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# Effects of Aminoglycosides and Spectinomycin on the Synthesis and Release of Lipopolysaccharide by *Escherichia coli*

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**The effects of aminoglycosides and spectinomycin on lipopolysaccharide (LPS) synthesis and release by *Escherichia coli* were studied. LPS synthesis was previously reported to be regulated by the stringent control mechanism. In agreement with this, the control of LPS synthesis in amino acid-deprived *relA*$^+$ cells was relaxed by spectinomycin, a proven stringent control antagonist, but not by kanamycin, an agent which is ineffective as a stringent control antagonist. The other stringent control antagonists tested (gentamicin, tobramycin, and, to a lesser extent, amikacin) unexpectedly failed to relax the control of LPS synthesis, and this was subsequently shown to be due to their inhibitory action on LPS synthesis. The release of LPS by nongrowing (amino acid-deprived and antibiotic-treated) bacteria was stimulated only under conditions in which the control of LPS synthesis was relaxed.**

The syntheses of several classes of macromolecules (e.g., stable RNA, phospholipids, and peptidoglycan) are inhibited by a mechanism known as the stringent response when *relA*$^+$ strains of *Escherichia coli* are subjected to amino acid deprivation (1). In contrast, this control is relaxed and these macromolecules continue to be synthesized in amino acid-deprived *relA* mutants. Two novel nucleotides, guanosine 5′-triphosphate 3′-diphosphate (pppGpp) and guanosine 5′-diphosphate 3′-diphosphate (ppGpp), accumulate during amino acid deprivation in *relA*$^+$ bacteria but not in *relA* mutants, and these compounds may be involved in mediating the stringent response. Several antibiotics which inhibit eubacterial ribosome function also inhibit the stringent response in amino acid-deprived *relA*$^+$ bacteria, apparently by preventing the accumulation of pppGpp and ppGpp (1, 2, 5). These stringent control antagonists include chloramphenicol and various aminoglycosides.

We have previously shown (6) that the synthesis of lipopolysaccharide (LPS) in *E. coli* and *Salmonella typhimurium* is subject to stringent control. Thus, LPS synthesis is inhibited during amino acid deprivation in *relA*$^+$ strains, but no inhibition occurs in *relA* mutants under the same conditions. Furthermore, the control of LPS synthesis in amino acid-deprived *relA*$^+$ cells can be relaxed by treatment with chloramphenicol.

Knox et al. (4) first reported that *E. coli* releases LPS into the growth medium during amino acid deprivation. Rothfield and Pearlman-Kothencz (8) subsequently showed that LPS release also occurs during normal growth and that the inhibition of protein synthesis by amino acid deprivation or by chloramphenicol treatment stimulates the rate of LPS release. We have recently confirmed (6) that the slow release of LPS is characteristic of the normal growth process and that chloramphenicol stimulates this release. However, in our experience, amino acid deprivation stimulates LPS release only in *relA* mutant strains and not in *relA*$^+$ bacteria. These results may mean that LPS release is stimulated in nongrowing bacteria only when the control of LPS (and phospholipid) synthesis is relaxed, e.g., by chloramphenicol treatment or by introducing a mutation into *relA*. This stimulation in the rate of LPS release may be attributable to the inability of nongrowing bacteria to accommodate the excess cell envelope material being synthesized, as already proposed by Russell (9). Our objective in this study was to investigate further the regulation of LPS synthesis and release in antibiotic-treated *E. coli*. We report the effects of several aminoglycosides and the aminocyclitol spectinomycin on these processes. Our results generally support the aforementioned correlation between LPS release and LPS synthesis. Furthermore, we show that certain aminoglycosides inhibit LPS synthesis by an undetermined mechanism.

## MATERIALS AND METHODS

**Bacterial strains and general cultural conditions.** The isogenic *E. coli* K-12 strains VC210 (*thi-1 lysA23 galE15 zfj*::Tn*5 rpsL*) and VC211 (*thi-1 lysA23 galE15 zfj*::Tn*5 relA2 rpsL*) have been described previously (6). They were grown in M9 minimal medium containing 50 mM glycerol as the carbon source and supplemented with thiamine (0.5 μg/ml) and L-lysine (50 μg/ml). Since *galE* mutants are galactose sensitive (7), galactose was added to the medium exactly as described previously (6) in experiments involving the determination of LPS synthesis. Cultures were grown at 37°C in a gyratory water bath shaker, and growth was followed with a Klett-Summerson colorimeter by using a blue filter.

**LPS synthesis and release.** Detailed procedures for LPS synthesis and release have been published previously (6). Briefly, amino acid deprivation was achieved by either omitting L-lysine from the medium or by adding L-valine to 500 μg/ml (to cause isoleucine deprivation). Unless indicated otherwise, the following antibiotics were added at the indicated concentrations (2 times the MICs): chloramphenicol, 8 μg/ml; spectinomycin, 64 μg/ml; kanamycin, 16 μg/ml; amikacin, 8 μg/ml; gentamicin, 2 μg/ml; tobramycin, 4 μg/ml. All antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.). To determine the effects of these treatments on LPS synthesis (Fig. 1 through 3), 0.5-ml samples taken from the various cultures at the indicated times were pulse-labeled for 10 min with 1 μCi of D-[1-$^3$H]galactose (10.4 Ci/mmol; Amersham Corp., Oakville, Ontario, Canada), and the amounts of radiolabel incorporated into cold trichloroacetic acid-insoluble fractions were determined.

The effects of the same treatments on the release of LPS were determined on cultures growing in M9 medium con-

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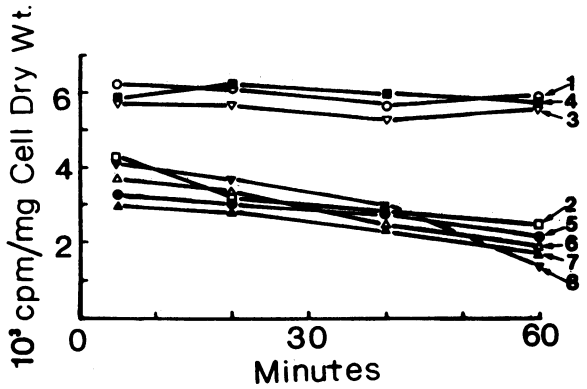


FIG. 1. Effects of antibiotics on LPS synthesis in strain VC210 *relA*$^+$. Numerals indicate the following: untreated control (curve 1); isoleucine-deprived control (curve 2); and a series of isoleucine-deprived cultures treated with chloramphenicol (curve 3), spectinomycin (curve 4), kanamycin (curve 5), gentamicin (curve 6), amikacin (curve 7), and tobramycin (curve 8). Samples taken at the indicated times were pulse-labeled with [$^3$H]galactose, and the amounts of radiolabel incorporated into LPS were determined.

taining 1 μCi (4.1 μg) of [$^3$H]galactose per ml (Fig. 4). The cells in samples taken at the indicated times were collected by centrifugation, and the amounts of radiolabeled trichloroacetic acid-insoluble material in the cell-free and cell-bound fractions were determined. In one set of experiments (Fig. 5), the effects of antibiotic treatment on the release of prelabeled (old) cell-bound LPS were determined. For this purpose, bacteria were labeled with [$^3$H]galactose for 120 min (1.5 doubling dilutions). Labeling was terminated, and the release of labeled LPS was then followed as described previously (6).

## RESULTS

**Effects of aminoglycosides and spectinomycin on LPS synthesis.** As discussed below, all of the antibiotics used in this study except kanamycin were previously shown to be effective inhibitors of the stringent response by several criteria, and these properties were verified for the concentrations used in this study (data not shown). Thus, we tested the effects of these agents on LPS synthesis during the stringent response, fully expecting that they would, with the exception of kanamycin, relax the control of LPS synthesis. The situation is more complex than that (Fig. 1 and 2).

The effects of the various agents on LPS synthesis in amino acid-deprived cells of strain VC210 *relA*$^+$ are shown in Fig. 1. As described previously (6), amino acid deprivation inhibited LPS synthesis by about 50%, and chloramphenicol treatment restored LPS synthesis to a rate equivalent to that observed in growing cells. Chloramphenicol was therefore used in all experiments as a control to illustrate the effects of a model stringent control antagonist. Consistent with our expectations, spectinomycin was as effective as chloramphenicol in relaxing the control of LPS synthesis, whereas kanamycin was ineffective. However, amikacin, gentamicin, and tobramycin also failed to restore LPS synthesis in amino acid-deprived cells. These latter results were unexpected, in view of the previous data (2, 5) indicating that these aminoglycosides are stringent control antagonists, but results of the experiments described below provide some insight into the basis for these results.

The effects of these same antibiotics on LPS synthesis in amino acid-deprived cells of the isogenic *relA* mutant strain

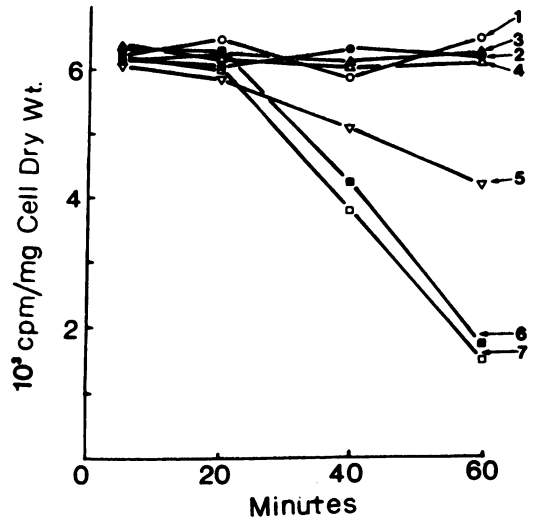


FIG. 2. Effects of antibiotics on LPS synthesis in strain VC211 *relA*. LPS synthesis was determined as described in the legend to Fig. 1 on an untreated culture (curve 1); an isoleucine-deprived culture (curve 2); and a series of isoleucine-deprived cultures treated with spectinomycin (curve 3), kanamycin (curve 4), amikacin (curve 5), tobramycin (curve 6), and gentamicin (curve 7).

VC211 are shown in Fig. 2. In this case, the control of LPS synthesis was completely relaxed during amino acid deprivation, as reported previously (6). With LPS synthesis being fully relaxed, we could readily determine whether any of the antibiotics had inhibitory effects on LPS synthesis. Thus, in agreement with previous results (6), chloramphenicol had no inhibitory effect on this relaxed LPS synthesis (data not shown). Kanamycin and spectinomycin also had no apparent inhibitory effect on LPS synthesis under these conditions. On the other hand, tobramycin and gentamicin exhibited strong inhibitory effects after 20 min of treatment, and amikacin showed a weaker, but significant, inhibitory activity. The inhibition of LPS synthesis by these agents is sufficient to explain why they did not exhibit any apparent relaxing effect on the control of LPS synthesis in strain VC210 *relA*$^+$ (Fig. 1); i.e., any relaxing effect that these agents may have had on LPS synthesis would have been negated by their inhibitory activities. For the sake of clarification, we again emphasize that these agents are stringent control antagonists when measured in ways other than LPS synthesis, e.g., by determining their effects on the syntheses of ppGpp, RNA, or peptidoglycan (2, 6).

The effects of treatments with growth inhibitory levels of antibiotics alone (i.e., without concomitant amino acid deprivation) on LPS synthesis were determined. Identical results were obtained with strains VC210 *relA*$^+$ (Fig. 3A) and VC211 *relA* (Fig. 3B). Spectinomycin and kanamycin, like chloramphenicol (data not shown) (6), had no inhibitory effect on LPS synthesis. On the other hand, LPS synthesis was inhibited in a time-dependent fashion by amikacin, gentamicin, and tobramycin (not tested in the case of strain VC211). These results therefore indicate that these antibiotics, by themselves, generally have the same effects as they do in amino acid-deprived bacteria.

**Effects of aminoglycosides and spectinomycin on LPS release.** In an effort to determine whether there was a correlation between LPS synthesis and the release of LPS by nongrowing bacteria, the effects of amino acid deprivation and treatment with selected antibiotics on the accumulation

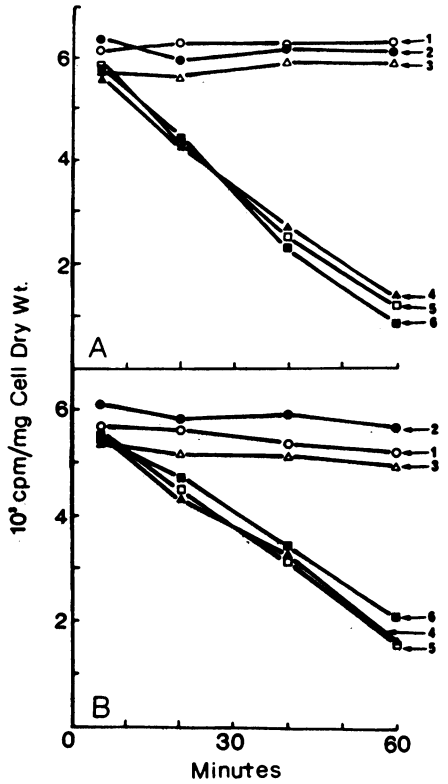


FIG. 3. Effects of treatments with antibiotics alone (i.e., without concomitant amino acid deprivation) on LPS synthesis in strains VC210 *relA*$^+$ (A) and VC211 *relA* (B). LPS synthesis was determined as described in the legend to Fig. 1 on untreated control cultures (curves 1) and a series of cultures treated with spectinomycin (curves 2), kanamycin (curves 3), gentamicin (curves 4), amikacin (curves 5), and tobramycin (curves 6).

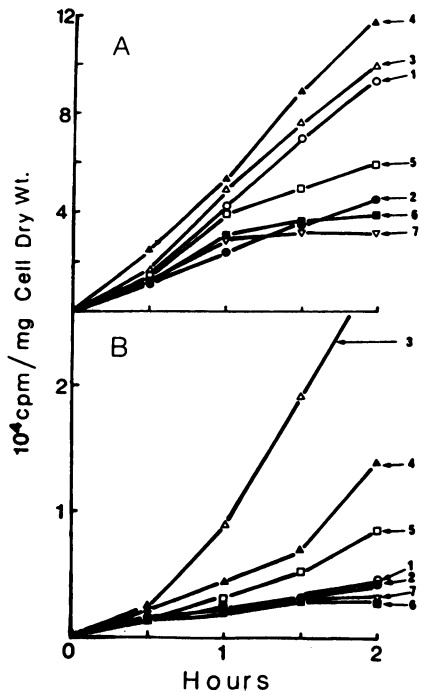


FIG. 4. Effects of antibiotics on the synthesis and release of LPS by strain VC210 *relA*$^+$. The following cultures were labeled with [$^3$H]galactose: untreated control (curves 1); lysine-deprived control (curves 2); and a series of lysine-deprived cultures treated with chloramphenicol (curves 3), spectinomycin (curves 4), amikacin (curves 5), tobramycin (curves 6), and gentamicin (curves 7). The cultures were sampled at the indicated times, and the amounts of radioactivity incorporated into the cell-bound (A) and culture supernatant (B) LPS fractions were determined.

of cell-bound LPS and cell-free LPS were determined. In these experiments, the various cultures were incubated continuously with [$^3$H]galactose, and the accumulation of radiolabeled LPS in the cell-bound and cell-free fractions was quantified at the times indicated in Fig. 4. In support of results already presented, the results in Fig. 4A indicate that amino acid deprivation of *relA*$^+$ cells inhibits the accumulation of cell-bound LPS by about 50% and that the addition of chloramphenicol abolishes this inhibitory effect. Spectinomycin was consistently slightly more effective than chloramphenicol in this regard. The rate of accumulation of cell-bound LPS by amino acid-deprived cells was stimulated by amikacin, but the degree of stimulation was small and was not sufficient to restore LPS synthesis to the levels seen in growing cells. This slight stimulation may represent a combination of relaxing and inhibitory effects exhibited by amikacin on LPS synthesis, although the relaxing effect could never be demonstrated conclusively by the method described in the legend to Fig. 1. Gentamicin and tobramycin initially had little or no effect on the rate of LPS accumulation by amino acid-deprived cells, but both antibiotics completely inhibited accumulation after 1 h of treatment. The results presented in Fig. 4B confirm those of previous reports (6, 8), showing that cell-free LPS is released at a slow rate during the normal growth of *E. coli*. Furthermore, amino acid deprivation had no additional effect on LPS release, but chloramphenicol treatment stimulated release by about fivefold. Spectinomycin and amikacin caused 2-fold and 1.5-fold increases in cell-free LPS accumulation after 2 h, respectively. On the other hand, tobramycin and gentamicin appeared to inhibit LPS release. The data in Fig. 4 therefore generally support the hypothesis that LPS release by nongrowing bacteria is associated with ongoing LPS synthesis. The only apparent anomaly was the finding that spectinomycin did not stimulate LPS release at least as effectively as chloramphenicol, and at the moment we are unable to explain this.

The experiment for which the results are presented in Fig. 4B was designed to examine the release of newly synthesized LPS and indicated that tobramycin and gentamicin apparently inhibit this process. Additional experiments were run to determine whether these antibiotics may affect the release of old LPS. In such experiments, the cell-bound LPS fraction was labeled before the cells were subjected to antibiotic treatment (6). Growing cells released the prelabeled LPS at a slow rate, and the addition of a growth inhibitory level of chloramphenicol to such a culture resulted in a stimulation in the rate of LPS release (Fig. 5). However, a similar treatment with gentamicin did not stimulate LPS release and, in fact, appeared to have an inhibitory effect.

## DISCUSSION

The antibiotics selected for this study were shown previously (2, 5), by various criteria, to be effective stringent control antagonists, with the exception of kanamycin, which has little or no activity in this regard. For example, they prevent the accumulation of pppGpp and ppGpp in amino

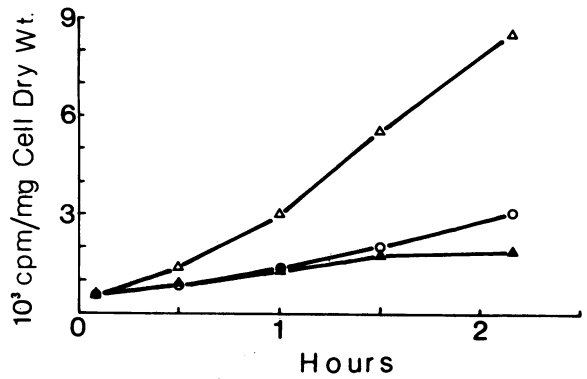


FIG. 5. Effect of antibiotics on the release of prelabeled LPS by strain VC210 *relA*$^+$. Bacteria were prelabeled with [$^3$H]galactose, and the amounts of radiolabeled LPS in the culture supernatants were determined at the indicated times. Symbols: ○, untreated control; △, chloramphenicol-treated; ▲, gentamicin-treated.

acid-deprived *relA*$^+$ strains of *E. coli* and concomitantly relax the control of stable RNA transcription and peptidoglycan synthesis (2, 5). They also relieve the RelA-dependent inhibition of the antibiotic-induced autolysis mechanism (5). Thus, as expected, spectinomycin relaxed the control of LPS synthesis in amino acid-deprived *relA*$^+$ cells, whereas kanamycin did not. On the other hand, most of the aminoglycosides tested (gentamicin, tobramycin, and, to a lesser extent, amikacin) unexpectedly failed to relax the control of LPS synthesis. It should be emphasized that the inability of kanamycin to relax the control of LPS synthesis was apparently due to its relatively poor ability to prevent pppGpp and ppGpp accumulation in amino acid-deprived *relA*$^+$ bacteria (2). In contrast, gentamicin, tobramycin, and amikacin were very effective in preventing pppGpp and ppGpp accumulation; but they nevertheless failed to relax LPS synthesis because they actually inhibited LPS synthesis. Amikacin actually appeared to have a combined relaxing and inhibitory effect on LPS synthesis (Fig. 4). The aminoglycosides are known to have pleiotropic effects on bacteria (3), and the observed inhibition of LPS synthesis, apparently reported here for the first time, reflects still another example of a secondary activity associated with some of these antibiotics. The mechanistic basis for the inhibition of LPS synthesis by these agents is not known and is currently under investigation. The reported (3) membrane damage caused by aminoglycosides could conceivably affect LPS synthesis. On the other hand, these aminoglycosides do not inhibit peptidoglycan synthesis (5), and it is not clear why the postulated membrane damage would not similarly affect the membrane-associated steps in peptidoglycan synthesis.

The inhibitory effects of certain aminoglycosides on LPS synthesis were also observed when *E. coli* was treated with these agents alone, i.e., without concomitant amino acid deprivation (Fig. 3). The syntheses of those macromolecular species which are normally under stringent control usually continue uninhibited under these conditions. For example, the syntheses of stable RNA and peptidoglycan are unaffected by treatments with chloramphenicol or any of the aminoglycosides used in this study (2, 5). The basis for this macromolecular accumulation during growth inhibition is not certain; it could be due simply to the fact that pppGpp and ppGpp do not accumulate during treatment with these antibiotics (2), or there may be a more complicated reason. In any case, the results in Fig. 3 indicate that the ability of agents such as amikacin, tobramycin, and gentamicin to inhibit LPS synthesis was not dependent on concomitant amino acid deprivation.

Our interest in this problem stems mainly from the observation that LPS synthesis may continue under certain conditions in growth-inhibited enterobacteria, e.g., during antibiotic treatment (6, 8). We were interested in testing the hypothesis that LPS release is stimulated under these same conditions. The data in Fig. 4 support the hypothesis, although the relationships between the rates of synthesis and the amounts of LPS released were not in perfect quantitative agreement. Thus, those agents which permitted synthesis to continue in the absence of cell growth (i.e., chloramphenicol, spectinomycin, and, to a lesser extent, amikacin) also stimulated LPS release. In contrast, agents such as tobramycin and gentamicin which inhibit LPS synthesis do not stimulate LPS release and, indeed, appear to inhibit release. It is possible that the stimulation of LPS release under these conditions may be due to the inability of nongrowing bacteria to accommodate the excess cell envelope material on their surfaces, as proposed by Russell (9). We consider the observation reported here to be important in view of the possible relevance of cell-free LPS in microbial pathogenesis (9).

## ACKNOWLEDGMENTS

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