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Lysis of Nongrowing *Escherichia coli* by Combinations of β-Lactam Antibiotics and Inhibitors of Ribosome Function

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It is generally assumed that only actively growing bacteria are killed by inhibitors of peptidoglycan synthesis. Several exceptional examples are described here. As expected, ampicillin did not lyse nongrowing, amino acid-deprived cultures of *relA*$^+$ strains of *Escherichia coli*, but the subsequent addition of several ribosome inhibitors (chloramphenicol, tetracycline, gentamicin, and kanamycin) caused various degrees of lysis in such ampicillin-treated cultures. Of the antibiotics tested, only streptomycin was ineffective in this regard. Peptidoglycan synthesis has been shown to be inhibited in amino acid-deprived *relA*$^+$ bacteria by the stringent control mechanism (E. E. Ishiguro and W. D. Ramey, J. Bacteriol. 127:1119–1126, 1976), and the ribosome inhibitors tested here relaxed peptidoglycan synthesis to various degrees under these conditions. The relative lysis-inducing activities of the ribosome inhibitors on ampicillin-treated, amino acid-deprived bacteria were directly correlated to their relative activities as stringent control antagonists. This phenomenon was not dependent on amino acid deprivation. Cultures treated with growth inhibitory levels of the various ribosome inhibitors alone were lysed by ampicillin, apparently because peptidoglycan synthesis continues uninhibited when growth is arrested by treatment with ribosome inhibitors. These results indicate that autolysis can be triggered by the inhibition of peptidoglycan synthesis occurring in the absence of wall expansion; i.e., active cell growth is unnecessary.

It is generally assumed that β-lactam antibiotics and other inhibitors of peptidoglycan synthesis kill only actively growing bacteria. For example, *Escherichia coli* cells which are deprived of a required amino acid, or other growth factors, develop tolerance to β-lactam antibiotics, and this forms the basis for the classic penicillin enrichment technique for the isolation of auxotrophic mutants (3, 8). We recently described an interesting exception which we call chloramphenicol-dependent lysis (7a). In this case, the addition of chloramphenicol to amino acid-deprived cultures of *E. coli* which were treated with β-lactam antibiotics, D-cycloserine, or moenomycin resulted in lysis. Furthermore, amino acid deprivation was not essential for this phenomenon; i.e., cultures inhibited with chloramphenicol alone were subsequently lysed when treated with inhibitors of peptidoglycan synthesis.

Peptidoglycan synthesis in *E. coli* is regulated by the stringent control mechanism (6, 7). Thus, amino acid deprivation results in the rapid accumulation of guanosine 5′-diphosphate 3′-diphosphate (ppGpp), a putative mediator of stringent control (1, 4), and in the concomitant inhibition of peptidoglycan synthesis in *relA*$^+$ strains. On the other hand, *relA* mutants do not accumulate ppGpp during amino acid deprivation, and peptidoglycan synthesis continues (relaxed control). We considered three observations to be important in formulating a possible mechanistic basis for chloramphenicol-dependent lysis. First, the tolerance to inhibitors of peptidoglycan synthesis during amino acid deprivation is exhibited by *relA*$^+$ bacteria but not by *relA* mutants; i.e., cultures of amino acid-deprived *relA* mutant strains undergo lysis when treated with β-lactam antibiotics, D-cycloserine, or moenomycin (5, 7a). Second, chloramphenicol is known to antagonize stringent control by preventing the accumulation of ppGpp in amino acid-deprived *relA*$^+$ cells (1), and chloramphenicol therefore causes relaxation of peptidoglycan synthesis under these conditions (7). Third, it is also noteworthy that peptidoglycan synthesis is not inhibited in cells treated with chloramphenicol alone, i.e., without concomitant amino acid deprivation (7). These points are discussed further below in terms of a possible mechanism for chloramphenicol-dependent lysis.

In this study, we screened several other inhibitors of protein synthesis for their abilities to lyse penicillin-treated amino acid-deprived *relA*$^+$ *E. coli*. We show that the various ribosome inhibitors tested caused lysis with various degrees of efficacy which were directly related to their relative activities as antagonists of stringent control. We also show that lysis caused by combinations of these ribosome inhibitors and β-lactam antibiotics was not dependent on concomitant amino acid deprivation and may be dependent on the growth medium used.

MATERIALS AND METHODS

***E. coli* K-12 strains and growth conditions.** *E. coli* K-12 W3110 was a wild-type strain obtained from B. J. Bachmann of the *E. coli* Genetic Stock Center, Yale University, New Haven, Conn. Strain LD5 (*thi lysA dapD rpsL*) was described previously (7). The bacteria were grown in M9 minimal medium containing 0.2% glucose and, for strain LD5, required growth factors as previously described (7). Viable cell counts were determined by plating on tryptic soy agar (Difco Laboratories, Detroit, Mich.). In one set of experiments (see Fig. 5), bacteria were also grown in tryptic soy broth (TSB; Difco Laboratories) and in M9 medium containing 0.2% glucose and 0.1% Casamino Acids (certified grade; Difco Laboratories). These media contained 50 μg of *meso*-DAP (diaminopimelic acid) per ml for growth of strain LD5. Cultures were incubated in a water bath shaker at 37°C.

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Culture turbidity was determined in a Klett-Summerson colorimeter with a blue filter for the M9 media and a green filter for TSB. The general procedures for the experiments were previously described (7). The bacteria were grown to a density of 5×10^8 cells per ml and then subjected to amino acid deprivation or antibiotic treatments as specified below. Lysine deprivation of strain LD5 was achieved by transferring washed bacteria to M9 minimal medium lacking L-lysine (7). Alternatively, both LD5 and W3110 were deprived of isoleucine by adding L-valine (500 μg/ml) to M9 medium (10). In complex media, amino acid deprivation was achieved by the addition of 500 μg of serine hydroxamate per ml.

Determination of RNA and peptidoglycan synthesis. The synthesis of peptidoglycan was assayed in strain LD5 (*dapD lysA*). The assays were done in M9 minimal medium containing 1.5 μg (3.3 μCi) of [G-^{3}H]DAP ([^{3}H]DAP; Amersham Corp., Arlington Heights, Ill.) per ml. The incorporation of [^{3}H]DAP into cold trichloroacetic acid-insoluble cell fractions was determined as previously described (7). RNA synthesis was assayed in the same manner, except that the assay medium contained 0.5 μg (0.08 μCi) of [2-^{14}C]uracil (Amersham) per ml instead of [^{3}H]DAP.

Determination of peptidoglycan hydrolysis. Strain LD5 was labeled in M9 minimal medium containing 1 μg (0.67 μCi) of [^{3}H]DAP per ml. Labeling was terminated after 70 min by adding unlabeled DAP to the culture at 50 μg/ml. The culture was subjected to amino acid deprivation and antibiotic treatment 15 min later. To determine whether the various treatments resulted in the solubilization of peptidoglycan, the amounts of radioactivity in the peptidoglycan fractions of the treated cultures were monitored as follows. At the indicated times, 0.5-ml samples of the cultures were drawn by a pipette directly into tubes containing 0.5 ml of boiling 8% sodium dodecyl sulfate. After 30 min of boiling, the sodium dodecyl sulfate-insoluble fractions (containing peptidoglycan) of each sample were collected on Millipore type HA membrane filters (0.45-μm pore diameter; Millipore Corp., Bedford, Mass.). The filters were rinsed with distilled water, dried, and then counted in a toluene-based scintillation cocktail by using a Beckman LS 3145T liquid scintillation counter.

Antibiotics. Chloramphenicol, tetracycline, gentamicin, kanamycin, streptomycin, ampicillin, cephalothin, cephaloridine, benzylpenicillin, and D-cycloserine were obtained from the Sigma Chemical Co., St. Louis, Mo. Moenomycin was the kind gift of G. Huber (Hoechst Aktiengesellschaft, Frankfurt am Main, Federal Republic of Germany). The MICs of the antibiotics were determined by preparing two-fold serial dilutions of the drugs in TSB. The media were inoculated with the test organism to yield 10^5 cells per ml. The presence or absence of growth was determined after 16 h of incubation at 37°C. The MIC of streptomycin was 8 μg/ml for strain W3110. Strain LD5 was streptomycin resistant (*rpsL*). The MICs of other drugs were identical in both strains; those relevant to the results presented below were (in micrograms per milliliter) ampicillin, 4; chloramphenicol, 4; gentamicin, 1; tetracycline, 1; and kanamycin, 8.

RESULTS

Experiments with amino acid-deprived bacteria. The effects of various inhibitors of protein synthesis on isoleucine-deprived cultures of strain W3110 which were treated with ampicillin at 40 μg/ml (equivalent to 10 times the MIC) are shown in Fig. 1. As shown previously (7a), such cultures were tolerant to ampicillin but were lysed when ampicillin was combined with chloramphenicol. Tetracycline and gentamicin were as effective as chloramphenicol in inducing lysis. However, kanamycin was significantly less effective, and streptomycin was essentially ineffective. In all cases, the decreases in culture turbidity were correlated with microscopic examinations which showed the appearance of large numbers of obviously lysed cells. Identical results were obtained with strain LD5 deprived of either isoleucine or lysine. Furthermore, ampicillin could be replaced by other β-lactam antibiotics (benzylpenicillin, cephalothin, and cephaloridine), D-cycloserine, or moenomycin.

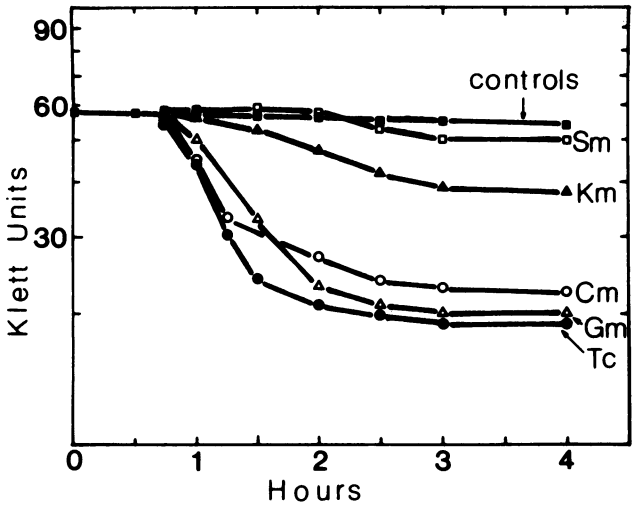

FIG. 1. Effects of ampicillin and various inhibitors of ribosome function on isoleucine-deprived cells of strain W3110. Ampicillin (40 μg/ml) was added to a series of isoleucine-deprived cultures at 10 min, and the following were added at 20 min: chloramphenicol, 100 μg/ml (○); tetracycline, 25 μg/ml (●); gentamicin, 50 μg/ml (△); kanamycin, 50 μg/ml (▲); and streptomycin, 100 μg/ml (□). An untreated amino acid-deprived control culture and controls which were treated with each of the ribosome inhibitors alone (not shown) gave the same results as the control treated with ampicillin alone (■).

The effects of the various ribosome inhibitors alone or in combination with ampicillin on the viability of isoleucine-deprived cells of strain W3110 in the experiment described in the legend to Fig. 1 are shown in Table 1. The viable cell count increased 1.7-fold after 2 h of isoleucine deprivation. Ampicillin treatment caused no change in the viable cell count over the same time period. In comparison, the viable cell counts decreased about 10^2-fold when ampicillin was combined with chloramphenicol or tetracycline. Thus, the observed decreases in the turbidities of cultures treated with these drug combinations (Fig. 1) were correlated with decreases in viable cell counts. It should be noted that kanamycin, streptomycin, and gentamicin by themselves were highly bactericidal both in normal growing cultures and in amino acid-deprived cultures, and their effects when combined with ampicillin were therefore not examined.

The decreases in the turbidity of amino acid-deprived cultures which were treated with combinations of β-lactam antibiotics and ribosome inhibitors were also correlated with peptidoglycan hydrolysis. A typical experiment is shown in Fig. 2A and 2B. A strain LD5 culture which was prelabeled with [^{3}H]DAP was divided into five portions. Culture 1 was an untreated control. The radioactivity in the peptidoglycan fraction of this culture remained constant throughout the experiment. Culture 2 was treated with ampicillin, and the

TABLE 1. Effect of ampicillin and various ribosome inhibitors on the viability of isoleucine-deprived cultures of strain W3110[a]

Treatment	No. of viable cells per ml
Untreated control	3.3×10^8
Ampicillin	1.8×10^8
Chloramphenicol	2.8×10^8
Chloramphenicol + ampicillin	4.4×10^6
Tetracycline	1.5×10^8
Tetracycline + ampicillin	2.9×10^6

[a] These data were obtained from the experiment described in the legend to Fig. 1. The cultures treated with streptomycin and gentamicin were not tested. The viable cell counts shown were determined after 120 min of treatment. The initial viable count was 1.9×10^8 cells per ml.

decrease in the turbidity of this culture was accompanied by solubilization of 65% of the labeled peptidoglycan. The other cultures (3, 4, and 5) were simultaneously subjected to isoleucine deprivation and ampicillin treatment. Culture 3, which received no additional treatment, showed no loss of label from the peptidoglycan fraction. In contrast, 20% of the label from the peptidoglycan fraction was lost if such cultures were also treated with either chloramphenicol (culture 4) or gentamicin (culture 5). Thus, the ribosome inhibitors caused peptidoglycan hydrolysis in ampicillin-treated amino acid-deprived cultures, although more extensive hydrolysis was observed in growing cultures which were treated with ampicillin.

The various ribosome inhibitors were tested for ability to antagonize stringent control. The effects of these agents on RNA and peptidoglycan synthesis in amino acid-deprived *relA*$^+$ bacteria are shown in Fig. 3A and 3B, respectively. The activities of these drugs as stringent control antagonists were assessed on the bases of the relative degrees of relaxation of macromolecular synthesis in drug-treated cultures and on the lag times between drug addition and the onset of relaxed macromolecular synthesis. The results indicate that (i) tetracycline, gentamicin, and chloramphenicol were equally effective in antagonizing stringent control, (ii) kanamycin was less effective, and (iii) streptomycin was the least effective. Thus, we concluded that the abilities of these ribosome inhibitors to cause autolysis of amino acid-deprived *relA*$^+$ bacteria when combined with ampicillin treatment (Fig. 1) appear to be directly correlated to their relative activities as stringent control antagonists.

Strain W3110 was used (Fig. 3A) to illustrate the relative ineffectiveness of streptomycin in relaxing RNA synthesis; strain LD5 was not used for this purpose because it is streptomycin resistant (*rpsL*). However, it should be noted that the other ribosome inhibitors had identical effects on RNA synthesis in amino acid-deprived cultures of both LD5 and W3110. Of course, peptidoglycan synthesis was not examined in W3110 because of the inability to label peptidoglycan specifically in this strain.

Experiments without amino acid deprivation. In the experiment described in the legend to Fig. 4, the growth of strain LD5 cultures was arrested by treatment with various ribosome inhibitors, and ampicillin was added 20 min later. In every case, lysis was initiated within 30 min after the addition of ampicillin. Thus, the lysis of nongrowing *E. coli* cells by the combined action of ampicillin and the various ribosome inhibitors was not dependent on concomitant

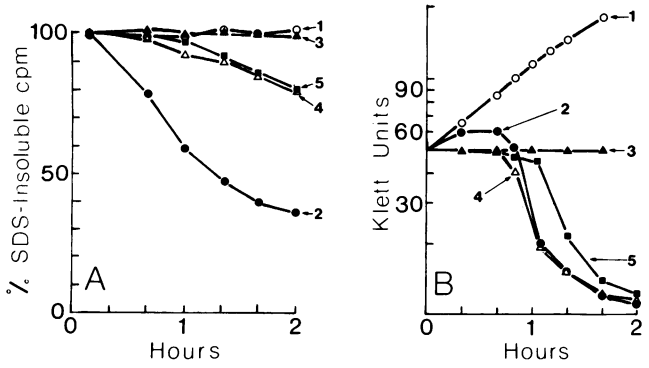

FIG. 2. Effects of various treatments on the hydrolysis of peptidoglycan in strain LD5. A culture growing in M9 minimal medium, which was prelabeled with [^{3}H]DAP, was divided into five portions: culture 1, untreated control; culture 2, treated with 40 μg of ampicillin per ml; cultures 3, 4, and 5, isoleucine deprived and ampicillin treated (40 μg/ml). Cultures 4 and 5 also received chloramphenicol (100 μg/ml) and gentamicin (50 μg/ml), respectively. At the indicated times, the levels of sodium dodecyl sulfate-insoluble radioactivity (A) and the culture turbidities (B) were determined. In panel A, 100% was equal to 17,000 cpm.

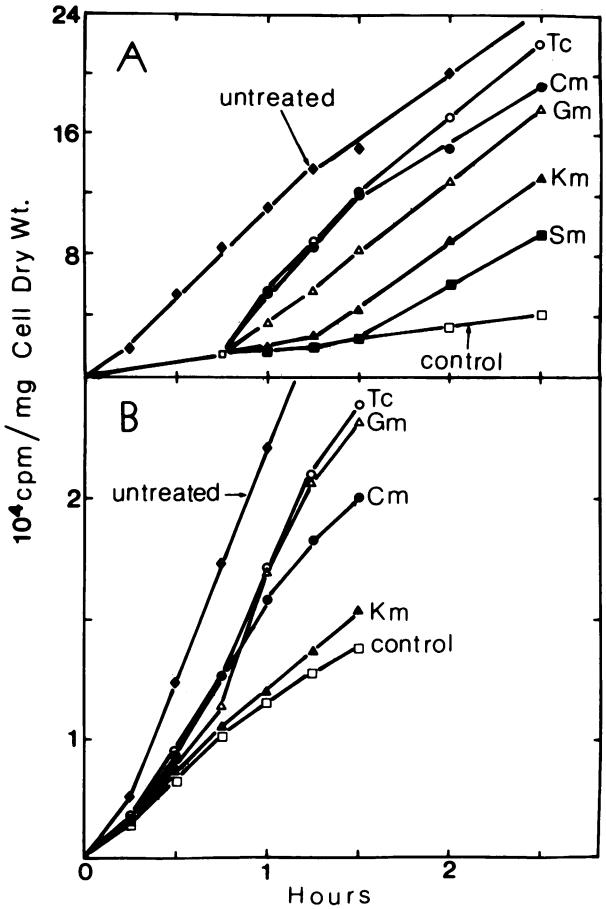

FIG. 3. Effects of various ribosome inhibitors on RNA synthesis in strain W3110 (A) and peptidoglycan synthesis in strain LD5 (B) during amino acid deprivation. The following were added at the concentrations given in the legend to Fig. 1 to isoleucine-deprived cultures at 40 min (A) and 25 min (B): tetracycline (○), chloramphenicol (●), gentamicin (△), kanamycin (▲), and streptomycin (■) (not tested in panel B). Normally growing controls (◆) and untreated isoleucine-deprived controls (□) are shown for comparison.

amino acid deprivation. Furthermore, all of the ribosome inhibitors tested were equally effective in causing lysis when combined with ampicillin under these conditions, in contrast to their differential effects on amino acid-deprived bacteria (Fig. 1). In an effort to reconcile these results, we determined the effects of the various ribosome inhibitors on peptidoglycan synthesis in strain LD5 cultures which were not amino acid deprived. Peptidoglycan synthesis continued in the presence of all of these agents at rates equal to that in an untreated control culture (Fig. 5). This was in contrast to their differential effects on the relaxation of peptidoglycan synthesis in amino acid-deprived cultures (Fig. 3B). Therefore, these results were consistent with the proposal made above; i.e., the lysis-inducing activities of the various drug combinations were directly related to the relative rates of peptidoglycan synthesis occurring in the drug-treated bacteria.

Effect of growth medium. In Fig. 6, we present preliminary data indicating that the phenomenon of lysis caused by combinations of β-lactam antibiotics and ribosome inhibitors was growth medium dependent. The combined effects of chloramphenicol and ampicillin on strain LD5 cultures which were subjected to amino acid deprivation in three different media are shown in Fig. 6A. Similar experiments were performed without imposing amino acid deprivation (Fig. 6B). M9 minimal medium was used in all of the experiments described in the preceding sections. The modification of this medium by the addition of 0.1% Casamino Acids did not alter the effects of the chloramphenicol-ampicillin combination (Fig. 6A and 6B). However, the lysis induced by these agents was markedly reduced when the experiments were performed in TSB.

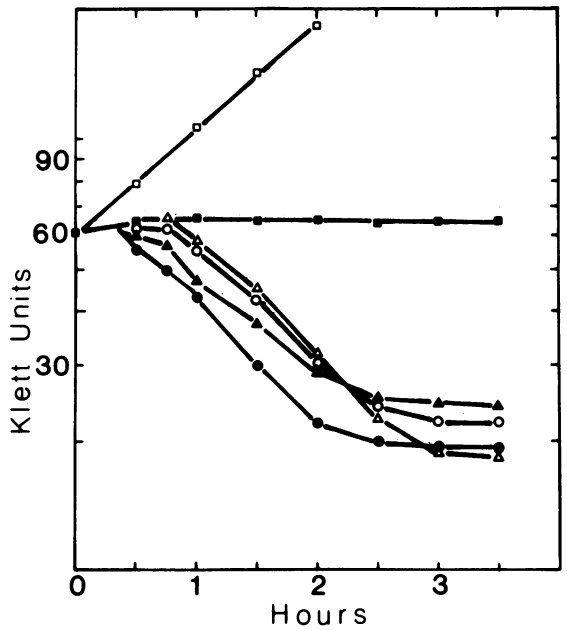

FIG. 4. Effects of ampicillin on strain LD5 cultures treated with various ribosome inhibitors. LD5 cultures were inhibited with tetracycline (○), chloramphenicol (●), kanamycin (△), and gentamicin (▲) at 0 min at the concentrations given in the legend to Fig. 1, and ampicillin (40 μg/ml) was added at 20 min. The controls treated with each of the ribosome inhibitors alone all gave identical results and are represented by a single curve (■). An untreated control (□) is also shown.

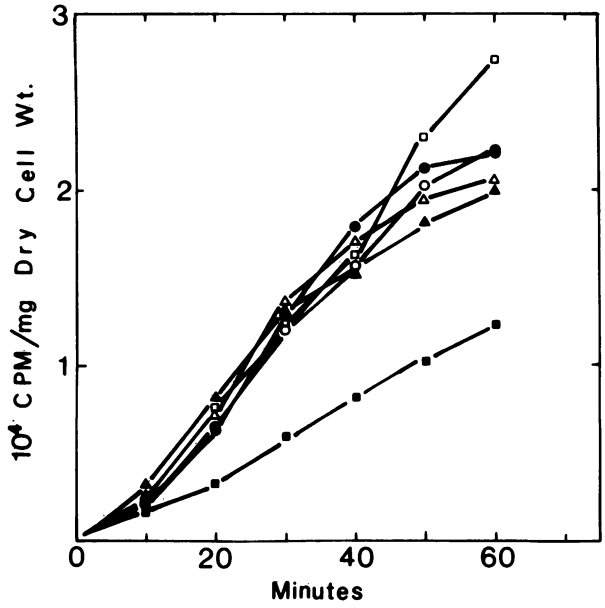

FIG. 5. Effects of ribosome inhibitors on peptidoglycan synthesis. Peptidoglycan synthesis was assayed in strain LD5 cultures treated with chloramphenicol (○), tetracycline (●), kanamycin (△), and gentamicin (▲) at the concentrations given in the legend to Fig. 1. Peptidoglycan synthesis in an untreated growing control culture (□) and in an isoleucine-deprived culture (■) are shown for comparison.

DISCUSSION

The inhibition of peptidoglycan synthesis apparently deregulates the activities of the cellular peptidoglycan hydrolases, and this ultimately results in autolysis (9). The mechanism of this process is not known. Amino acid-deprived *relA*$^+$ *E. coli* develops tolerance to lysis induced by β-lactam antibiotics and other inhibitors of peptidoglycan synthesis (5, 7a). In our view, the lysis tolerance of nongrowing bacteria is related to the reduced rate of peptidoglycan synthesis occurring in such cells, e.g., the inhibition of peptidoglycan synthesis caused by stringent control in amino acid-deprived *relA*$^+$ cells. However, our data indicate that, contrary to popular belief, active cell growth is in fact not essential for autolysis induced by inhibitors of peptidoglycan synthesis. Nongrowing cells apparently can be lysed provided that active peptidoglycan synthesis is occurring in such cells, e.g., by treating amino acid-deprived cells with stringent control antagonists as described above. It is premature to discuss the implications of this finding on phenomena such as the synergistic or antagonistic activities of ribosome inhibitors on the action of inhibitors of peptidoglycan synthesis. For example, the lysis of nongrowing *E. coli* was apparently growth medium dependent. The basis for this is not known, and further work on this aspect is clearly necessary.

This study extended our previous observations on the phenomenon of chloramphenicol-dependent autolysis (7a) by showing that other inhibitors of protein synthesis can also cause lysis of amino acid-deprived *relA*$^+$ bacteria which have been treated with inhibitors of peptidoglycan synthesis. As already mentioned, chloramphenicol antagonizes stringent control by inhibiting the accumulation of ppGpp in amino acid-deprived *relA*$^+$ cells (1). Thus, we have proposed (7a) that chloramphenicol acted by relaxing peptidoglycan

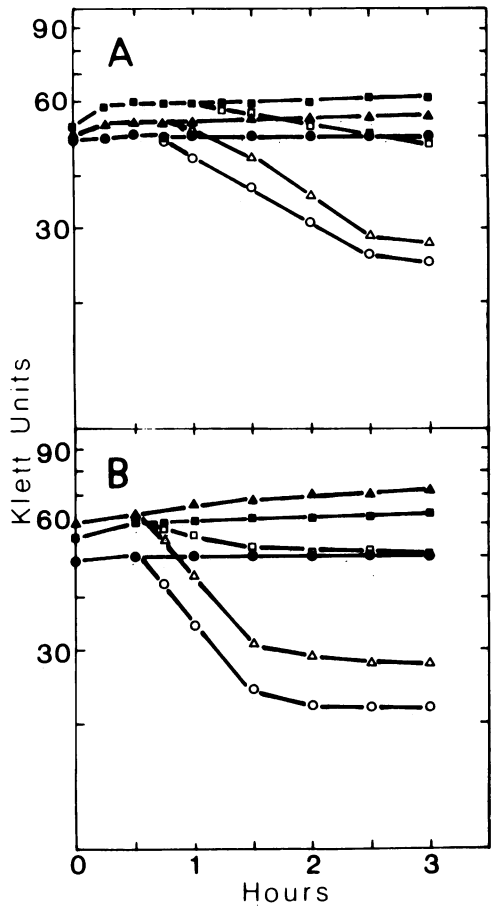

FIG. 6. Effect of growth medium on lysis induced by a combination of ampicillin and chloramphenicol. (A) Serine hydroxamate (500 μg/ml) was added to impose amino acid deprivation in duplicate cultures of strain LD5 in M9 minimal medium (○, ●), M9 medium containing 0.1% Casamino Acids (△, ▲), and TSB (□, ■). Ampicillin (40 μg/ml) and chloramphenicol (100 μg/ml) were added at 30 and 40 min, respectively, to one of the cultures in each set (open symbols). The other cultures (closed symbols) served as untreated controls. (B) The same experiment was performed without imposing amino acid deprivation; i.e., without the addition of serine hydroxamate. The various sets of media are represented by the same symbols as in panel A. Growth was arrested by adding chloramphenicol to both cultures in each set at 0 min. Ampicillin was added to one of the cultures in each set at 20 min (open symbols). The other cultures (closed symbols) served as controls.

synthesis during amino acid deprivation and that this was essential for the induction of autolysis. It is noteworthy that this proposal was based largely on observations indicating that treatment with inhibitors of peptidoglycan synthesis alone was sufficient to cause lysis of amino acid-deprived cells of *relA* mutant strains (5, 7a); i.e., chloramphenicol is not required for lysis in these cases because peptidoglycan synthesis is relaxed during amino acid deprivation as a consequence of the *relA* mutation. Further support for this proposed mechanism is presented here in the form of data showing that the relative efficacies of the various ribosome inhibitors as lysis-inducing agents when combined with ampicillin (Fig. 1) correlated directly with their relative activities as antagonists of the stringent control mechanism (Fig. 3A and 3B). Of the antibiotics tested, kanamycin and streptomycin were the agents which were the least effective for both properties. This result is consistent with a recent report (2) showing that kanamycin and streptomycin do not inhibit the synthesis of ppGpp in amino acid-deprived *relA*$^+$ cells.

Turbidimetric and microscopic determinations indicated that the treatments with the most effective stringent control antagonists were as effective in causing lysis of amino acid-deprived cultures as treatments with β-lactam antibiotics alone were in causing lysis of growing cultures (e.g., see Fig. 2). When possible, we also showed that the stringent control antagonists caused loss of viability of ampicillin-treated amino acid-deprived cells (Table 1). However, peptidoglycan hydrolysis assays indicated that peptidoglycan hydrolysis was markedly more extensive in growing cultures treated with ampicillin than in amino acid-deprived cultures treated with combinations of ampicillin and ribosome inhibitors (Fig. 2). Thus, it appears that lysis and loss of viability do not necessarily require extensive peptidoglycan hydrolysis.

The lysis induced by combinations of ribosome inhibitors and ampicillin was not dependent on concomitant amino acid deprivation. Furthermore, lysis occurred equally well with all of the ribosome inhibitors under these conditions, apparently because peptidoglycan synthesis was not inhibited. It is therefore interesting to note that peptidoglycan synthesis in wild-type *E. coli* was not obligatorily coupled to growth and continued in the absence of growth under at least one set of conditions, namely during treatment with ribosome inhibitors.

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