) are resistant to 20 μg and the class II types (e.g. G11e1) to 40 μg of D-ampicillin per ml in such a test.

TABLE 7. *Summary of mapping*

Cross	Donor	Recipient	Selection	No. of recombinants tested	Relative frequency[a]							
					Pro$^+$	His$^+$	Trp$^+$	PyrD$^+$	Cho-s[b]	Cho-r[b]	Bio$^+$	Gal$^+$
1	G11e1	D21	His$^+$/str-r	184		100	21		11			
			Trp$^+$/str-r	184		28	100		41			
2	G11e1	RE149	Bio$^+$/str-r	62					26		100	93
			Gal$^+$/str-r	92					5		49	100
3	G11a1	RE149	Bio$^+$/str-r	92							100	97
			Gal$^+$/str-r	92							50	100
4	G11e1	MC100	Trp$^+$/str-r	92			100		42			5
			Gal$^+$/str-r	92			9		20			100
5	G11e1	MS31	Trp$^+$/str-r	92			100	28	17			1
			Ura$^+$/str-r	92			45	100	45			2
			Gal$^+$/str-r	92			10	13	2			100
6	HfrH	D21-C19	Pro$^+$/str-r	92	100		21			35		
			Trp$^+$/str-r	92	29		100			55		
7	KL16	D21-C19	Trp$^+$/str-r	88	1		100			56		
			Pro$^+$/str-r	88	100		28			40		

[a] The selected marker was given a value of 100. The figures shown are the relative frequency of appearance of unselected donor markers among the selected recombinants.

[b] Presence of the class II mutation was also checked by measuring ampicillin resistance of the recombinants. There was complete agreement between response to cholate and ampicillin resistance of all recombinants.

G11a1 and G11e1 carrying the *trp* or *pro-trp* region were isolated by mixing 5×10^7 donor cells (G11a1 or G11e1) with 5×10^8 recipient cells (*recA* derivatives of D11 or D21, strains D114 and D214, respectively) per ml. After 30 min at 37 C, the cells were concentrated 10 times, shaken thoroughly to break all pairs, and spread on different selective media with streptomycin used to counter-select the donor strain. The frequency of F′ clones obtained was 2×10^{-8} to 10^{-7} per input donor. When selected for Trp$^+$, about 90% of the clones also contained *pro*$^+$. The former is a late and the latter an early marker on the G11 chromosome. Purified clones were tested for ampicillin resistance. The results are summarized in Table 8. In the experiment with D214 as recipient, the F′ clones were tested for cholate resistance; all clones with F′ factors derived from G11a1 were found to be resistant whereas those with F′ factors from G11e1 were sensitive to cholate. Corresponding results were obtained when ampicillin resistance was tested. It should be observed that *recA*, as other mutations that significantly reduce growth rate of the cells, results in a decreased ampicillin resistance. However, a comparison between the corresponding heterogenote pairs (e.g., D214/F′-G11a1 versus D214/F′-G11e1) shows that the class II mutation in G11e1 when present in the F′ factor increases ampicillin resistance by 50 to 100%. We also observed that when G11e1 was the donor all F′ clones were mucoid. The crosses with strain D114 as recipient were the exception, since in this case about 20% of the F′ clones from both donors were considerably more resistant to ampicillin than the majority of the F′ clones. Presumably the F′ factor in those cases also contained the *ampA* gene (at 82 min on the *E. coli* K-12 chromosome). The sizes of the F′ factors were not determined since they were transferred poorly when the secondary F′ clones were tested for F′ donor capacity. However, they could be cured with acriflavine.

DISCUSSION

Genetics. The class II mutation in G11e1 gives pleiotropic effects on the phenotype of the cells (43). In a previous paper (43), other mutations in the *pro-trp* region on the K-12 chromosome were surveyed. The phenotype of G11e1 resembles that of mutants in the *tolIII–tolIV* gene(s) (also denoted *tolA*, reference 51) described by Nomura and Witten (42) and by Nagel de Zwaig and Luria (36). These mutations are cotransducible with *gal* (at 17 min on the *E. coli* K-12 chromo-

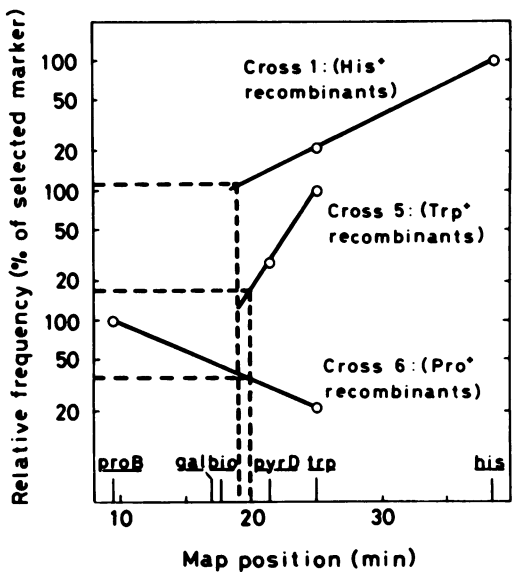

FIG. 4. *Results of crosses 1, 5, and 6 (Table 7). The strains were crossed by the gradient method. His+ recombinants were selected in cross 1, Trp+-recombinants in cross 5, Pro+-recombinants in cross 6. The recombinants were tested for unselected markers. The frequency of cholate-sensitive recombinants in crosses 1 and 5 and the frequency of cholate-resistant recombinants in cross 6 are indicated by the horizontal dotted lines. Their intersections with the respective gradient lines give the approximate map position of the class II mutation in G11e1.*

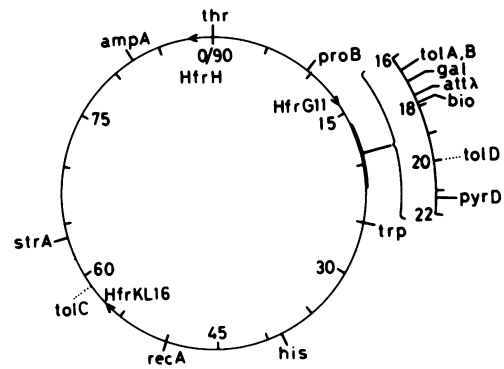

FIG. 5. *Genetic map of Escherichia coli. Hfr origins and relevant genetic markers of strains used in this paper, as well as the main colicin tolerance loci tolA, B, and C, are inserted according to Taylor (51). The approximate map position (20 min) of the mutation described in this paper is indicated by the temporarily adopted gene symbol tolD.*

some). However, our genetic experiments with the class II mutation in G11e1 (Table 7, Fig. 4) show that the corresponding gene is located several minutes from *gal*. Hence, this gene cannot be *tolIII–tolIV* but a new *tol* gene. The slightly increased chromosomal and episomal resistance to chloramphenicol in G11e1 resembles that of mutants in the *cmlA* and *cmlB* loci. These genes are located in the region *bio-pyrD* (46, 47), i.e., in the same part of the chromosome as the class II mutation in G11e1.

Because colicin tolerance is the most striking effect on the phenotype of the class II mutation in strain G11e1, we propose that the corresponding gene is denoted *tolD* until the exact gene product is known.

An interesting finding was the dominance of the *tolD* mutation in partial diploids (Table 8). This may indicate that the mutation concerns a regulatory gene rather than a structural gene.

Nature of the colicin receptors. Guterman and Luria (18) reported that LPS from wild-type *E.*

TABLE 8. *Amplicillin and cholate resistance of F'-trp+ clones*

Recipient strain	*Rec* allele of recipient	Donor of F'-trp	Ampicillin resistance of donor	No. of clones tested	Ampicillin resistance (μg/ml)[a]	Response to cholate[b]	Colony morphology[c]
D11	+	—		1	2	r	n
D114	*recA*	—		1	1	NT	n
D114	*recA*	G11a1	Class I	19	1	NT	n
D114	*recA*	G11a1	Class I	5	10	NT	n
D114	*recA*	G11e1	Class II	18	1.5	NT	Mucoid
D114	*recA*	G11e1	Class II	6	>10	NT	Mucoid
D21	+	—		1	20	r	n
D214	*recA*	—		1	10-15	r	n
D214	*recA*	G11a1	Class I	46	10-15	r	n
D214	*recA*	G11e1	Class II	46	20	s	Mucoid

[a] All resistance tests were made by replication. Single-cell tests were not very accurate in this case since the efficiency of plating is rather low with *recA* strains.

[b] Tested on LA plates. The resistant (r) clones grew on 40 to 50 mg of sodium cholate per ml, whereas the upper limit for the sensitive (s) clones was 5 to 10 mg/ml; NT, not tested.

[c] Donor strain G11a1 has normal (n) colony morphology; G11e1 is mucoid.

coli B and *E. coli* K-12 can protect cells from killing by colicin B. Table 5 shows a similar result for colicin E2. However, as Guterman and Luria pointed out, these data do not show that the LPS contains colicin receptors. It is quite probable that the protecting effect is due to nonspecific coating of the cells with LPS. In the experiments reported in Table 5, a large excess of LPS was required to give any protecting effect; the solution contained, per milliliter: LPS, from about 10^{10} cells and about 3×10^8 cells. LPS itself had no colicin E2-inactivating effect. This finding makes the role of LPS as receptor of colicin E2 even more uncertain.

Biochemical basis of colicin tolerance. We suggest that at least two types of colicin-tolerant mutants exist, target (membrane) mutants and mutants with a changed envelope which reduces the probability of an adsorbed colicin molecule to reach its true "receptor" (target).

At least two of the many *tol* mutants of *E. coli* K-12 reported seem to be true "colicin pathway" (target) mutants. One is *refII*, causing tolerance to colicin E2 only, described by Holland and Threlfall (24). This mutation is concerned with the activity of a deoxyribonuclease. The other is a colicin E1-tolerant mutant whose cytoplasmic membrane is resistant to the action of colicin E1 (5).

Šmarda and Taubeneck (49) showed that some colicins adsorb directly to the cytoplasmic membrane of certain bacterial L forms. However, with colicin-sensitive bacteria, there seems to be a significant degree of nonlethal adsorption of colicin molecules in their envelopes (23). About 3,000 colicin molecules may be adsorbed by one bacterium. This amount of colicin corresponds to 30 killing units (33), i.e., only one out of 100 adsorbed colicin molecules finally reaches its target site.

Many disturbances in the composition or the steric organization of the cell wall might lead to increased trapping or repulsion of various molecules, e.g., colicins. Some *tol* mutants and our colicin-tolerant penicillin spheroplasts (experiment 5, Table 3) may thus have envelopes which reduce the probability of lethal interaction between an adsorbed colicin molecule and a target site. This can also explain why different *tol* mutants often are killed by the actual colicin to a certain extent (*see* Fig. 1 and 2, and references 22 and 31).

The importance of envelope organization is emphasized by the fact that divalent cations could restore colicin sensitivity in the tolerant strain G11e1 and that this phenotypic reversion was not an all-or-none phenomenon (Table 2). Divalent cations are known to act by cross-linking various components of the gram-negative cell envelope (3, 11, 17). Carson and Eagon (8) showed that such links (ionic bonds) are of importance for cell envelope rigidity of *Pseudomonas aeruginosa*. G11e1 has a slightly reduced osmotic stability compared to G11a1, but Mg^{2+} ions strengthened the cells (43).

However, the suggested role of a change in LPS structure in causing the colicin tolerance of the mutant G11e1 could not be seen to be supported by chemical analyses of the polysaccharide part of its LPS (Table 4). Our experiments also failed to show adsorption of phage T4 to *E. coli* K-12 LPS. This is in contrast to the reports that phage T4 can be adsorbed to LPS from *E. coli* B (53, 55). The discrepancy between in vivo and in vitro results for *E. coli* K-12 may be due to different conformation of the LPS molecule in the two states.

Cell wall polymer biosynthesis proceeds in the absence of protein synthesis, but requires energy. Transition from sensitivity to tolerance in two temperature-dependent *tol* mutants was also shown to require energy but not protein synthesis (42). In this experiment, a defective (changed) cell envelope might have been synthesized during incubation after temperature shift. Other temperature-dependent *tol* mutants seem to require protein synthesis for conversion in either direction (37). Also in our shift experiments with Mg^{2+} ions, the change from sensitivity to tolerance was a rather slow process, and the time required for the opposite change was somewhat longer (Fig. 3). This also indicates that synthesis of a modified envelope after shift of medium is necessary for transition.

In conclusion, we believe that the increasing number of loci on the *E. coli* chromosome which control the action of phages, colicins, and different toxic substances actually often concern the cell envelope as such, rather than specific targets or transport systems for these attacking agents.

ACKNOWLEDGMENTS

The technical assistance of Ann-Sofie Kjellström is gratefully acknowledged.

This investigation was supported by grants from the Swedish Natural Science Research Council (Dnr 2453) and the Swedish Cancer Society (nr 68:44 and 69:47).

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