

## Effect of R-Factor-Mediated Drug-Metabolizing Enzymes on Survival of *Escherichia coli* K-12 in Presence of Ampicillin, Chloramphenicol, or Streptomycin

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*Escherichia coli* K-12 carrying the R-factor R1 is resistant to ampicillin, chloramphenicol, and streptomycin. This resistance is due to R-factor-coded enzymes that metabolize the drugs. The effects of these enzymes on the survival of cell populations were studied in the presence of high concentrations of antibiotics. For all three antibiotics there were considerable inoculum effects. For ampicillin and chloramphenicol, the inoculum effect was due to detoxification of the medium, whereas streptomycin was not significantly metabolized. The survival of the population in the presence of streptomycin was due to the presence of resistant mutants. At high concentrations of ampicillin or chloramphenicol, the surviving cells were not mutants. Survival in the presence of ampicillin is presumably due to variations in resistance during the cell division cycle. The rate of acetylation of chloramphenicol decreased with time and was zero after 1 to 2 h. Treatment with high concentrations of chloramphenicol did not cause lysis of the cells but partially opened the outer membrane, causing excretion of  $\beta$ -lactamase.

Gram-negative bacteria may be resistant to antibiotics due to the presence of R-factors containing structural genes for drug-metabolizing enzymes. The R-factor R1 confers resistance to ampicillin, chloramphenicol, kanamycin, streptomycin, and sulfonamides (25). The mechanism of R1-mediated resistance is known in three cases; ampicillin is hydrolyzed by  $\beta$ -lactamase (19), chloramphenicol is acetylated (10), and streptomycin is adenylylated (22). In a previous communication (28), the specific activities of R1-mediated  $\beta$ -lactamase, chloramphenicol acetylase, and streptomycin adenylylase were measured in a strain of *Escherichia coli* K-12; the streptomycin adenylylase activity was much lower than that of the other two enzymes. Now we present a study of the effects of these three enzymes on the survival of populations of R1-carrying *E. coli* K-12 cells growing in liquid medium containing high concentrations of ampicillin, chloramphenicol, or streptomycin. For ampicillin and chloramphenicol, survival of the population was due to detoxification of the medium by the corresponding enzymes. The activity of streptomycin adenylylase was too low to cause any detectable decrease in the concentration of streptomycin in the medium during growth, but mutants were selected that were more resistant to streptomycin than normal R1 cells.

### MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** The *E. coli* K-12 strain G11a1 (*ilv*, *metB*) was used in this study (9). The R-factor R1 mediates resistance to ampicillin, chloramphenicol, sulfonamides, streptomycin, and kanamycin (25). R1 was transferred as previously described (27), giving strain G11a1-R1. In the bioassay of streptomycin, the *E. coli* K-12 strain G11 (*ilv*, *metB* [reference 39]) was used.

We used Bertani LB medium (4) supplemented with Vogel and Bonner medium E (42), 1  $\mu$ g of thiamine per ml, and 0.2% glucose. LB medium was solidified with 1.5% agar (LA plates). Soft agar (SA) consisted of Difco nutrient broth (1.3%), 0.6% agar, and 2.5 mM  $\text{CaCl}_2$ . DIL solution contained Difco nutrient broth (0.1%) and NaCl (0.5%).

The experiments were performed at 37 C. The bacteria were cultivated on a rotary shaker, and growth was recorded by optical density (OD) readings in a Klett-Summerson colorimeter with filter W66. In the exponential phase in LB medium, 100 Klett units corresponded to  $4 \times 10^8$  cells/ml or 250  $\mu$ g of cell substance (dry weight) per ml. Total cell counts were performed in a Bürker counting chamber.

**Materials.** *D*-Ampicillin was kindly donated by Astra, Södertälje, Sweden; streptomycin sulfate and chloramphenicol were donated by Kabi AB, Stockholm, Sweden and Ercopharm A/S, Vedbaek, Denmark, respectively. Sulfonamide was obtained from Fluka AG, Buchs, Switzerland, and membrane filters were from Sartorius-Membranfilter GmbH, Göttingen, West Germany.

**Determinations of resistance.** For single-cell

tests, the bacteria were grown in LB medium 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 highest drug concentration permitting 100% cell survival (27).

In tube dilution tests, exponentially growing bacteria were diluted and added to test tubes containing 5 ml of LB medium and different concentrations of the drug to be tested. After incubation overnight, the tubes were scored for turbidity.

**Induction of  $\beta$ -galactosidase.** The ability of a culture to form  $\beta$ -galactosidase was tested in the following way. Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG,  $5 \times 10^{-4}$  M, Sigma Chemical Co., St. Louis, Mo.) was added to a 5-ml culture growing exponentially in glucose-free LB medium. Incubation was continued for 30 min, after which the cells were harvested by centrifugation. The cells were resuspended in 1 ml of minimal medium E without any carbon source and lysed by the addition of 2 drops of toluene. The activity of  $\beta$ -galactosidase was measured as described by Pardee et al. (31).

**Analytical methods.** Hydrolysis of ampicillin was assayed by measuring the formation of ampicilloic acid by using an automated microiodometric method (20).

Chloramphenicol and streptomycin were determined by bioassay (18) by using the *E. coli* K-12 strains G11a1 (chloramphenicol) and G11 (streptomycin) as indicator strains. The test strain was grown in LB medium to a cell density of  $4 \times 10^8$  cells/ml and diluted 100 times in NaCl (0.9%, wt/vol). A 0.1-ml portion of this dilution was added to melted SA (45 C) and poured onto LA plates. The culture samples were always filtered free of cells by using 23-mm membrane filters before antibiotic concentrations were determined. Paper disks (5-mm diameter) were soaked in the solutions to be tested and applied to the agar surface. Zones of growth inhibition were measured after 16 h of incubation at 37 C and were compared with those obtained with known standards in the same experiment. In all cases, four disks were used per sample and standard. A standard curve for streptomycin is given in Fig. 1, which also shows the standard deviation of zone diameters. This deviation was  $\pm 0.1$  mm, giving an accuracy of  $\pm 3\%$  for the bioassay.

The bioassay of chloramphenicol is about 10 times less sensitive than the bioassay of streptomycin.

$\beta$ -lactamase, chloramphenicol acetylase, and streptomycin adenylase were measured as previously described (28). One unit of enzyme is defined as the amount of enzyme that metabolizes 1  $\mu$ mol of substrate per min at 37 C. Protein was measured according to Lowry et al. (21).

## RESULTS

**Resistance test.** Strain G11a1-R1 was tested for antibiotic resistance and the activity of the corresponding drug-metabolizing enzymes. Table 1 shows that single-cell resistance on LA plates was (per ml): ampicillin, 75  $\mu$ g; chloramphenicol, 300  $\mu$ g; and streptomycin, 10

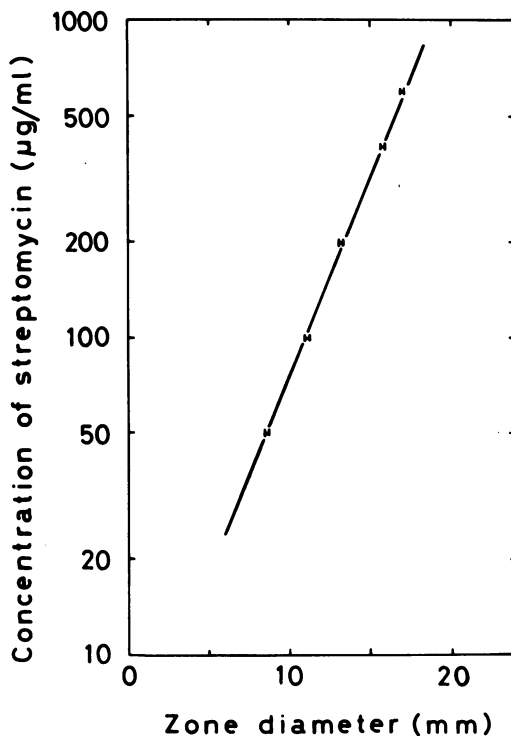


FIG. 1. Standard curve for streptomycin determined by bioassay using *E. coli* K-12 strain G11 as indicator. The standard deviation is given in the figure.

$\mu$ g. The activity of the streptomycin-inactivating enzyme was much less than that of  $\beta$ -lactamase and chloramphenicol acetylase.

Resistance to ampicillin, chloramphenicol, and streptomycin was measured in tube dilution tests. There was a considerable inoculum effect for all three antibiotics tested, particularly for ampicillin and streptomycin. At small inocula, resistance to ampicillin and chloramphenicol was the same as on plates, although resistance to streptomycin was considerably higher in LB medium than on LA plates. LB medium but not LA medium is supplemented with medium E. The addition of medium E to LA plates raised streptomycin resistance to the same level as found at small inocula in LB medium, presumably by increasing the buffering capacity (1, 6, 8, 33, 43) and the salt concentration (3, 7, 17) of the medium.

**Effect of ampicillin on growing cultures of G11a1-R1.** G11a1-R1 was grown in the logarithmic phase on a rotary shaker in LB medium. At a density of  $4 \times 10^8$  cells/ml, the culture was diluted fivefold with 37 C LB medium containing ampicillin. Incubation was continued, and OD was recorded. At intervals, samples were taken for viable count and for determination of

TABLE 1. Resistance to antibiotics and activity of the corresponding enzymes

Strain	Single-cell resistance on LA plates ( $\mu\text{g/ml}$ )				Enzyme activity (units per mg of protein)		
	D-Ampicillin	Chloramphenicol	Streptomycin	Sulfonamide	$\beta$ -lactamase <sup>a</sup>	Chloramphenicol acetylase <sup>b</sup>	Streptomycin adenyllylase
G11a1	15	3	1	100	0.006	$10^{-3}$	$<3 \times 10^{-5}$
G11a1-R1 <sup>c</sup>	75	300	10	300	1.50	9.2	$3.8 \times 10^{-4}$

<sup>a</sup> D-Ampicillin (1.5 mM) was used as substrate.

<sup>b</sup> Measured in strain EC1005 (28).

<sup>c</sup> R1 was transferred from RC711 to G11a1. RC711 has been described by Meynell and Datta (25).

ampicilloic acid formed. Rapid lysis and killing of the cells as well as rapid hydrolysis of ampicillin were recorded at high concentrations of ampicillin. However, growth resumed, and the time when viable count started to increase coincided with the time when all ampicillin was hydrolyzed. Growth resumed eventually even at the highest concentration of ampicillin tested (Fig. 2). The survivors after growth in 8,000 of  $\mu\text{g}$  D-ampicillin per ml were shown not to be mutants by repeated growth of such survivors in the presence of different ampicillin concentrations as well as by single-cell tests. Samples were analyzed in a Bürker counting chamber. At the time when the minimum viable count was reached, all cells present in the cultures were filaments. The number of filaments present was twice as high as the viable count determined by the soft-agar overlay technique. To check that the filaments were induced by ampicillin and not present in the culture before addition of ampicillin, the length was measured at the time when the minimum viable count was reached. Assuming a generation time of 25 to 30 min, the filaments were too short to have been present before the addition of ampicillin.

**Effect of chloramphenicol on growing cultures of G11a1-R1.** Experiments analogous to those described above for ampicillin were performed with chloramphenicol (Fig. 3). At intermediate concentrations of chloramphenicol, growth was immediately inhibited but resumed when chloramphenicol was completely metabolized. At higher concentrations of chloramphenicol, the metabolism rate gradually decreased and was zero after 1 to 1.5 h of incubation. At such high concentrations of chloramphenicol, growth never resumed. The continuous reduction in the rate of acetylation might have been due to product inhibition of the enzymes. However, the *in vitro* chloramphenicol acetylation was not inhibited by the cell-free medium obtained by centrifugation of a culture incubated for 4 h in the presence of 2,500  $\mu\text{g}$  of chloramphenicol per ml. This medium should

contain about 5 mM of acetylated chloramphenicol. During these 4 h there was neither any decrease in the chloramphenicol acetylase activity nor any excretion of this enzyme from the cells. Furthermore, when cells were incubated in the presence of 2,000  $\mu\text{g}$  of chloramphenicol per ml for 2 h, centrifuged, washed in LB medium, and then resuspended in LB medium containing the same amount of chloramphenicol, no acetylation of the drug was observed.

Viable count decreased with time (Fig. 4), but the rate of killing was much lower than in the ampicillin experiments (cf. Fig. 2). Washing the cells with DIL solution on membrane filters did not increase the colony count of cells grown in the presence of chloramphenicol. The R-factor R1 codes for a  $\beta$ -lactamase that is located in the periplasm (19). There was a considerable excretion of this enzyme from the cells treated with chloramphenicol. However, there was very little loss of protein from the cells, indicating that the decrease in OD and the leakage of  $\beta$ -lactamase were not due to cell lysis. The ability to form  $\beta$ -galactosidase by induction with IPTG was also measured at 500, 800, 1,000 and 2,400  $\mu\text{g}$  of chloramphenicol per ml. At all these concentrations, the inducibility was completely lost less than 1 min after the addition of chloramphenicol. The capacity to form  $\beta$ -galactosidase was completely restored when the drug disappeared from the medium.

The survivors from the experiments of Fig. 3 were tested for chloramphenicol resistance on plates. Their resistance was identical to that of the original G11a1-R1 culture.

**Effect of streptomycin on growing cultures of G11a1-R1.** Experiments analogous to those described above for ampicillin and chloramphenicol were performed with streptomycin (Fig. 5). After the addition of streptomycin, growth (OD and viable count) continued as in the control. After a time, which was shorter the higher the concentration of streptomycin, growth was inhibited, followed by a decrease in OD of the culture and killing of the cells.

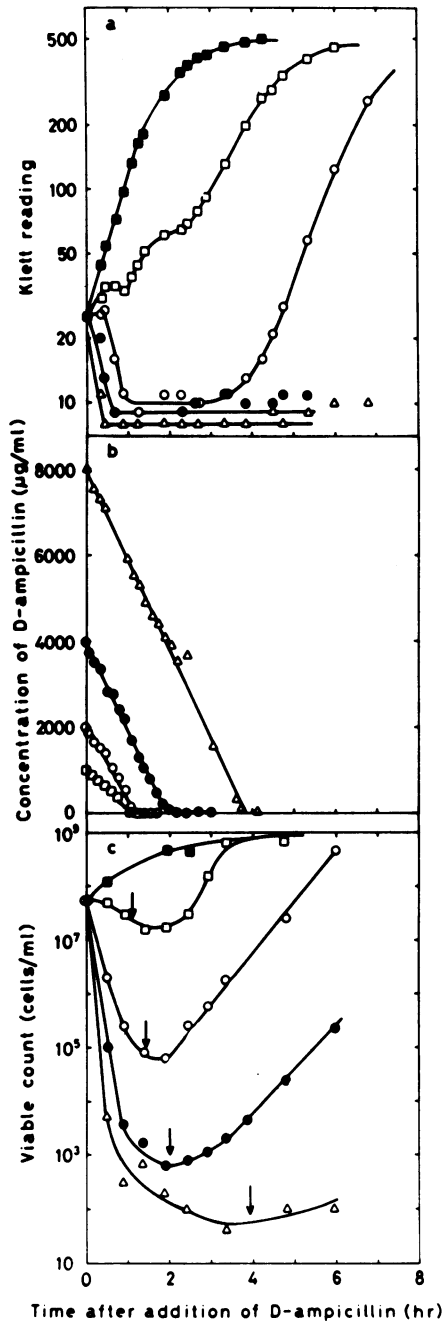


FIG. 2. Effect of D-ampicillin on *E. coli* G11a1-R1. The cells were pregrown in LB medium on a rotary shaker at 37 C. At zero time (25 Klett units, corresponding to 10<sup>8</sup> cells/ml) ampicillin was added in the following concentrations (μg/ml): 0 (■); 1,000 (□); 2,000 (○); 4,000 (●); and 8,000 (Δ). (a) OD of the cultures. (b) Concentration of D-ampicillin in the medium. (c) Viable count of the cultures.

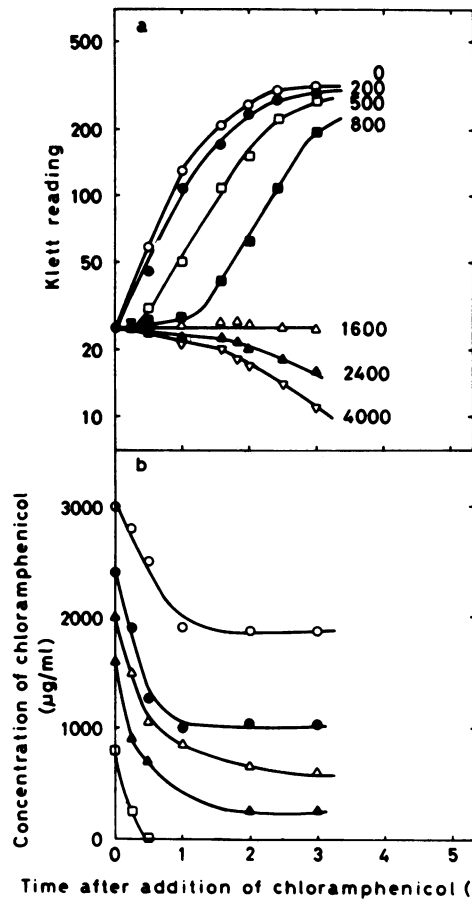


FIG. 3. Effect of chloramphenicol on *E. coli* G11a1-R1. The experiment was performed as described in Fig. 2. Concentrations of chloramphenicol tested are indicated as μg/ml in the figure. (a) OD of the cultures. (b) Concentration of chloramphenicol in the cultures determined by bioassay using strain G11a1 as test organism.

However, after further incubation, viable count as well as OD again started to increase.

R-factor-mediated resistance to streptomycin has been shown to be due to R-factor-coded enzymes that adenylate or phosphorylate the antibiotic (29, 30, 41, 44). However, there was no reduction in the concentration of streptomycin in the medium even after 20 h of incubation. The bioassay used is not very accurate but will detect reductions by 10% without difficulty (see Fig. 1).

Thus, resumption of growth was not due to detoxification of the medium. Viable count was determined on LA plates and on LA plates containing 100 μg of streptomycin per ml. The cultures from the start contained a fraction of

about  $10^{-5}$  of cells with increased streptomycin resistance (Fig. 6). The same frequency of mutants was found when 10 clones of G11a1-R1 were examined. In the presence of streptomycin, this fraction grew uninhibitedly, whereas the normal G11a1-R1 cells were rapidly killed. At 400  $\mu\text{g}$  of streptomycin per ml, even the normal cells resumed some growth after 3 h, probably due to decrease in the pH of the culture (1, 6, 8, 33, 43). Under similar conditions, resistance was five times higher at pH 6.0 than at pH 8.0.

The cultures from the experiment of Fig. 5 were grown to the stationary phase and then tested for streptomycin resistance of the individual cells. The resistance level was 10  $\mu\text{g}/\text{ml}$  for the control culture. However, when 300  $\mu\text{g}$  of streptomycin per ml was included in the LB medium, 9% of the surviving cells were resistant to 100  $\mu\text{g}$  of streptomycin/ml. This resistant

fraction increased with increasing concentrations of streptomycin. The results, summarized in Fig. 7, show that there was a class of resistant mutants growing at 100 to 150  $\mu\text{g}$  of streptomycin per ml. At 5,000  $\mu\text{g}$  of streptomycin in the pregrowth medium, we obtained a fraction of 7% of the population that was resistant to at least 400  $\mu\text{g}/\text{ml}$ . Thus, the resumed growth in Fig. 5 was due to mutants with a higher streptomycin resistance than G11a1-R1.

## DISCUSSION

Cells containing the R-factor R1 are resistant to antibiotics due to R-factor-mediated drug-metabolizing enzymes. Ampicillin is hydrolyzed by  $\beta$ -lactamase (19), chloramphenicol is acetylated (10), and streptomycin is adenylylated (22). In a previous paper we showed that there is a linear correlation between single-cell resistance and enzyme production (28), indicating that the survival of a cell in the presence of antibiotic is dependent on the presence of the corresponding enzyme.

Tube dilution tests showed that a population of cells survives much higher concentrations of ampicillin, chloramphenicol, and streptomycin than a single cell. This strong inoculum effect is further discussed below.

**Effect of ampicillin.** Cells containing the R-factor R1 produce a  $\beta$ -lactamase that hydrolyzes ampicillin (19). The enzyme is constitutive and has no co-factor requirement. Growth in the cultures resumed at the time when all ampicillin had been hydrolyzed (Fig. 2c). Analogous findings have been reported for *Pseudomonas pyocyanea* (34) and *Staphylococcus aureus* (23, 35). Hence, the survival of the

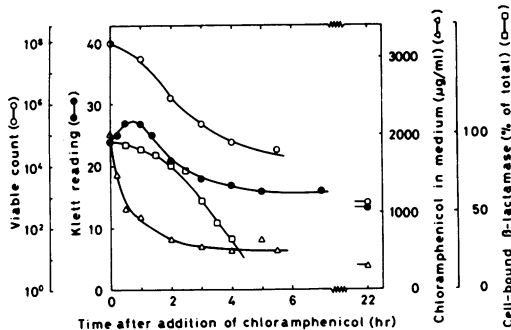


FIG. 4. Effect of addition of 2,000  $\mu\text{g}$  of chloramphenicol per ml to G11a1-R1 on viable count, OD, chloramphenicol concentration in the medium, and cell-bound  $\beta$ -lactamase. Experimental conditions were as in Fig. 3.

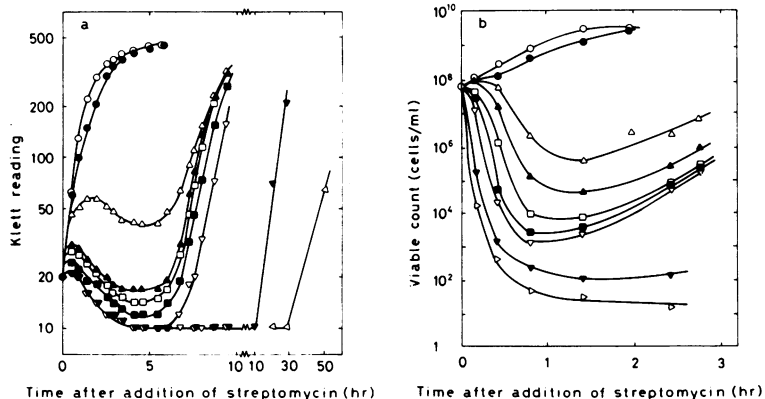


FIG. 5. Effect of streptomycin on growth of *E. coli* G11a1-R1. The experiment was performed as described in Fig. 2. Concentrations of streptomycin tested ( $\mu\text{g}/\text{ml}$ ): 0 (O); 300 (●); 400 ( $\Delta$ ); 700 ( $\blacktriangle$ ); 1,000 ( $\square$ ); 1,500 ( $\blacksquare$ ); 2,000 ( $\nabla$ ); 5,000 ( $\blacktriangledown$ ); and 10,000 ( $\triangledown$ ). (a) OD of the cultures. (b) Viable count of the cultures.

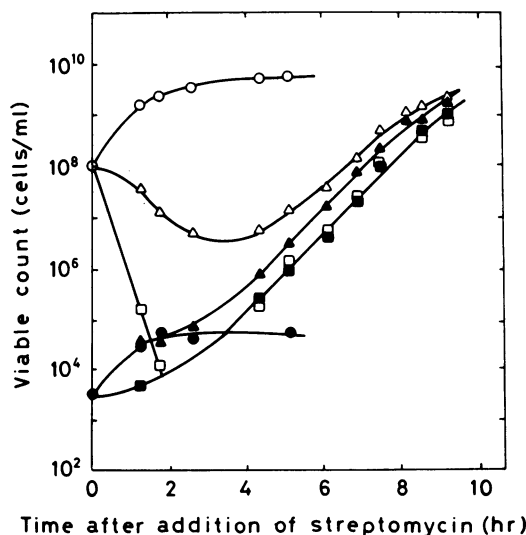


FIG. 6. Effect of streptomycin on selection of mutants of G11a1-R1 with increased resistance to streptomycin. Experimental conditions were as in Fig. 5. Streptomycin concentrations ( $\mu\text{g/ml}$ ): 0 (circles), 400 (triangles) and 2,000 (squares). Viable count was measured on LA plates (open symbols) and on LA plates containing 100  $\mu\text{g}$  of streptomycin per ml (closed symbols).

population is due to detoxification of the medium.

There are several possible explanations of why some of the cells survive even in the presence of high concentrations of ampicillin: (i) some of the cells contain an increased number of R-factor copies per cell, (ii) the population contains highly resistant mutants, or (iii) cells in a certain stage of the division cycle are less susceptible to killing by ampicillin.

A cell containing an increased number of R-factor copies should contain a proportionally increased  $\beta$ -lactamase activity and hence a proportionally increased ampicillin resistance (26, 28). However, about 80 times more copies of R1 than the average cell contains would be required to confer a resistance of 8,000  $\mu\text{g}$  of ampicillin/ml. There is very little likelihood that the statistical variations in the number of plasmid copies can be that great.

The mutant theory was ruled out by analysis of the survivors obtained at high concentrations of ampicillin.

The final possibility proposed was that cells at a certain stage of the cell division cycle are less susceptible to killing by ampicillin. There are great variations in the penicillin susceptibility of synchronously growing *E. coli* during the

division cycle, with maximum susceptibility 10 min before the time of cell division (15, 24). It is also known that penicillins induce the formation of filaments (16). A microscope examination of the cultures grown in the presence of high concentrations of ampicillin showed that the majority of the cells present lysed rapidly. The few remaining living cells were filaments. The size of the filaments indicated that they were not formed before the addition of ampicillin. There was a good correlation between viable count and the number of filaments.

We favor the conclusion that at high concentrations of ampicillin some cells survive due to variations in penicillin susceptibility during the cell division cycle. The survivors form filaments and, when the ampicillin is completely hydrolyzed, they resume normal growth.

**Effect of chloramphenicol.** The R-factor R1 codes for an enzyme that acetylates chloramphenicol (10). The inactivating enzyme requires acetyl-coenzyme A (28, 37), i.e., an intact energy metabolism. The rate of the inactivation of chloramphenicol decreased with time and became zero after 1 to 2 h of incubation. Why the rate of acetylation of chloramphenicol decreases is not obvious. Gale and Paine (12) and Gale and Folkes (11) found that very high concentrations of chloramphenicol have no effect on the respiration of glucose. Hahn et al. (14) have shown that phosphorylation in glucose catabolism is unaffected by chloramphenicol. Hence, the formation of acetyl-coenzyme A should not be reduced by chloramphenicol. However, some quantitative estimation of the

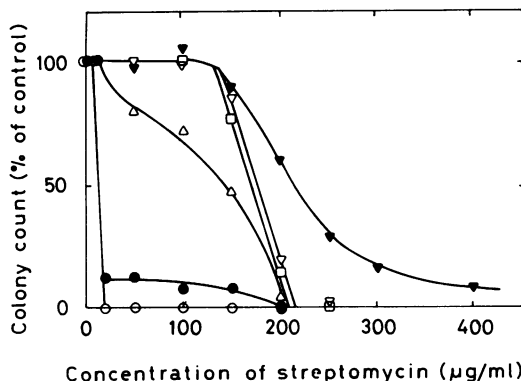


FIG. 7. Resistance to streptomycin of G11a1-R1 grown in the presence of different concentrations of streptomycin. The cultures from Fig. 5 were taken after incubation over night, diluted  $10^6$ -fold, and spread on LA containing streptomycin. Concentrations of streptomycin ( $\mu\text{g/ml}$ ) in pregrowth medium: 0 ( $\circ$ ), 300 ( $\bullet$ ), 400 ( $\Delta$ ), 1,000 ( $\square$ ), 2,000 ( $\nabla$ ) and 5,000 ( $\blacktriangledown$ ).

metabolism of chloramphenicol may be relevant to the present discussion. During the first 15 min (half a generation), about 600  $\mu\text{g}$  of chloramphenicol was acetylated per ml (Fig. 4); this is in accordance with the acetylase activity measured in vitro (Table 1). If we assume that the final product is the diacetate (38), this corresponds to a consumption of about 4  $\mu\text{mol}$  of adenosine 5'-triphosphate (ATP) per ml. About 10.5 g of cellular dry weight is normally obtained per mole of ATP (2, 13, 36), and the acetylation of 600  $\mu\text{g}$  of chloramphenicol energetically corresponds to the formation of 40  $\mu\text{g}$  of cell substance. At the time of addition of chloramphenicol, the cell concentration was about 70  $\mu\text{g}$  dry weight per ml. Hence, the acetylation of chloramphenicol consumes all energy that was available for growth. The result also shows that, in the initial stage, energy metabolism has its normal capacity even in the presence of these huge concentrations of chloramphenicol. Thus, acetylation of chloramphenicol introduces a heavy load on the energy metabolism of the cells. It is reasonable to suggest that this load on the energy metabolism creates difficulties for the cells to supply themselves with the maintenance energy required; e.g., this could lead to losses of acetyl-coenzyme A or some other essential component (cf. below).

The enzyme chloramphenicol acetylase is cytoplasmic (40) and was not lost from the chloramphenicol-treated cells. The continuous reduction in the rate of acetylation could be due to product inhibition of the enzyme. However, no product inhibition was found at the concentrations of acetylated chloramphenicol present in the medium (5 mM).

The decrease in OD observed in the presence of chloramphenicol was not due to leakage of substances from the cells, because very little extracellular protein was found even after 4 h of incubation. Hence, there was no major lysis of the cells. This was verified by the fact that protein synthesis (measured as induction of  $\beta$ -galactosidase) resumed its normal level immediately after the chloramphenicol had been metabolized. Hence, there was no irreversible distortion of protein synthesis. However,  $\beta$ -lactamase was excreted to a large extent. This enzyme is periplasmic (19), and its excretion is consistent with the finding that chloramphenicol treatment leads to the loss of outer membrane complex (lipopolysaccharide, phospholipid, and membrane protein [32]). The bactericidal effects of chloramphenicol have been discussed by Brock (5).

Growth resumed when chloramphenicol was

completely metabolized, and the survivors were not mutants. Hence, the survival of the population in the presence of chloramphenicol is due to detoxification of the medium.

**Effect of streptomycin.** No inactivation of streptomycin was detected in vivo, although R1 codes for an enzyme that metabolizes streptomycin (28). The enzyme activity is very low but is still of importance for the survival of the cells in the presence of streptomycin (see Table 1). In the presence of high concentrations of streptomycin, there was rapid killing of the cells. However, after 1 to 2 h of incubation, the viable count started to increase. This resumption of growth was due to mutants present in the population. These mutants were about 10 times as resistant to streptomycin as was the parent strain. They will be further discussed in an accompanying paper (22).

In conclusion, the R-factor R1 has a drastic effect on the survival of the population in the presence of high concentrations of ampicillin, chloramphenicol, and streptomycin. In the two first cases, survival is due to detoxification of the medium, whereas in the presence of streptomycin, more resistant mutants are selected.

#### ACKNOWLEDGMENTS

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