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Direct Correlation between Overproduction of Guanosine 3′,5′-Bispyrophosphate (ppGpp) and Penicillin Tolerance in *Escherichia coli*

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The penicillin tolerance exhibited by amino acid-deprived *Escherichia coli* has been previously proposed to be a consequence of the stringent response. Evidence indicating that penicillin tolerance is directly attributable to guanosine 3 ,5 -bispyrophosphate (ppGpp) overproduction and not to some other effect of amino acid deprivation is now presented. Accumulation of ppGpp in the absence of amino acid deprivation was achieved by the controlled overexpression of the cloned *relA* gene, which encodes ppGpp synthetase I. The overproduction of ppGpp resulted in the inhibition of both peptidoglycan and phospholipid synthesis and in penicillin tolerance. The minimum concentration of ppGpp required to establish these phenomena was determined to be 870 pmol per mg (dry weight) of cells. This represented about 70% of the maximum level of ppGpp accumulated during the stringent response. Penicillin tolerance and the inhibition of peptidoglycan synthesis were both suppressed when ppGpp accumulation was prevented by treatment with chloramphenicol, an inhibitor of ppGpp synthetase I activation. Glycerol-3-phosphate acyltransferase, the product of *plsB*, was recently identified as the main site of ppGpp inhibition in phospholipid synthesis (R. J. Heath, S. Jackowski, and C. O. Rock, J. Biol. Chem. 269:26584–26590, 1994). The overexpression of the cloned *plsB* gene reversed the penicillin tolerance conferred by ppGpp accumulation. This result supports previous observations indicating that the membrane-associated events in peptidoglycan metabolism were dependent on ongoing phospholipid synthesis. Interestingly, treatment with β-lactam antibiotics by itself induced ppGpp accumulation, but the maximum levels attained were insufficient to confer penicillin tolerance.

In *Escherichia coli*, amino acid deprivation results in the coordinate inhibition of a variety of metabolic activities. This phenomenon, known as the stringent response, probably represents a means of enhancing the survival of bacteria during periods of starvation (see reference 4 for a review). Amino acid-deprived *E. coli* rapidly accumulate guanosine 3′,5′-bispyrophosphate (ppGpp). The stringent response is thought to be mediated by ppGpp. The synthesis of ppGpp during the stringent response is catalyzed by ppGpp synthetase I, a ribosome-associated enzyme encoded by the *relA* gene, which is activated by amino acid starvation. The stringent response can be prevented (i.e., relaxed) by inhibiting the synthesis of ppGpp. This may be accomplished either by introducing a mutation in the *relA* gene or by treating amino acid-deprived *relA*$^+$ bacteria with certain ribosome inhibitors, e.g., chloramphenicol, which apparently interfere with the activation of RelA.

The intracellular levels of ppGpp in *E. coli* are inversely related to growth rate (22). The growth rate-dependent synthesis of ppGpp is catalyzed by ppGpp synthetase II, which has been identified as the product of the *spoT* gene (9, 29).

Cell wall peptidoglycan metabolism is inhibited during the stringent response. One key site of inhibition in peptidoglycan synthesis was identified as the terminal step in peptidoglycan polymerization corresponding to the activities of the penicillin-binding proteins (20). In addition to the peptidoglycan polymerases, the expansion of peptidoglycan during cell growth is thought to require the activities of certain peptidoglycan hydrolases (see references 10 and 11 for reviews on this topic). The mechanisms by which β-lactam antibiotics kill bacteria are directly attributable to the activities of peptidoglycan hydrolases. The β-lactam antibiotics apparently uncouple the hydrolase activities from their normal roles in peptidoglycan synthesis, and the uncoupled hydrolases, in turn, cause the bacteriolysis which is characteristic of β-lactam treatment. It is well known that amino acid-deprived bacteria are penicillin tolerant; the procedures for the penicillin selection of auxotrophic mutants devised by Lederberg and Zinder (15) and by Davis (6) in 1948 are based on this fact. This form of penicillin tolerance has since been shown to apparently represent another consequence of the stringent response (7, 14). It has been attributed to the inhibition of both the peptidoglycan polymerase and peptidoglycan hydrolase activities during amino acid deprivation (18).

Membrane phospholipid synthesis in *E. coli* is also inhibited during the stringent response (23). We have recently shown that the β-lactam-induced bacteriolysis of amino acid-deprived bacteria resulting from the relaxation of the stringent response is dependent on ongoing phospholipid synthesis; i.e., the inhibition of relaxed phospholipid synthesis results in penicillin tolerance (21). We have proposed that the activities of both the peptidoglycan polymerases and hydrolases are obligatorily coupled to phospholipid synthesis.

The present work was based on the observation that the inhibition of peptidoglycan metabolism and the development of penicillin tolerance during the stringent response are both consequences of the inhibition of phospholipid synthesis. Our objective was to demonstrate a direct correlation between these phenomena and the accumulation of ppGpp. To accomplish this, we employed a derivative of a recombinant plasmid recently constructed by Svitil et al. (25) which carries the *relA*

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gene fused to a *tac* promoter. With this plasmid, the overproduction of ppGpp could be induced in a controlled fashion with isopropyl-β-D-thiogalactopyranoside (IPTG); i.e., the degree of ppGpp overproduction was directly related to the concentration of IPTG used for the induction of *relA*. An important consideration was that this method permitted us to bypass the use of amino acid deprivation, which has, up to now, been the traditional way to induce ppGpp accumulation. We show here that the induction of ppGpp accumulation resulted in the inhibition of both phospholipid synthesis and peptidoglycan synthesis and in penicillin tolerance. Furthermore, the dependence of these phenomena on ppGpp concentration was demonstrated. While this study was in progress, Heath et al. (8) reported that ppGpp overproduction resulting from the induction of the recombinant plasmids constructed by Svitil et al. (25) caused the inhibition of both fatty acid and phospholipid synthesis and that these inhibitory effects could be relieved by the overexpression of the *plsB* gene (which encodes *sn*-glycerol-3-phosphate acyltransferase [PlsB]). We report here that the overexpression of PlsB eliminated the ppGpp-dependent penicillin tolerance. This supports the proposal that ppGpp mediates penicillin tolerance through the inhibition of phospholipid synthesis.

MATERIALS AND METHODS

Plasmids. Plasmids pALS10 and pALS14 carry the complete *relA* gene and an inactive truncated derivative, respectively, which are fused to *tac* promoters (25). They were obtained from A. Svitil and J. Zyskind. For our experiments on penicillin tolerance, it was necessary to inactivate the β-lactamase activity encoded on pALS10 and pALS14. To accomplish this, we took advantage of the unique *Pvu*I site within the β-lactamase gene. The Ω interposon, which encodes spectinomycin or streptomycin resistance, was cut from plasmid pHP45Ω (19) with *Sma*I and ligated into the *Pvu*I sites of pALS10 and pALS14, giving rise to plasmids pDR24 and pDR25, respectively. Plasmids pRJ10 and pRJ12, which carry the complete *plsB* gene and an inactive truncated derivative of *plsB*, respectively (8), were obtained from C. O. Rock.

***E. coli* K-12 strains.** Strain CF1693 (Δ*relA251*::*kan* Δ*spoT207*::*cat*) was obtained from M. Cashel (29). VC7 (*thi-1 lysA23 rpsL109*) was from our laboratory collection. Strains VC891 and VC892 were derivatives of VC7 carrying plasmids pDR24 and pDR25, respectively. Strain VC7000 was constructed by transducing the Δ*relA251*::*kan* allele from CF1693 into VC7. VC7001 (Δ*relA* Δ*spoT*) was constructed by transducing the Δ*spoT207*::*cat* allele from CF1693 into VC7000. Transductions were performed with bacteriophage P1*vir* by the method of Miller (17). Strains VC7004 and VC7005 were derivatives of strain VC891 carrying plasmids pRJ10 and pRJ12, respectively.

Growth conditions. Bacteria were grown in the modified M56LP medium of Bell (1) in experiments involving radiolabeling with $^{32}P_i$. M9 minimal medium (12) was used in all other experiments. The media contained 0.2% glucose and the required growth factors. The exceptions were strains VC7004 and VC7005, which were grown in M9 medium containing 0.2% glucose, 25 μg of thiamine per ml, and 0.1% Casamino Acids (Difco Laboratories, Detroit, Mich.). All cultures were incubated in a 37°C water bath shaker as described previously (12). Spectinomycin (25 μg per ml) and kanamycin (50 μg per ml) were used to maintain plasmid-bearing strains. Culture turbidity was measured at 420 nm with a Beckman DU-64 spectrophotometer. The accumulation of ppGpp resulted in biphasic growth; i.e., the growth-inhibitory effects of ppGpp occurred 40 to 60 min after induction of *relA*. The doubling times reported for such cultures (see Table 1) were estimated by extrapolation of the growth curves after the maximum levels of ppGpp were attained. Cell dry weights were determined turbidimetrically on the basis of a standard curve. Viable cell counts were determined by plating serial dilutions of culture samples in triplicate on tryptic soy agar (Difco Laboratories). The plates were counted after 16 h of incubation at 37°C. The stringent response was provoked by adding L-valine to the medium at 500 μg per ml to induce isoleucine deprivation. IPTG was added where indicated to induce expression of the *relA* or truncated *relA* genes on plasmids pDR24 and pDR25, respectively. Chloramphenicol was added at 100 μg per ml to prevent activation of RelA expressed from plasmid pDR24 (25).

Treatment with β-lactam antibiotics. The general procedures for treatment with β-lactam antibiotics have been previously described (14, 18, 21). Unless specified otherwise, ampicillin was used at 50 μg per ml and cephaloridine was used at 10 μg per ml. The MICs of ampicillin and cephaloridine were 5 and 6 μg per ml, respectively. Lysis constants were calculated from the lysis curves, assuming that lysis obeyed first-order kinetics (16).

Liquid scintillation counting. Radioactive samples were counted in Ready Flow III liquid scintillation cocktail (Beckman Instruments Inc., Mississauga, Ontario, Canada), with a Beckman LS 8100 liquid scintillation counter.

Assay of peptidoglycan synthesis. Peptidoglycan synthesis was assayed by measuring the incorporation of [2,6-^{3}H]*meso*-2,6-diaminopimelic acid into cold trichloroacetic acid-insoluble fractions as described previously (12). Briefly, cultures were grown for three doublings to a density of 4×10^8 cells per ml. At this point, [2,6-^{3}H]*meso*-2,6-diaminopimelic acid was added to a final concentration of 0.2 μg per ml (10 μCi/μg). At the designated times, 100-μl samples of the cultures were applied to Whatman 3MM filter paper disks. The disks were immersed in ice-cold 5% trichloroacetic acid, rinsed twice in cold 95% ethanol, dried, and subjected to liquid scintillation counting (12).

Assay of phospholipid synthesis. Bacteria were grown for two doublings in M56LP low-phosphate medium to a density of 2×10^8 cells per ml. $^{32}P_i$ (5 μCi/ml) was added, and the culture was incubated for an additional 45 min before incorporation of ^{32}P into the phospholipid fraction was measured. Phospholipids were extracted with chloroform-methanol (1:2) by the method of Bligh and Dyer (2). The amounts of radioactivity in 200-μl aliquots of the chloroform fractions were determined by liquid scintillation counting.

Quantification of ppGpp. Cultures grown in M56LP low-phosphate medium were labeled with $^{32}P_i$ (40 μCi/ml). Samples (200 μl) of the cultures were extracted with 20 μl of 11 M formic acid on ice for 30 min. The samples were centrifuged to remove cell debris, and 10- to 20-μl aliquots of the extracts were applied to polyethyleneimine cellulose F thin-layer chromatography plates (Merck). Chromatograms were developed in 1.5 M KH_2PO_4. The separated nucleotides were visualized by autoradiography. The ppGpp spots were cut out and quantified by liquid scintillation counting. For each ppGpp sample, the radioactivity in an equal amount of material from the area between the ppGpp and GTP spots was subtracted as a background value. The values are expressed as pmol of ppGpp per mg (dry weight) of cells. In our experiments, a culture with an A_{420} of 1.0 had 1.15 mg (dry weight) of cells per ml. In the experiment described in Fig. 4, the cultures were uniformly labeled for three generations. Each of the experiments was performed three times, and the values reported in Fig. 4 are the averages of these experiments.

Materials. All antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.), except norfloxacin, which was provided by Merck Sharp & Dohme Research Laboratories (West Point, Pa.). $^{32}P_i$ (carrier free, 10 mCi per ml) was obtained from Amersham (Oakville, Ontario, Canada). [2,6-^{3}H]*meso*-2,6-diaminopimelic acid (30 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, Mo.). Restriction endonucleases, T4 DNA ligase, and IPTG were from Boehringer Mannheim (Laval, Quebec, Canada).

RESULTS

Overproduction of ppGpp inhibits both phospholipid and peptidoglycan synthesis. The plasmid pDR24 in strain VC891 contained the *relA* gene fused to a *tac* promoter. Therefore, the addition of IPTG to a culture of VC891 resulted in the induction of *relA* and in the overproduction of ppGpp as previously described (25). Figure 1 shows the effects of ppGpp overproduction by VC891 on growth (A), phospholipid synthesis (B), and peptidoglycan synthesis (C). If ppGpp accumulation was induced by isoleucine deprivation, growth, phospholipid synthesis, and peptidoglycan synthesis were inhibited as expected (compare curves a and b in each panel). The induction of the *relA* gene on pDR24 had the same general inhibitory effects, but the degrees of inhibition showed a dependence on IPTG concentration. For example, Fig. 1 shows that 50 μM IPTG was close to the minimum concentration required to cause inhibition of growth, of phospholipid synthesis, and of peptidoglycan synthesis (curves d), whereas 25 μM IPTG exhibited almost no inhibitory activity (curves c). On the other hand, 100 μM IPTG caused more severe inhibitory effects, which were comparable to the effects of the stringent response (Table 1).

Overproduction of ppGpp causes ampicillin tolerance. Figure 2 summarizes the effects of ampicillin on strains VC891 and VC892. A growing culture of strain VC891 was susceptible to ampicillin-induced lysis (Fig. 2, compare curves a and b). The treatment of VC891 with 50 μM IPTG to induce the overproduction of ppGpp resulted in the inhibition of growth (Fig. 2, curve c) and in the development of tolerance to ampicillin-induced lysis (Fig. 2, curve d). When chloramphenicol was added to an IPTG-treated culture to inhibit the activation of RelA (25), the susceptibility of VC891 to ampicillin-induced lysis was restored (Fig. 2, curve e). In contrast to VC891, strain

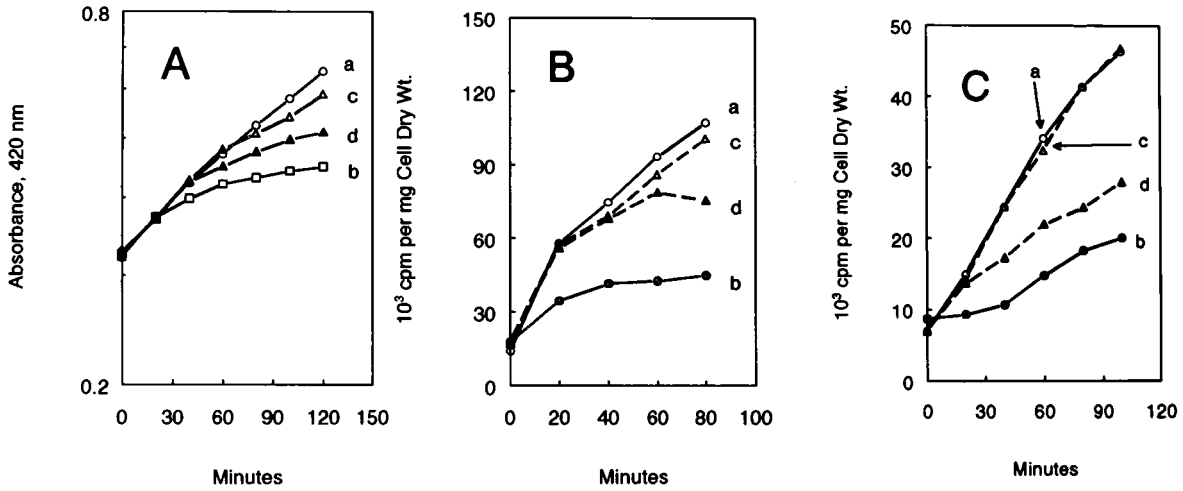

FIG. 1. Effects of ppGpp overproduction on growth (A), phospholipid synthesis (B), and peptidoglycan synthesis (C) in strain VC891. The cultures used for curves a were untreated controls. The cultures used for curves b were subjected to isoleucine deprivation. The cultures used for curves c and d were treated with 25 and 50 μM IPTG, respectively.

VC892, which carries pDR25 (with a truncated inactive derivative of the *relA* gene), was prone to ampicillin-induced lysis even in the presence of 100 μM IPTG (Fig. 2, curve f).

The minimum concentration of IPTG required to cause ampicillin tolerance was determined. Table 1 shows that growth of strain VC891 was progressively more inhibited as the concentration of IPTG was increased. For example, 50 μM IPTG caused a threefold increase in doubling time, and a concentration of 100 μM inhibited growth to a level characteristic of the stringent response. Table 1 also shows that 50 μM IPTG was sufficient to induce ampicillin tolerance, as determined by turbidimetric measurements. This is verified in Fig. 3, which shows the effects of different concentrations of IPTG on the viability of ampicillin-treated cultures of strain VC891. IPTG at 50 μM not only protected ampicillin-treated cells from lysis as measured turbidimetrically (Table 1) but also prevented loss of cell viability (Fig. 3). Lower concentrations of IPTG had no such protective effect. Therefore, the minimum amount of IPTG required to cause ampicillin tolerance was approximately 50 μM.

Quantification of ppGpp. The maximum levels of ppGpp accumulated by strains VC7 and VC891 were quantified, and these results are shown in Fig. 4. Under our experimental conditions, the steady-state concentration of ppGpp in a normal exponential-phase culture of strain VC7 was determined to be about 35 pmol/mg (dry weight) of cells (sample a, Fig. 4). The addition of IPTG to cultures of VC891 resulted in the maximum accumulation of ppGpp after about 40 min of treatment. Treatments with 50 and 25 μM IPTG resulted in peak accumulations of ppGpp amounting to 870 and 720 pmol/mg (dry weight) of cells, respectively (samples b and c, Fig. 4). These concentrations are significantly lower than the amount accumulated during the stringent response. For example, isoleucine deprivation resulted in an increase in the ppGpp level to 1,200 pmol/mg (dry weight) of cells (sample d, Fig. 4). These results indicate that the minimum concentration of ppGpp required for ampicillin tolerance was about 870 pmol/mg (dry weight) of cells.

Treatment with β-lactam antibiotics causes ppGpp accumulation. During the course of the ppGpp quantification experiments, we discovered that the treatment of growing cultures of VC7 with ampicillin resulted in the accumulation of ppGpp. Figure 5 shows that the time course of ppGpp accumulation was dependent on ampicillin concentration. Furthermore, the

TABLE 1. Effects of pGpp overproduction on growth and ampicillin-induced bacteriolysis of strain VC891

Treatment	Doubling time (h)	Lysis constant
None	2.1	$(6.7 \pm 0.4) \times 10^{-3}\text{min}^{-1}$
25 μM IPTG	3.2	$(4.0 \pm 0.2) \times 10^{-3}\text{min}^{-1}$
50 μM IPTG	6.0	No lysis
100 μM IPTG	17.0	No lysis
Isoleucine deprivation	17.0	No lysis

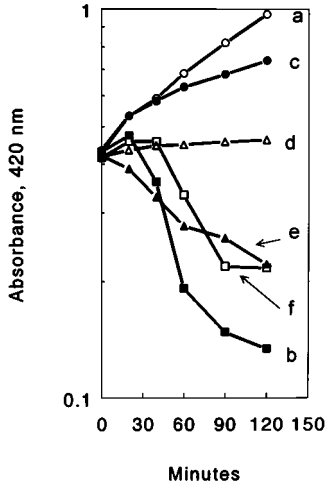

FIG. 2. Effect of ppGpp overproduction on ampicillin tolerance. Culture a of strain VC891 represents an untreated control. A portion of this culture was treated with ampicillin (b). Three other portions were treated with 50 μM IPTG at −15 min to induce the accumulation of ppGpp. One of these received no further treatment (c); beginning at 0 min, the other two were treated with ampicillin (d) or a combination of ampicillin and chloramphenicol (e). For comparison, a culture of strain VC892 was treated with 100 μM IPTG at −15 min and then with ampicillin at 0 min (f). The ampicillin and chloramphenicol concentrations used here were 50 and 100 μg per ml, respectively.

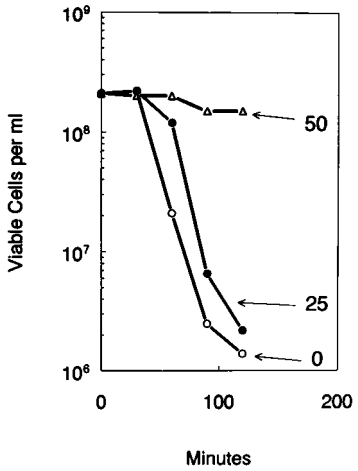

FIG. 3. Effect of ppGpp overproduction on viability of ampicillin-treated cultures of strain VC891. Three cultures were treated with 0, 25, and 50 μM IPTG (as indicated) at −15 min. They were then treated with 50 μg of ampicillin per ml beginning at 0 min, and viable cell counts were determined as indicated.

accumulation of ppGpp continued up to the point of lysis induction. In the experiment whose results are shown in Fig. 5, the ppGpp determinations were terminated when the cultures began to lyse, except in the case of the culture which was treated with the sublethal dose of 3 μg of ampicillin per ml. The largest accumulation observed was about 400 pmol of ppGpp per mg (dry weight) of cells, which was induced by 80 min of treatment with the lethal dose of 10 μg of ampicillin per ml. It is noteworthy that this level represents about half of the minimum amount of ppGpp required to confer ampicillin tolerance (compare samples b and e in Fig. 4). The accumulation of ppGpp was also observed during treatment with sublethal amounts of ampicillin. For example, Fig. 5 shows that growth in the presence of 3 μg of ampicillin per ml resulted in an accumulation of 160 pmol of ppGpp per mg (dry weight) of cells after 90 min; the maximum level of accumulation in this

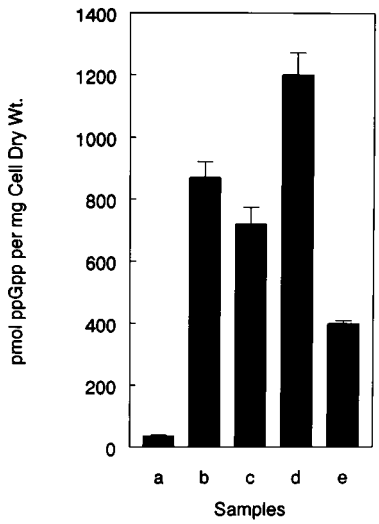

FIG. 4. Quantification of ppGpp. Sample a represents the steady-state level of ppGpp in an untreated culture of strain VC7. The other samples were from cultures of strain VC891 which were treated as follows: 50 μM IPTG (b); 25 μM IPTG (c); isoleucine-deprived (d); 10 μg of ampicillin per ml (Fig. 5). The data are the average values from three experiments.

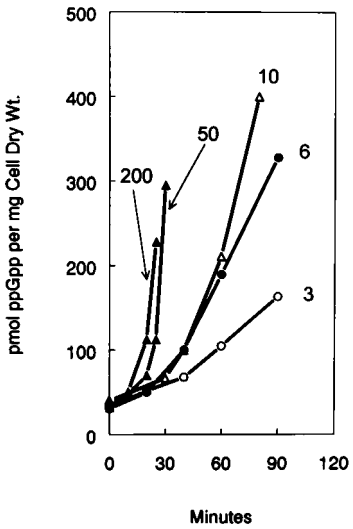

FIG. 5. Accumulation of ppGpp by strain VC7 in response to ampicillin treatment. At 0 min, cultures were treated with ampicillin at the concentrations (in micrograms per milliliter) represented by the numerals on the curves. Quantification of ppGpp was terminated at the first sign of lysis, except in the case of the culture which was treated with 3 μg per ml, which is sublethal.

case is not known, since measurements were not made beyond this point. Additional experiments indicated that cephaloridine also caused the accumulation of ppGpp, but rifampin and norfloxacin did not.

Strains VC7000 (Δ*relA*) and VC7001 (Δ*relA* Δ*spoT*) were used in order to identify the ppGpp synthetase which was responsible for the observed accumulation of ppGpp during ampicillin treatment. Neither strain exhibited ppGpp accumulation under these conditions (data not shown). This was to be expected in the case of VC7001 (Δ*relA* Δ*spoT*), because this strain is incapable of synthesizing ppGpp. The fact that VC7000 (Δ*relA*) also did not exhibit ppGpp accumulation indicates that ppGpp synthetase I was responsible for the ampicillin-induced ppGpp synthesis.

Reversal of ppGpp-induced ampicillin tolerance by overexpression of PlsB. Heath et al. (8) have recently reported that the overexpression of *plsB*, which encodes *sn*-glycerol-3-phosphate acyltransferase, reversed the ppGpp-induced inhibition of phospholipid synthesis in experiments similar to those described here. Furthermore, we have shown that lysis induced by treatment with β-lactam antibiotics or by other inhibitors of peptidoglycan synthesis is dependent on phospholipid synthesis (21). We therefore tested the effects of *plsB* overexpression on IPTG-induced ampicillin tolerance. Two derivatives of strain VC891 were constructed for this purpose. Strain VC7004 carried plasmid pRJ10 and consequently overproduced PlsB. The second strain, VC7005, carried plasmid pRJ12 and overproduced an inactive truncated derivative of PlsB. Both strains were grown in the presence of 50 μM IPTG to induce ppGpp synthesis and were then subjected to ampicillin treatment. Figure 6 shows that strain VC7005, like its parent VC891, was ampicillin tolerant (curve d). In contrast, strain VC7004 was susceptible to ampicillin-induced lysis (Fig. 6, curve b). Therefore, overexpression of PlsB reversed the ampicillin tolerance associated with the accumulation of ppGpp.

DISCUSSION

We have previously proposed that ppGpp is involved in the inhibition of peptidoglycan synthesis (12) and the development

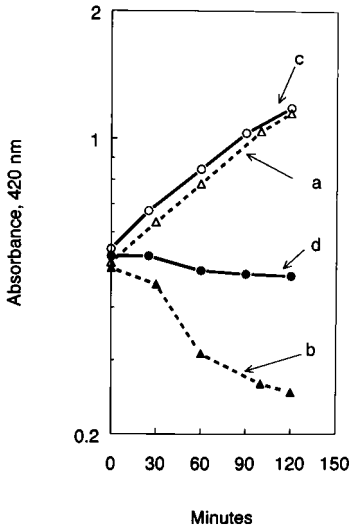

FIG. 6. Effect of overexpression of PlsB on ppGpp-induced ampicillin tolerance. Cultures of strain VC7004 were either untreated (a) or treated with 50 μM IPTG and 50 μg of ampicillin per ml (b). For comparison, cultures of VC7005 were either untreated (c) or treated with 50 μM IPTG and 50 μg of ampicillin per ml (d).

of penicillin tolerance (14) in *E. coli*. The bases for these proposals were arguably circumstantial; i.e., these proposals were based primarily on the observed association of these phenomena with the stringent response and their suppression when the stringent response was relaxed. In this study, we have demonstrated for the first time a direct relationship between the accumulation of ppGpp and the regulation of peptidoglycan metabolism. To accomplish this, we induced the accumulation of ppGpp directly through the controlled overexpression of the cloned *relA* gene, and we thereby bypassed the necessity to employ amino acid deprivation. One important conclusion from this study was that the inhibition of peptidoglycan synthesis and the development of penicillin tolerance were both directly associated with the accumulation of ppGpp. The effects of ppGpp on these processes were concentration dependent. Furthermore, the minimum effective concentrations of ppGpp in both cases were identical; the required concentration was about 30% lower than the maximum concentration accumulated during the stringent response.

Another major objective of this study was to further establish previous observations (21) which indicated that peptidoglycan metabolism requires ongoing phospholipid synthesis. While our work was in progress, Heath et al. (8) reported that phospholipid synthesis in *E. coli* was inhibited when ppGpp accumulation was induced by the overexpression of RelA. We have confirmed their result and, in addition, have shown that the concentration of ppGpp which was necessary to inhibit phospholipid synthesis was approximately the same as that required to inhibit peptidoglycan synthesis and to induce penicillin tolerance. The work of Heath et al. (8) indicated that ppGpp negatively controlled both fatty acid and phospholipid biosynthesis at the level of *sn*-glycerol-3-phosphate acyltransferase. The inhibition of de novo fatty acid and phospholipid synthesis caused by the accumulation of ppGpp was accompanied by the accumulation of long-chain acyl-acyl carrier proteins, i.e., the end products of fatty acid synthesis and the substrates for PlsB. Furthermore, the overexpression of *plsB* prevented the inhibition of both fatty acid and phospholipid synthesis and the accumulation of long chain acyl-acyl carrier proteins. We have demonstrated here that the overproduction of PlsB abolished ppGpp-associated penicillin tolerance. This provides still further support for our proposal (21) that peptidoglycan metabolism and penicillin-induced bacteriolysis are dependent on ongoing phospholipid synthesis.

Interestingly, treatment of *E. coli* with either ampicillin or cephaloridine (the only β-lactams tested) resulted in the accumulation of ppGpp. The kinetics of ppGpp accumulation were directly dependent on the concentrations of the β-lactam employed. It is known that intracellular levels of ppGpp are inversely related to growth rate (22). Therefore, the simplest explanation for these observations would be one based on the fact that treatment with β-lactam antibiotics resulted in the inhibition of growth, with the degree of inhibition being directly dependent on β-lactam concentration. This inhibitory effect on growth could, in turn, have been responsible for the induction of ppGpp accumulation. However, our continuing studies on this phenomenon suggest that the mechanism is apparently more complex than this. For example, although norfloxacin and rifampin were at least as effective as inhibitors of growth, they failed to induce ppGpp accumulation. On the other hand, it is unlikely that this effect is specifically associated with β-lactam agents. Gramicidin and polymyxin have been shown previously to cause ppGpp accumulation, and this was attributed to the abilities of these antibiotics to interfere with ppGpp degradation by an as-yet-uncharacterized mechanism (5). Another unexpected result was the RelA dependence of the β-lactam-induced ppGpp accumulation. As already noted, the growth rate-controlled synthesis of ppGpp has been attributed to SpoT or ppGpp synthetase II (9, 29), whereas the RelA activity has long been considered as being dedicated to sensing amino acid deprivation (4). The link between amino acid limitation and treatment with β-lactam agents is not obvious, and this matter clearly requires further investigation. Finally, the maximum levels of ppGpp accumulated during the course of treatment with β-lactam antibiotics, while significant, were insufficient to induce tolerance to β-lactam antibiotics. For example, the highest concentration of ppGpp achieved during ampicillin treatment was at least twofold lower than the minimum concentration required for the induction of ampicillin tolerance (Fig. 4).

A correlation between ppGpp accumulation and amdinocillin resistance has been previously demonstrated for *E. coli* (3, 13, 26–28), and it has been of interest to determine whether this phenomenon is related to the ppGpp-dependent penicillin tolerance described here. Amdinocillin is a β-lactam agent which specifically inhibits penicillin-binding protein 2 (PBP 2). The inhibition of PBP 2 results in the production of spherical cells and is lethal when bacteria are grown in rich medium; these observations indicate that PBP 2 is essential for the lateral elongation of cell wall peptidoglycan (24). However, PBP 2 is dispensable when the ppGpp levels in *E. coli* are elevated; such bacteria are in fact resistant to amdinocillin (3, 27). For example, bacteria continue to grow as spherical cells in the presence of amdinocillin when the stringent response is partially induced (3, 27). Joseleau-Petit et al. (13) have recently quantified the minimum concentration of ppGpp required to confer amdinocillin resistance and have found this value to be about 140 pmol of ppGpp per A_{600}. This value is over sevenfold lower than the minimum amount of ppGpp required to induce penicillin tolerance as determined in this study; i.e., the minimum level of ppGpp for penicillin tolerance was determined to be 870 pmol/mg (dry weight) of cells or about 1,000 pmol per A_{420}. It is also noteworthy that the proposed role of ppGpp in amdinocillin resistance is very different from our hypothesis concerning phospholipid synthesis and peptidoglycan metabo-

lism. The inactivation of PBP 2 results in the inhibition of cell division, which can be reversed by the introduction of a multicopy plasmid carrying *ftsZ* (28). Furthermore, amdinocillin-resistant spherical cells have diameters which are almost four times larger than those of their normal rod-shaped counterparts, and it is thought that such cells may require more FtsZ to carry out septation (13). It has, therefore, been suggested that the role of ppGpp in amdinocillin resistance may be to stimulate the production of FtsZ, possibly at the level of transcription of *ftsZ*. On the bases of the amounts of ppGpp required and the proposed modes of action, we conclude that the ppGpp-dependent amdinocillin resistance studied by D'Ari's group is not directly related to the phenomenon of penicillin tolerance described here.

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