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Resistance of *Escherichia coli* to Penicillins

VIII. Physiology of a Class II Ampicillin-Resistant Mutant

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Class II ampicillin-resistant mutants of *Escherichia coli* are defined as having a twofold increase in penicillinase-mediated ampicillin resistance when determined by colony formation tests on plates. In this paper, one class II mutant has been compared to its parent strain. In liquid medium, the mutant was less resistant than the parent strain both in the absence and in the presence of R1 and R-factor mediating penicillinase activity. The penicillinase activity was found to be almost completely bound to the cells in the parent strain, whereas it was excreted to a great extent in the class II mutant strain. In liquid medium, resistance was well correlated to the cell-bound penicillinase activity, whereas the excreted penicillinases were also of great importance for survival on ampicillin plates. The mutant also had a changed resistance to a great number of other antibacterial drugs. The mutant was found to be more sensitive than the parent strain to osmotic shock, especially when treated with ethylenediaminetetraacetic acid or washed with sodium ions. However, the osmotic stability was restored by the presence of 1 mM Mg^{2+} ions. The class II mutant was more sensitive than the parent strain to sodium cholate, and it adsorbed the phages T4 and T3-1 at a slower rate than did the parent strain. The two strains adsorbed T6 at the same rate. The class II phenotype could be gradually reversed by increasing concentrations of divalent cations. The pleiotropic changes in the phenotype are apparently unrelated to the specific targets for the antibacterial agents tested. They are secondary consequences of a cell envelope mutation. The findings indicate that the class II mutation mediates a structural change in the lipopolysaccharide of the cell envelope.

Ampicillin-resistant mutants of *Escherichia coli* K-12 can be grouped into several classes. Class I mutants are mutated in the *ampA* gene which increases resistance (11) and penicillinase activity (6) of the wild-type cell by a factor of about 10. *ampA* is located at 82 min and is cotransducible with *purA* (10). Mutants belonging to class II show a twofold increase in ampicillin resistance of wild-type cells as well as of cells carrying *ampA* or episomal penicillinase genes (23) but do not contain an increased amount of penicillinase (4). Mutants of this type have been assumed to be mutated in a second gene, previously designated *ampB*. However, genetic experiments (*unpublished data*) have revealed that mutations in several loci can give the class II phenotype. In this paper, we present physiological evidence that one class II mutant has a defective cell envelope.

MATERIALS AND METHODS

Bacterial strains, phages, media, and growth conditions. The *E. coli* K-12 strains used are listed in Table 1. The R factor R1 carries resistance to ampicillin, chloramphenicol, sulfonamides, streptomycin, and kanamycin (19). R1 was transferred as described previously (23).

The phages T3-1, T4, T5, and T6 were propagated on G11. This strain cannot function as a host of T3, but, by using G11a1, a mutant, T3-1, was isolated which can grow on all G11 strains (Boman, *personal communication*). In all experiments with T4, 2.5 mM $CaCl_2$ was included. T-phage stocks were prepared in LB medium. Phages were added to the bacteria at a multiplicity of 1. After lysis, the culture was shaken with chloroform and centrifuged. G11 was used as the indicator bacterium for titration of the T phages. Stocks of phage λ were prepared by ultraviolet induction of G11a1. After lysis, chloroform was added. AB311 was used as the indicator for phage λ.

The minimal medium used was medium E described by Vogel and Bonner (29). It was supplemented with 0.2% glucose, 1 μg of thiamine per ml, and 25 μg of the L epimer of the required amino acids per ml. The complete medium used was LB of Bertani (3) supplemented with medium E (but containing 0.2% glucose). It was solidified with 1.5% agar (LA

TABLE 1. *E. coli K-12 strains used and their relevant characters*

Strain	Origin	Ampicillin resistance		Auxotrophic markers[b]	Response to str[b]
		Resistance class	Phenotype[a]		
G11	Stent and Brenner (26)	Wild type	amp-s	*ilv, metB*	s
G11a1[c]	Eriksson-Grennberg et al. (11)	Class I	amp-10	*ilv, metB*	s
G11e1[c]	Eriksson-Grennberg et al. (11)	Class II	amp-20	*ilv, metB*	s
D1	RC711 of Meynell and Datta (19)	Wild type	amp-s	*his, pro, trp*	s
AB311	Taylor and Adelberg (27)	Wild type	amp-s	*thr, leu*	r

[a] See reference 23.

[b] Abbreviations: *amp*, ampicillin; *his*, histidine; *ilv*, isoleucine-valine; *leu*, leucine; *met*, methionine; *pro*, proline; r, resistance; s, sensitivity; *str*, streptomycin; *thr*, threonine; *trp*, tryptophan. The capital letter after *met* refers to the genetic map of Taylor and Trotter (28).

[c] G11a1 is a spontaneous ampicillin-resistant class I mutant isolated from G11, and G11e1 is a spontaneous ampicillin-resistant class II mutant isolated from G11a1. G11a1 and G11e1 both contain the *ampA* gene.

plates). LA plates usually contained 2.5 mM $CaCl_2$. Soft agar (SA) consisted of Difco Nutrient Broth (1.3%), 0.6% agar, and 2.5 mM $CaCl_2$.

Unless otherwise stated, the experiments were performed at 37 C. The bacteria were cultivated on a rotary shaker, and growth was recorded by optical density readings by using a Klett-Summerson colorimeter with filter W66. In the exponential phase in LB medium, 100 Klett units corresponded to 4×10^8 cells/ml.

Materials. DL-Ampicillin (α-aminobenzyl penicillin, with a 2:3 ratio between the D and L epimers) and penicillin G (benzyl penicillin) were kindly provided by AB Astra, Södertälje, Sweden; chloramphenicol, D-cycloserine, and streptomycin sulfate were provided by AB Kabi, Stockholm, Sweden; and bacitracin was provided by Novo Industri A/S, Copenhagen, Denmark. Actinomycin D was obtained from Merck Sharp and Dohme, Rahway, N.J.; crystal violet was from Merck, Darmstadt, Germany; kanamycin was from AB Ferrosan, Malmö, Sweden; novobiocin was from Merck Sharp and Dohme, Philadelphia, Pa.; and sodium cholate was from Kebo, Stockholm, Sweden. Lysozyme (3× crystallized from egg white, 35,000 Sigma units/mg) was obtained from Sigma Chemical Co., St. Louis, Mo.

Determination of resistance. The bacteria to be tested by the single-cell test were grown in LB on a rotary shaker and harvested in the exponential growth phase. About 200 cells were spread on LA plates containing different amounts of the drug to be tested. The resistance level is defined as the maximal drug concentration permitting 100% cell survival (23).

Analytical methods. The automatic iodometric method for the determination of penicillin-β-lactamase activity and the bioassay for the determination of penicillin concentration, by using *Sarcina lutea* as the test organism, have been described previously (6).

RESULTS

We have studied the effects of one mutation that gives the class II phenotype. The experiments were performed by comparing the class II mutant G11e1 with its parent strain G11a1 (Table 1). G11e1 was isolated on an ampicillin plate (11), and its resistance to D-ampicillin and penicillin G is twice as high as that of G11a1 (Table 2). In some experiments, the R factor R1 was introduced into both strains.

Growth characteristics and antibiotic resistance. The class II strain G11e1 had a reduced growth rate compared to G11a1; the generation times in LB medium were 32 and 24 min, respectively, on the basis of optical density measurements. The experiment illustrated in Fig. 1 shows that G11e1 was more sensitive to elevated incubation temperatures than was G11a1. Furthermore, G11e1 had a tendency to form mucoid colonies on rich medium.

In the experiment shown in Fig. 2 and 3, exponentially growing cultures in LB were diluted at zero time to 10^8 cells/ml into a number of subcultures containing different concentrations of DL-ampicillin. Figure 2 shows optical density, and Fig. 3 shows viable count as a function of incubation time in the presence of DL-ampicillin. G11e1 and G11e1-R1 were considerably less resistant than G11a1 and G11a1-R1. We also determined resistance by serial dilution tests. The result obtained at very dilute inocula shows that resistance of the single cell is less for the class II strain than for the parent strain (Table 2). Finally, we included the levels of resistance to ampicillin and penicillin G on LA plates. The results of the various resistance tests are summarized in Table 2. G11e1 and G11e1-R1 were more resistant to ampicillin on plates, but less resistant in liquid medium, than G11a1 and G11a1-R1, respectively.

In a previous paper (6), we reported that resistance in liquid medium is better correlated

TABLE 2. *Resistance of the class II mutant and the parent strain to some antibacterial agents*

Resistance determination	Resistance (μg/ml)			
	Without R factor		With R factor	
	G11a1	G11e1	G11a1-R1	G11e1-R1
Serial dilution test with				
DL-Ampicillin[a]	15	10	100	50
Sodium cholate[b]	5	1		
Phenol[b]	1,500	500		
Growth experiments with DL-ampicillin[c]				
Lysis induced	30	20	1,000	600
Single-cell colony formation on plates with[d]				
DL-Ampicillin	25	50	250	500
Penicillin G	150	250	200	400
Kanamycin	0.5	1.0	700	2,000
Streptomycin	0.6	1.0	10	30
Chloramphenicol	3	6	200	600
D-Cycloserine	30	20		
Crystal violet	6	12		
Bacitracin	400	400		
Novobiocin	40	90		
Sodium cholate	50	4		
Actinomycin D	r[e]	r[e]		

[a] Inoculum size was about 10 cells/ml. Turbidity was scored after incubation at 37 C overnight.

[b] Log cells in LB (4×10^8 cells/ml) were diluted 100-fold into tubes with LB and the agent to be tested. Turbidity was scored after 4 hr at 37 C.

[c] Ampicillin was added to an exponentially growing culture at a cell density of 10^8 per ml (Fig. 2).

[d] Resistance is given as the highest concentration in LA at which all cells plated gave rise to colonies. About 200 cells were plated.

[e] Grew well on 10 μg/ml; no higher concentrations were tested (25).

with penicillin hydrolysis than is resistance on plates. Therefore, we determined the penicillinase activity of the cells and studied penicillin hydrolysis in growing cultures. The penicillinases produced by *ampA* strains and by R1-carrying strains are normally cell-bound and located in the periplasm. However, Table 3 shows that about 20% of the chromosomally mediated penicillinase and about 75% of the R1 enzyme were excreted by G11e1 and G11e1-R1. In growing cultures, ampicillin was hydrolyzed at a considerably higher rate by G11e1-R1 than by G11a1-R1 (Fig. 4). This is due to the fact that the Michaelis constant is much higher for growing R1 cells than for the free enzyme (6). But, even in the case of G11e1-R1, a rather long time was required to reduce the concentration of ampicillin to nonlethal values.

We also tested G11a1 and G11e1 for resistance to a number of other agents (Table 2). G11e1 showed unchanged resistance to bacitracin, lowered resistance to D-cycloserine, and doubled resistance to crystal violet and novobiocin. Table 2 also shows that altered resistance was not

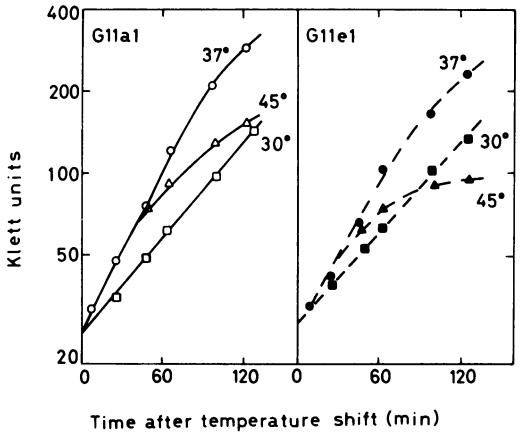

FIG. 1. *Effect of incubation temperature on growth of G11a1 (left) and G11e1 (right). The cells were grown in LB medium to a cell density of 5×10^8 per ml at 37 C and then at zero time were diluted fivefold in LB prewarmed to 30, 37, and 45 C. Incubation was continued. and optical density was recovered.*

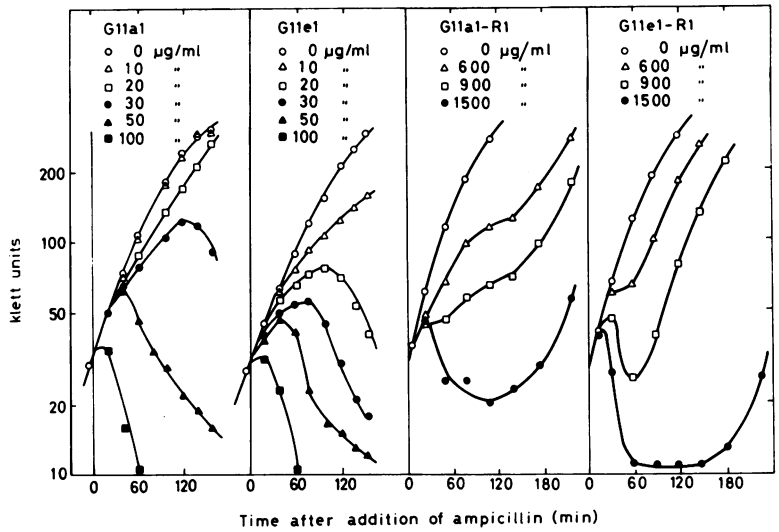

FIG. 2. *Growth curves of G11a1, G11e1, G11a1-R1, and G11e1-R1 cells in the presence of* DL*-ampicillin. The cells were grown in LB medium on a rotary shaker at 37 C. At zero time (cell density about* 10^8 *per ml), ampicillin was added as indicated in the figure.*

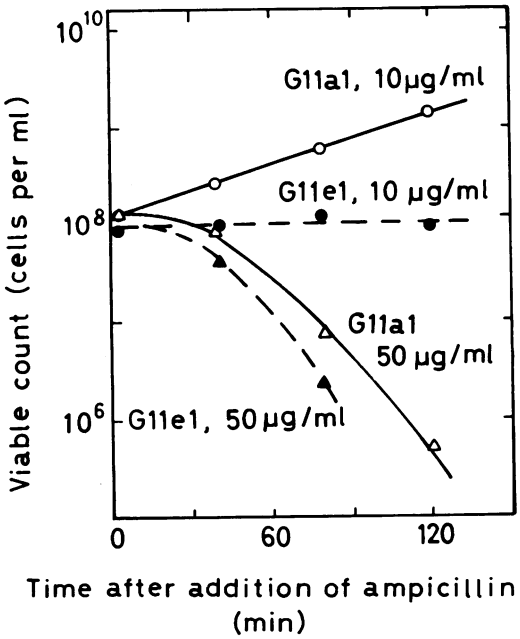

FIG. 3. *Effect of addition of* DL*-ampicillin on viable count of G11a1 and G11e1. Experimental conditions were as in Fig. 2. Ampicillin was added as indicated in the figure.*

specific for cell wall antibiotics. The resistance levels for kanamycin, streptomycin, and chloramphenicol were increased by a factor of two or three in G11e1. This was also the case in G11e1-R1 as compared to G11a1-R1, in which the R factor itself makes the cells resistant to these agents.

TABLE 3. *Localization of penicillinase in the class II mutant and in the parent strain*

Strain	Penicillinase (units/mg, dry weight)[a]	
	Cell-bound	In medium
G11a1	0.052	0.001
G11e1	0.035	0.009
G11a1-R1	0.124	0.015
G11e1-R1	0.048	0.143

[a] The cells were grown in minimal medium plus glucose. At a cell density of 0.3 mg (dry weight) per ml, the cells were centrifuged and resuspended in phosphate buffer (0.05 M, *p*H 7.5). The penicillinase activity was measured in the cell fraction and in the cell-free medium by using penicillin G as substrate. Strains with chromosomally mediated resistance hydrolyze penicillin G 37 times faster than D-ampicillin (6), whereas R1 strains hydrolyze both penicillins at approximately the same rate (14).

Stability of the cell envelope. The fact that the class II strain excreted part of its penicillinase indicates that the mutation affects the cell envelope. Therefore, we measured the osmotic stability of the cells. A comparison of G11a1 and G11e1 showed that a one-centrifugation wash with 0.9% NaCl and resuspension in distilled water gave rise to a significant killing of G11e1 (Table 4). When distilled water was used for

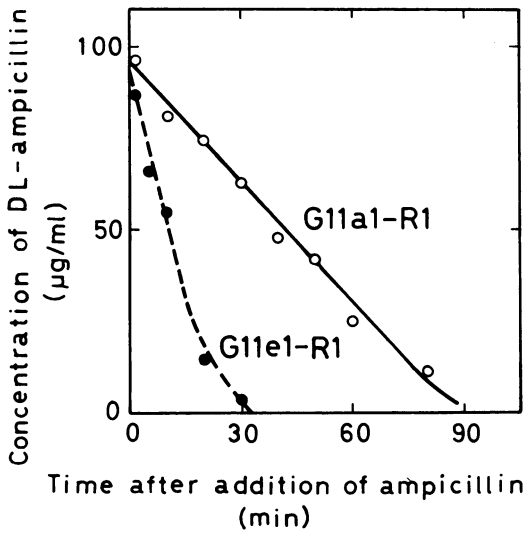

FIG. 4. *Hydrolysis of* DL-*ampicillin by cultures of G11a1-R1 and G11e1-R1 growing exponentially in LB medium. At zero time (cell density about* 10^8 *per ml), ampicillin was added. The concentration of ampicillin was determined by bioassay with Sarcina lutea as the test organism.*

washing, the cell killing was quite moderate. The cells were more susceptible to killing when washed at higher temperatures, whereas they were almost completely stable at 0 C. Killing was counteracted by the presence of 1 mM Mg^{2+} ions.

The degree of the osmotic shock given to cells can be varied by dilution in different concentrations of NaCl. Figure 5 shows that G11e1 was considerably more susceptible to osmotic shock than was G11a1. The presence of the R factor did not change this result.

The results in Table 4 show that Mg^{2+} ions had a protective effect against disruption of the cell envelope. It was therefore expected that, compared to the parent strain, G11e1 should be more sensitive to treatment with ethylenediaminetetraacetic acid (EDTA). This was found to be the case. Figure 6 shows the kinetics of killing obtained by incubation in 1 mM EDTA. G11e1 was killed very quickly for the first 30 min, and then the death rate leveled off. An incubation time of 30 min was therefore selected for an investigation of killing of the bacteria at different concentrations of EDTA. G11e1 was much more sensitive to EDTA than was G11a1 (Fig. 7). We also tried the "cold water wash" of Neu and Heppel (21). About 30% of the G11a1 cells survived this treatment, whereas the corresponding figure for G11e1 was only 0.2%.

Experiments with T-phages and phage λ. Earlier, we reported that efficiency of plating (EOP) for T4 was reduced on G11e1 as compared to G11a1 (5). Weidel, Koch, and Lohss (30) proposed that T3 and T4 have their receptors in the lipopolysaccharide part of the cell wall, whereas T5 and T6 adsorb to the lipoprotein [see review by Weidel and Primosigh (31)]. Therefore, we tested these four phages on G11a1 and G11e1. Table 5 shows that the EOP of T3-1 and T4 was reduced in G11e1. Table 5 also shows that the time required for T3-1 and T4 to lyse a culture was much longer for G11e1 than for G11a1. There was also a great difference in the rate by which G11e1 and G11a1 were killed. These effects are due to a reduced adsorption rate (Table 5 and Fig. 8). For T6, there was no difference between the strains in any of these four tests.

TABLE 4. *Effect of various treatments on survival of G11e1 and G11a1 when diluted into distilled water*

Treatment before dilution[a]	Temp	Survival[b]	
		G11a1	G11e1
	C		
None		0.70	0.27
Centrifugation and resuspension in supernatant fluid	37	0.80	0.29
One wash[c] with saline (0.9% NaCl)	37	0.93	1.2×10^{-2}
Two washes with saline	37	0.80	4.8×10^{-5}
Three washes with saline	37		2×10^{-6}
Three washes in distilled water	37		0.31
Two washes with saline plus 1 mM Mg^{2+}	37	1.00	0.11
Two washes with saline and then addition of 1 mM Mg^{2+} 10 min before dilution	37		1.6×10^{-3}
Two washes with saline	0	0.90	0.80
Two washes with saline	45	0.03	10^{-6}
None, diluted 1,000 ×	37	0.55	0.27

[a] Log cells at a cell density of about 5×10^8 cells per ml were diluted 100-fold into distilled water. Viable counts were made after 30 min at the temperature indicated.

[b] Ratio between viable count after and before the treatment.

[c] Washes were performed as follows. The cells were centrifuged and suspended in the wash medium to the initial volume and incubated for 15 min at the temperature indicated.

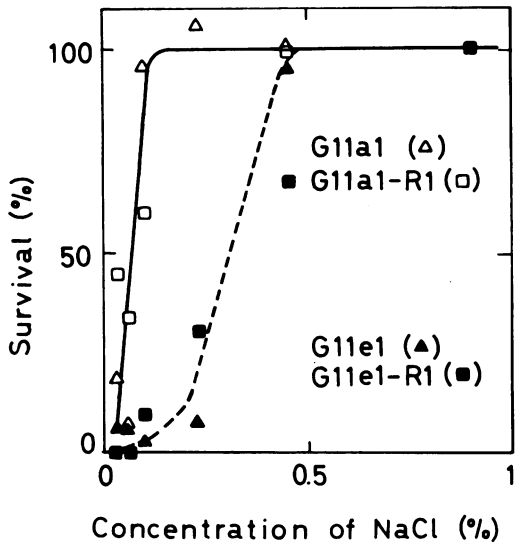

FIG. 5. *Effect of NaCl concentration on the survival of the class II mutant strain (closed symbols) and the parental strain (open symbols). Log cells grown in LB medium to a cell density of about 4 × 10⁸ cells/ml were harvested by centrifugation, washed twice with 0.9% NaCl, suspended in 0.9% NaCl, and then diluted 100-fold in different concentrations of NaCl. The diluted suspensions were incubated at 37 C for 30 min, after which viable count was determined.*

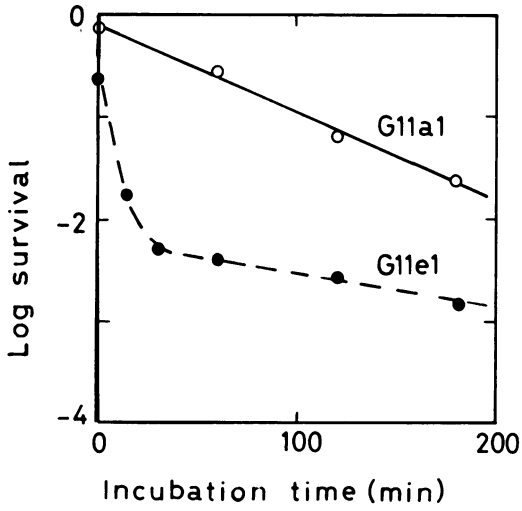

FIG. 6. *Kinetics of the lethal effect of 1 mM EDTA on G11a1 and G11e1 at 37 C. Log cells (4 × 10⁸ cells per ml) were diluted 100 times in distilled water plus 1 mM EDTA and incubated at 37 C. Viable counts were made at different times as indicated on the abscissa. Survival is defined as the ratio between the viable count so obtained and the viable count of the control that was diluted 100 times in 0.9% NaCl.*

The G11 strains are lysogenic for λ. Cultures of G11a1 contain more free phages than G11e1. In the exponential growth phase, the difference was 2- to 3-fold and in the stationary phase it was about 10-fold. This could be explained by the finding that the adsorption rate constant was two times higher for G11e1 than for G11a1.

Resistance to sodium cholate and triphenyltetrazolium chloride (TTC). It was also found that G11e1 showed an increased sensitivity to bile salts. Figure 9 shows that incubation with sodium cholate in LB medium at 37 C caused a rapid decrease in viability for G11e1, whereas G11a1 was able to grow. Addition of 20% sucrose did not rescue G11e1 from being killed by sodium cholate. Resistance to cholate was also determined in tube tests and on plates, and the results are included in Table 2. However, if the cells were converted into spheroplasts by treatment with EDTA and lysozyme, both strains were lysed by sodium cholate at the same efficiency (Fig. 10).

TTC is a substance which acts on the periplasmic membrane and forms a red insoluble formazan when reduced. When TTC was included in LA plates, we observed that G11e1 colonies

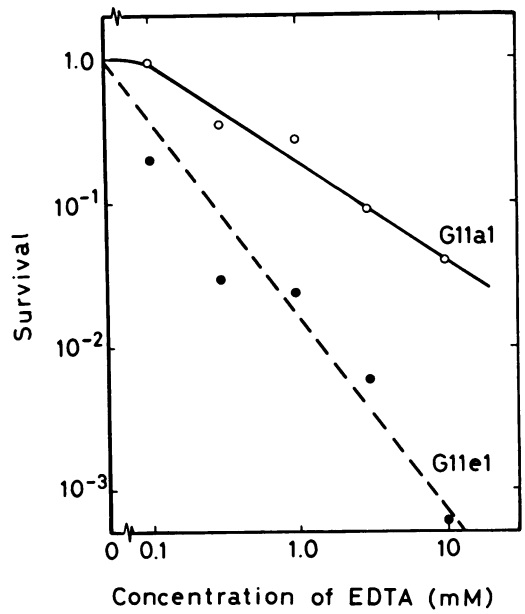

FIG. 7. *Effect of EDTA on survival of G11a1 and G11e1. Log cells (4 × 10⁸ cells/ml) were diluted 100-fold into distilled water containing EDTA and incubated for 30 min before viable count was determined. Survival is defined as the ratio between the viable count so obtained and the viable count of the control that was diluted 100 times in 0.9% NaCl and incubated for 30 min at 37 C.*

TABLE 5. *Adsorption and growth of T phages on G11a1 and G11e1*

Determination	Phage used	Strain	
		G11a1	G11e1
EOP[a]	T3-1	1	5×10^{-5}
	T4	1	10^{-2}
	T5	1	1
	T6	1	1
Time to lysis (min)[b]	T3-1	18	56
	T4	23	60
	T6	25	25
Adsorption rate constant (cm^3/min)[c]	T4	2.6×10^{-9}	6×10^{-11}
	T6	2.2×10^{-9}	1.7×10^{-9}

[a] Phages were mixed with bacteria in soft agar which was poured on LA plates and incubated at 37 C overnight. EOP (efficiency of plating) was defined as the ratio between the plaque count obtained with a given strain and with G11a1.

[b] Logarithmically growing cells were diluted in LB medium. Phages were added at a multiplicity of 5, and the optical density was measured. The period between the addition of phages and the highest value on the growth curve is defined as time to lysis.

[c] Adsorption rate constant (1) was determined from the slope of the curves of Fig. 8.

were colored red at 0.05 mg of TTC per ml, whereas 0.5 mg/ml was required to give the same degree of redness to G11a1 colonies. The resistance levels of LA plates were 1 and 5 mg of TTC per ml for G11e1 and G11a1, respectively.

Effect of divalent ions on the phenotypic properties of the class II mutant. Table 4 indicates that Mg^{2+} ions stabilized the cells of the class II mutant G11e1. The results in Table 6 show that higher concentrations of Ca^{2+} or Mg^{2+} ions reversed the phenotype of the class II mutant. Ampicillin resistance on plates decreased for both strains with increasing ion concentration, but the effect on G11a1 was quite moderate. None of the concentrations tested had any effect on the colony size, which indicates that the rather high concentrations of divalent ions tested did not significantly inhibit growth of the cells. G11a1 survived 5 mg of sodium cholate per ml to about the same extent at all concentrations of divalent ions tested, whereas the survival of G11e1 greatly increased by increasing ion concentration. Furthermore, Mg^{2+} ions decreased the adsorption of phage λ to G11e1 to the same rate as to G11a1. However, Mg^{2+} ions did not restore the ability of G11e1 to adsorb phage T4.

DISCUSSION

Resistance to ampicillin. Resistance to ampicillin was determined in various ways (Fig. 2, 3), and Table 2 shows that G11e1 and G11e1-R1 were less resistant in liquid medium than were G11a1 and G11a1-R1. The R1 enzyme was liberated from the cells to a considerable extent in the class II mutant strain G11e1-R1. The cell-bound R1 penicillinase activity is inhibited in growing cultures (6), and, consequently, ampicillin breakdown was much more rapid in growing cultures of G11e1-R1 than in those of G11a1-R1 (Fig. 4). The excreted penicillinase activity is of importance for the survival of the population as can be seen in Fig. 2, which shows that growth, after addition of ampicillin resumes its normal rate more rapidly in the case of G11e1-R1 than in the case of G11a1-R1. But lysis was more easily induced in the former strain, thus showing that resistance of the individual cell is better correlated

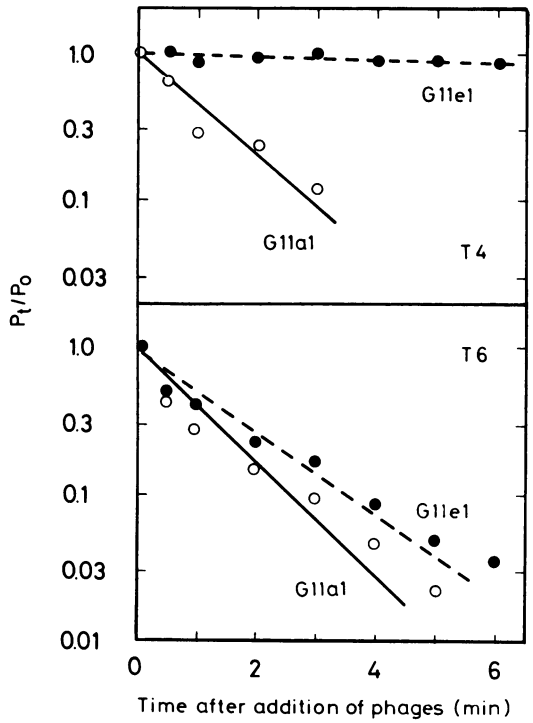

FIG. 8. *Kinetics of adsorption of phages T4 and T6 on G11a1 and G11e1. At zero time, exponentially growing bacteria (4×10^8 cells/ml) were mixed with phages at a multiplicity of about 2×10^{-4}. Samples were withdrawn at intervals and centrifuged, and free phages were assayed in the supernatant fluid by using strain AB311 as indicator on LA plates containing 100 μg of streptomycin per ml. The result was plotted as P_t/P_0 against time, where P_0 and P_t are the phage titers at times zero and t.*

to the cell-bound penicillinase activity (see tube dilution test in Table 2). If this activity is assumed to be evenly distributed in the whole cell wall volume and if ampicillin has free entrance to this space, a rough calculation based on the rate of hydrolysis by growing cells (Fig. 4) shows that all ampicillin in the cell wall can be hydrolyzed within 1 sec. This seems to be enough activity for the formation of a steep gradient in the concentration of ampicillin in the cell wall, thus reducing the concentration at the sensitive site to permissible values. A closer calculation is not

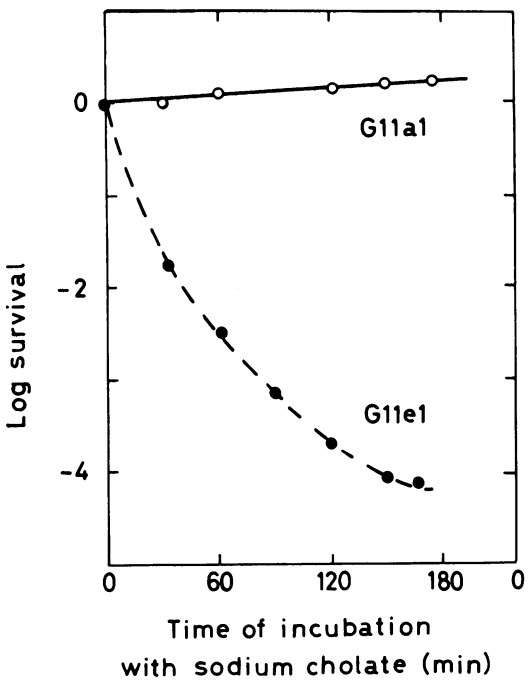

FIG. 9. *Effect of sodium cholate on survival of G11a1 and G11e1. Log cells (4 × 10⁸ cells per ml) were diluted 100-fold into LB medium containing 5 mg of sodium cholate per ml. Viable count was made at intervals during incubation on a rotary shaker at 37 C.*

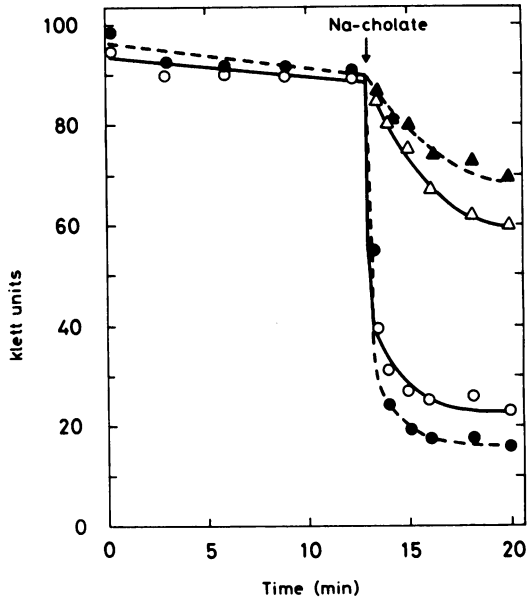

FIG. 10. *Effect of sodium cholate on EDTA-lysozyme spheroplasts of G11a1 (open symbols) and G11e1 (filled symbols). The cells were grown in LB and harvested at 4 × 10⁸ cells/ml by chilling and centrifugation. After washing, the cells were suspended in 0.05 M phosphate buffer (pH 7.4) containing 20% sucrose. At zero time, 1 mM EDTA and 20 μg of lysozyme per ml were added. After 13 min of incubation at 20 C, 1.25 mg (triangles) or 5 mg (circles) of sodium cholate per ml was added and turbidity was recorded.*

TABLE 6. *Effect of divalent cations*

Strain	Divalent ion	Test	Concn of divalent ions (M) added[a]				
			0.000	0.003	0.01	0.03	0.1
G11a1	Ca^{2+}	Ampicillin resistance on plates (μg/ml)[b]	25	25	15	10	10
G11e1	Ca^{2+}		40	40	25	10	10
G11a1	Mg^{2+}		20	20	20	25	10
G11e1	Mg^{2+}		35	30	25	20	10
G11a1	Ca^{2+}	Survival when incubated for 60 min in LB with 5 mg of sodium cholate per ml	0.50		0.57	0.70	0.48
G11e1	Ca^{2+}		0.0035		0.013	0.07	0.75

[a] The cell material was pregrown in the presence of the concentrations of divalent cations indicated. The same concentration was also included in the particular test.

[b] About 200 log-phase cells were spread on LA plates containing D-ampicillin.

possible, since diffusion into the cell wall in a shaken culture cannot be measured and the exact locations of the sensitive site and of the penicillinase molecules are unknown.

On plates, the excreted enzymes are presumably retained close to the cells, thus creating a zone of lower ampicillin concentration around the colonies. Like the R1 enzyme, the excreted chromosomally mediated penicillinase is more efficient in hydrolyzing penicillin than is the cell-bound enzyme (14, 15). This may explain why class II mutants are more resistant to ampicillin on plates than are the parent strains.

Identity of the mutation giving the class II phenotype. Preliminary mapping experiments indicate that the class II phenotype can be obtained by mutations in several loci in the *pro-trp* region. Many different mutations have been described that map in this region and that give at least some of the phenotypic characters described for G11e1. Mucoid colonies are obtained by the *capR* (18) and *capS* (17) mutations, but these cells form enormous amounts of capsular material in contrast to G11e1 (Steele and Boman, *in press*). Mutants that excrete periplasmic enzymes and that are sensitive to low osmotic pressure have been reported by Mangiarotti, Apirion, and Schlessinger (16), but these mutants are much more fragile than are G11e1 cells. However, the most striking similarity with the phenotype of G11e1 is shown by the *tolIII* and *tolIV* mutants described by Nomura and Witten (22) and by Nagel de Zwaig and Luria (20). These mutants were isolated as being tolerant to colicins of the E group but are also fragile and sensitive to cholate and EDTA. The corresponding genes map close to *gal*, and the gene order is *tol-gal-bio*. The class II mutation in strain G11e1 mediates tolerance to colicins E2 and E3 (*unpublished data*). However, this mutation seems to be located between *pyrD* and *bio* (*unpublished data*). Thus, it does not map in the *tolIII* or *tolIV* locus. Reeve (24) described some mutants with increased episomal and chromosomal chloramphenicol resistance. These resistance genes are located in the *pyrD-bio* region, and we cannot at present exclude the possibility that the class II mutation in G11e1 is similar to one of these.

Cell envelope of the class II mutant. G11e1 differed from G11a1 in many respects. Some of the differences were concerned with the stability of the cell, and others were concerned with the adsorption of certain phages and sensitivity to colicins (*unpublished data*). All of these data indicate that the mutation giving the class II phenotype is concerned with the cell envelope. An imbalance in the biosynthesis of the cell envelope is also indicated directly by the fact that colonies of G11e1 have a tendency to be mucoid and indirectly by the reduced growth rate of G11e1.

Phages T3-1 and T4, which show a reduced adsorption on G11e1, have receptors in the lipopolysaccharide part of the cell envelope, whereas T5 and T6, which adsorb equally well on G11a1 and G11e1, have receptors in other parts of the cell envelope (Table 5, Fig. 8) (30, 31). These findings indicate that G11e1 is changed in the lipopolysaccharide.

A reduced stability of G11e1 cells was observed in the osmotic shock experiments (Fig. 5, Table 4). There are data which show that the murein sacculus is not alone responsible for the stability of the cell envelope and that lipoproteins and lipopolysaccharides are also involved (7). Divalent cations seem to act by cross-linking these substances (2, 8, 9). If G11e1 cells have an altered lipopolysaccharide, this fact may explain their sensitivity to EDTA.

Bile salts are mild surface-active agents often used to dissolve biological membranes. The increased sensitivity to sodium cholate for G11e1 cells (Fig. 9) can therefore hardly be explained by the increased fragility of the cells. Rather, sodium cholate seems to pass the loose cell envelope and to obtain access to the membrane more easily. The same explanation can be applied to the TTC experiments. This view was supported by experiments in which the effect of sodium cholate on G11e1 was not counteracted by the addition of 20% sucrose. Furthermore, EDTA-lysozyme spheroplasts of G11e1 and wild-type cells were equally sensitive to cholate, indicating that the membrane was not changed in G11e1 (Fig. 10). The increased resistance to chloramphenicol, kanamycin, and streptomycin may also be due to a reduced diffusion through the cell envelope of these antibiotics which inhibit processes inside the plasma membrane. That a disturbance in the lipopolysaccharide of the class II mutant may cause permeability changes is in line with the observations that EDTA treatment of gram-negative bacteria causes a release of lipopolysaccharide (13), an increase in the permeability for a number of substances (12), and excretion of periplasmic enzymes (21). It is also in line with the findings that increasing concentrations of divalent cations can gradually reverse the class II phenotype (Table 6). We believe that this is due to a partial restoration of the steric organization or the charge of the envelope. However, no reversion by Mg^{2+} ions was observed for the adsorption of phage T4, which again indicates that the class II mutation of G11e1 mediates a change of the primary structure of the lipopolysaccharide.

Many mutations giving resistance to phages, colicins, antibacterial substances, etc. have been studied. They often show very pleiotropic effects on the phenotype of the cells. We believe that studies of many of these mutants give very little information about the action of the selective agents used for isolation. The phenotypic properties by which these mutants have been isolated are often secondary consequences of changes of the cell envelope. This also applies to the class II mutation which has no direct connection with the action of penicillin. A definite conclusion about the nature of the mutation in G11e1 and in other class II mutants must await the completion of direct studies on the composition of the cell surface which are in progress in this laboratory. These mutants may be of interest since very little genetic information about the cell envelope of *E. coli* is available.

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