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β-Lactam-Induced Bacteriolysis of Amino Acid-Deprived *Escherichia coli* Is Dependent on Phospholipid Synthesis†

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The penicillin tolerance of amino acid-deprived *relA*$^+$ *Escherichia coli* is attributed to the stringent response; i.e., relaxation of the stringent response suppresses penicillin tolerance. The β-lactam-induced lysis of amino acid-deprived bacteria resulting from relaxation of the stringent response was inhibited by cerulenin, or by glycerol deprivation in the case of a *gpsA* mutant (defective in the biosynthetic *sn*-glycerol 3-phosphate dehydrogenase). Therefore, β-lactam-induced lysis of amino acid-deprived cells was dependent on phospholipid synthesis. The lysis process during amino acid deprivation can be experimentally dissociated into two stages designated the priming stage (during which the interaction between the β-lactam and the penicillin-binding proteins occurs) and the β-lactam-independent lysis induction stage. Both stages were shown to require phospholipid synthesis. It has been known for some time that the inhibition of phospholipid synthesis is among the plethora of physiological changes resulting from the stringent response. These results indicate that the inhibition of peptidoglycan synthesis and the penicillin tolerance associated with the stringent response are both secondary consequences of the inhibition of phospholipid synthesis.

A variety of metabolic processes are coordinately inhibited in *Escherichia coli* during amino acid deprivation in a phenomenon which has been termed the stringent response (see reference 2 for a review). The stringent response represents a strategy designed to enhance survival during starvation stress. The process coincides with the rapid accumulation of guanosine 3′,5′-bispyrophosphate (ppGpp). The synthesis of ppGpp is catalyzed by ppGpp synthetase I, a ribosome-associated enzyme encoded by the *relA* gene, which is activated by amino acid deprivation. Therefore, the stringent response can be entirely eliminated (i.e., relaxed) by inhibiting the synthesis of ppGpp, e.g., by introducing a mutation in *relA* or by treatment of amino acid-deprived *relA*$^+$ bacteria with certain inhibitors of ribosome function, such as chloramphenicol, which apparently interfere with the activation of ppGpp synthetase I. The stringent response is thought to be mediated by ppGpp. However, little is known about how ppGpp could exhibit such a remarkable array of inhibitory activities as those observed during the stringent response.

The syntheses of phospholipids (22) and cell wall peptidoglycan (14) are inhibited during the stringent response. In the case of peptidoglycan synthesis, one key site of inhibition has been identified as the terminal step in peptidoglycan polymerization corresponding to the penicillin-sensitive incorporation of disaccharide-peptide units into wall peptidoglycan. The penicillin-sensitive peptidoglycan transpeptidation reaction is inhibited during the stringent response, as determined in in vitro assays with ether-permeabilized bacteria prepared from amino acid-deprived cultures; peptidoglycan transpeptidation activity is restored when the stringent response is inhibited by chloramphenicol treatment (13). The relaxed synthesis of peptidoglycan in vivo during amino acid deprivation, i.e., in either *relA* mutants or in *relA*$^+$ bacteria treated with chloramphenicol, is dependent on phospholipid synthesis (11), and this correlation has been extended to the in vitro assays of both penicillin-sensitive transpeptidase and D-alanine carboxypeptidase activities (12). Therefore, the terminal step in peptidoglycan synthesis is obligatorily coupled to phospholipid synthesis, and the inhibition of peptidoglycan synthesis during the stringent response is a direct consequence of the inhibition of phospholipid synthesis mediated by ppGpp.

Thirty years ago, Weidel and Pelzer (27) proposed that the growth of cell wall peptidoglycan required the coordinated activities of peptidoglycan synthetases and peptidoglycan hydrolases. The lethal action of β-lactam antibiotics in *E. coli* is based on the abilities of these antibiotics to deregulate or uncouple the activities of peptidoglycan hydrolases in some way which is not understood (see references 8, 9, and 23 for reviews). Tomasz et al. (24) demonstrated that penicillin inhibited bacterial growth but did not cause the characteristic lysis and cell death associated with penicillin treatment when the peptidoglycan hydrolase activities were compromised. This phenomenon, now known as penicillin tolerance, clearly established the fact that the killing action of β-lactam antibiotics involves a two-step process. In the first step, which we refer to here as the priming stage, the β-lactam antibiotics interact with their target penicillin-binding proteins (PBPs). The second step, which we call the lysis induction stage, involves the deregulation of peptidoglycan hydrolases and the consequent bacteriolytic response. Penicillin tolerance results if the second step of this process is somehow blocked.

The killing activities of β-lactam antibiotics are directly related to bacterial growth rate, and it is well known that slowly growing and nongrowing bacteria are penicillin tolerant. The

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† Dedicated to Uli Schwarz on the occasion of his 60th birthday in recognition of his invaluable contributions to the field.

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penicillin selection procedures devised by Lederberg and Zinder (16) and by Davis (4) to enrich cultures for auxotrophic mutants are based on this fact. It is now known that the penicillin tolerance exhibited by amino acid-deprived *E. coli* can be attributed to the stringent response (6, 15). Thus, amino acid deprivation results in penicillin tolerance in *relA*$^+$ bacteria but not in *relA* mutants or in chloramphenicol-treated *relA*$^+$ bacteria. We (18) have demonstrated that the ampicillin-induced lysis of amino acid-deprived cells can be experimentally dissociated into its two component stages: (i) an early ampicillin-dependent stage, i.e., the priming stage; and (ii) a later ampicillin-independent lysis induction stage. As discussed below (see Fig. 5), both stages are regulated independently by the stringent response. It is interesting and relevant that the activities of the PBPs remain high enough during the initial stages of the amino acid deprivation period to satisfy the requirements for priming, i.e., amino acid-deprived *relA*$^+$ cells can be primed if the β-lactam antibiotic is added early during amino acid deprivation. Therefore, the characteristic penicillin tolerance of such bacteria is primarily due to the inhibition of peptidoglycan hydrolase activities by the stringent response.

We show here that the lysis of amino acid-deprived *E. coli* induced by β-lactam antibiotics upon relaxation of the stringent response was dependent on phospholipid synthesis. Furthermore, both the priming and lysis induction stages exhibited this dependence.

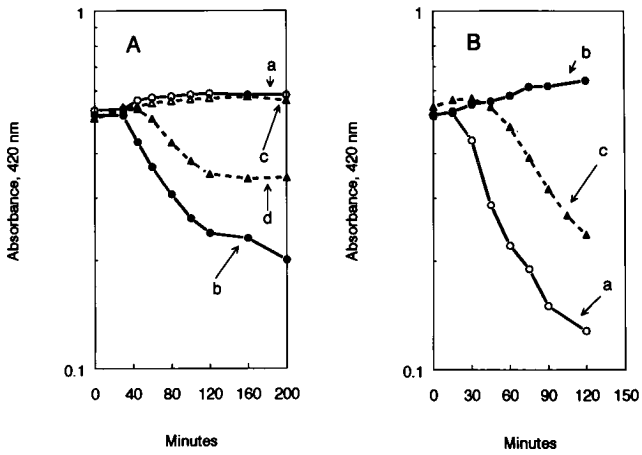

FIG. 1. Inhibition of ampicillin-induced lysis of amino acid-deprived bacteria by cerulenin. (A) At 0 min, a series of cultures of strain VC7 (*relA*$^+$) were subjected to isoleucine deprivation alone (a) or combined with chloramphenicol (100 μg/ml) to relax the stringent response (b). In addition to chloramphenicol, cultures c and d received cerulenin (50 μg/ml) and cerulenin (50 μg/ml) plus a mixture of palmitate and oleate (100 μg of each per ml), respectively. At 5 min, all cultures were treated with 50 μg of ampicillin per ml. (B) At 0 min, a series of cultures of strain VC8 (*relA*) were subjected to isoleucine deprivation. Culture a received no additional treatment; cultures b and c received cerulenin (50 μg/ml) and cerulenin (50 μg/ml) combined with a mixture of palmitate and oleate (100 μg of each per ml), respectively. At 5 min, all cultures were treated with 50 μg of ampicillin per ml.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains were derivatives of *E. coli* K-12. The isogenic pair, strains VC7 (*thi-1 lysA23 rpsL109 relA*$^+$) and VC8 (*thi-1 lysA23 rpsL109 relA2*), were from our laboratory collection. Strain VC58 was a *gpsA* derivative of strain VC8 (11). Bacteria were grown in M9 minimal medium containing 0.2% glucose and required growth factors in a 37°C water bath shaker as previously described (14) with the exception of experiments which involved the reversal of cerulenin inhibition (see Fig. 1 and 3B). In these experiments, the carbon source was 0.4% glycerol (5). To reverse cerulenin inhibition, the growth medium was supplemented with a mixture of potassium oleate and potassium palmitate (100 μg of each per ml, final concentration) dissolved in Brij 58 (20 μg/ml) as described by Goldberg et al. (5). Bacteria were grown in the fatty acid-supplemented medium for at least three generations before amino acid deprivation and cerulenin treatment were initiated. In these experiments, Brij 58 (20 μg/ml) was added to all media which were not supplemented with fatty acids. Isoleucine deprivation was achieved by adding valine to the medium at a final concentration of 500 μg/ml. Chloramphenicol at 100 μg/ml was used to relax the stringent response in amino acid-deprived strain VC7. Glycerol deprivation of the *gpsA* mutant, strain VC58, was performed as described previously (11). Culture turbidity was determined with a Beckman DU-64 spectrophotometer at 420 nm.

Antibiotic treatment. The dissociation of ampicillin-induced lysis into two stages was achieved by the method of Pisabarro et al. (18), with one modification. We have found that the priming stage could be more efficiently accomplished by treating the amino acid-deprived cells with 200 μg of ampicillin per ml (rather than 50 μg/ml as originally described) for 20 min. Other basic experimental procedures involving antibiotic treatment have been previously described (15). The agents were used at concentrations equal to approximately 10 times their MICs (imipenem, 20 μg/ml; cephaloridine, 60 μg/ml; cefsulodin, 100 μg/ml; D-cycloserine, 100 μg/ml; phosphonomycin, 150 μg/ml; and ampicillin, 50 μg/ml except in priming, for which 200 μg/ml was used as noted above). Cerulenin was used at 50 μg/ml.

Antibiotics. Moenomycin and imipenem were gifts from Hoechst Aktiengesellschaft (Frankfurt am Main, Germany) and Merck Sharpe & Dohme (Rahway, N.J.), respectively. All other β-lactam antibiotics, phosphonomycin, D-cycloserine, chloramphenicol, and cerulenin were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Requirement for phospholipid synthesis in lysis induced by β-lactam antibiotics. Isoleucine-deprived cultures of strains VC7 (*relA*$^+$) and VC8 (*relA*) were treated with ampicillin. As shown in Fig. 1A, ampicillin treatment of strain VC7 (*relA*$^+$) did not result in lysis (curve a) unless an antagonist of the stringent response such as chloramphenicol was present (curve b). In contrast, ampicillin by itself was sufficient to cause lysis of strain VC8 (*relA*) (Fig. 1B, curve a). These results confirm that the ampicillin-induced lysis of amino acid-deprived *E. coli* is regulated by the stringent response. As shown in Fig. 1A, the ampicillin-induced lysis which occurred upon relaxation of the stringent response in strain VC7 (*relA*$^+$) was inhibited by cerulenin (curve c). Furthermore, cerulenin also inhibited the ampicillin-induced lysis of isoleucine-deprived strain VC8 (*relA*), as indicated by curve b in Fig. 1B. In both strains, the inhibitory effect of cerulenin on lysis was eliminated by the addition of oleate and palmitate (curves d and c in Fig. 1A and B, respectively).

Cerulenin blocks fatty acid synthesis by inhibiting 3-ketoacyl-acyl carrier protein synthetases I and II (3, 26). Therefore, the inhibitory effects of cerulenin on lysis shown in Fig. 1 may indicate that the ampicillin-induced lysis of amino acid-deprived *E. coli* was dependent on fatty acid synthesis. An alternative explanation is that lysis required phospholipid synthesis which, in these cases, was inhibited as a consequence of fatty acid deficiency. In an effort to distinguish between these possibilities, the effect of ampicillin treatment was tested under conditions in which a direct block in phospholipid synthesis was imposed. For this purpose, we used strain VC58, a derivative of VC8 (*relA*) with a mutation in *gpsA* (which encodes the biosynthetic *sn*-glycerol 3-phosphate dehydrogenase [1]). Strain VC58 therefore requires glycerol or L-glycerol 3-phosphate for growth and phospholipid synthesis. Figure 2 shows the effect of ampicillin on cultures of VC58 which were subjected to isoleucine starvation in the presence and in the absence of glycerol. Ampicillin-induced lysis occurred when the glycerol requirement was satisfied. However, isoleucine-starved cells which were also deprived of glycerol were lysis tolerant. These results indicate that the ampicillin-induced lysis of amino acid-deprived *E. coli* was dependent on ongoing phospholipid synthesis.

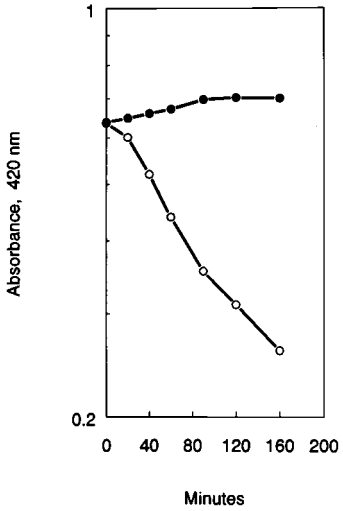

FIG. 2. Inhibitory effect of glycerol deprivation on ampicillin-induced lysis of strain VC58 (*relA gpsA*). Isoleucine-deprived cultures were treated with 50 μg of ampicillin per ml in the presence (○) and absence (●) of glycerol.

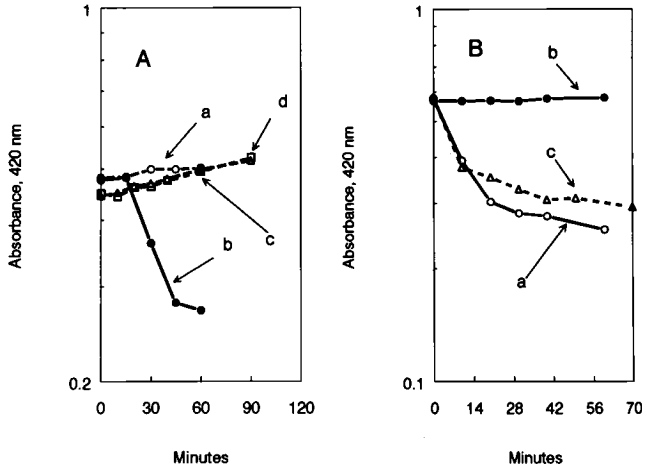

FIG. 3. Dependence of the priming stage on phospholipid synthesis. (A) Isoleucine-deprived cultures of strain VC 7 (*relA*$^+$) were primed for 20 min with 200 μg of ampicillin per ml in the presence (c and d) and absence (a and b) of cerulenin. At 0 min, the cells were washed free of ampicillin (and cerulenin in the cases of cultures c and d) and resuspended in isoleucine deprivation medium for determination of lysis induction. The subcultures primed in the absence of cerulenin received no further treatment (a) or 100 μg of chloramphenicol per ml to relax the stringent response (b). The subcultures primed in the presence of cerulenin received no further treatment (c) or 100 μg of chloramphenicol (d). (B) Isoleucine-deprived cultures of strain VC8 (*relA*) were primed with 200 μg of ampicillin per ml for 20 min with (b and c) or without (a) cerulenin. Culture c also received oleate and palmitate (100 μg of each per ml). At 0 min, priming was terminated, and the cells from each culture were resuspended in fresh isoleucine deprivation medium for determination of lysis induction.

The requirement for phospholipid synthesis in bacteriolysis of amino acid-starved bacteria was not restricted to the action of ampicillin. Identical results were obtained with the β-lactams benzylpenicillin, imipenem, cephaloridine, and cefsulodin. It is important to note that lysis induced by the non-β-lactam agents D-cycloserine, moenomycin, and phosphonomycin also exhibited a dependence on phospholipid synthesis. In these experiments, all agents were used at concentrations equal to 10 times their MICs. Therefore, it would appear that phospholipid synthesis is a general requirement for lysis induced by any antimicrobial agent which interferes with peptidoglycan synthesis.

Dependence of priming stage on phospholipid synthesis. As noted above, we have previously shown that the β-lactam-induced lysis of amino acid-deprived *E. coli* can be experimentally dissociated into its two component stages, termed priming and lysis induction (18). Figure 3 represents two experiments designed to determine whether the priming stage required phospholipid synthesis.

In the first of these experiments, a culture of strain VC7 (*relA*$^+$) was divided into two portions. Both portions were simultaneously subjected to isoleucine deprivation and ampicillin (200 μg/ml) treatment to initiate the priming stage. To determine the effect of inhibiting phospholipid synthesis on priming, one subculture was also treated with cerulenin. After 20 min, the cells from both subcultures were washed free of unbound ampicillin (and cerulenin in one case) to terminate the priming stage; they were resuspended in isoleucine starvation medium and tested for lysis induction. The cells which were treated with ampicillin in the absence of cerulenin were divided into two equal cultures represented by curves a and b in Fig. 3A. The culture receiving no further treatment did not exhibit lysis (curve a). In contrast, treatment of the second culture with chloramphenicol to relax the stringent response resulted in lysis induction (curve b), indicating that the ampicillin priming stage was successfully achieved. The cells which were treated with ampicillin in the presence of cerulenin were also divided into two cultures represented by curves c and d in Fig. 3A. Lysis was not observed in cells which did not receive further treatment (curve c). Furthermore, attempts to induce lysis of these cells by chloramphenicol treatment were unsuccessful (curve d). Therefore, cerulenin treatment inhibited the ampicillin priming stage.

In the second experiment to test whether phospholipid synthesis was required for the priming stage, we used strain VC8 (*relA*). In this case, three parallel isoleucine-deprived cultures were prepared and treated with ampicillin in either the presence (two of the cultures) or absence (one of the cultures) of cerulenin. In addition, a mixture of oleate and palmitate was added to one of the cerulenin-containing cultures. After the standard priming period of 20 min, the cells in all three cultures were washed and resuspended in fresh isoleucine starvation medium. As shown in Fig. 3B, the culture that was treated with ampicillin in the absence of cerulenin lysed without further treatment (curve a), indicating that the priming stage had been successfully achieved. In contrast, the cells which were treated with ampicillin in the presence of cerulenin did not lyse (curve b), again indicating that phospholipid synthesis was required during the priming stage. Furthermore, the presence of fatty acids during the priming stage to negate the inhibitory effects of cerulenin permitted priming to occur, as evidenced by lysis (curve c).

Dependence of lysis induction stage on phospholipid synthesis. To determine whether phospholipid synthesis was required for the lysis induction stage, we tested the ability of cerulenin to inhibit the lysis of cells which had been primed with ampicillin. Strain VC8 (*relA*) was deprived of isoleucine and treated with ampicillin at 0 min as shown in Fig. 4A. In agreement with previous results (18), priming was complete after 20 min of treatment, and the process of bacteriolysis was initiated at this time (curve a). The addition of cerulenin at 20 min completely prevented lysis (curve b). Furthermore, significant inhibition of lysis was also observed when cerulenin was added at 30 min, i.e., 10 min after the initiation of lysis (curve c). The same results were obtained when this experiment was

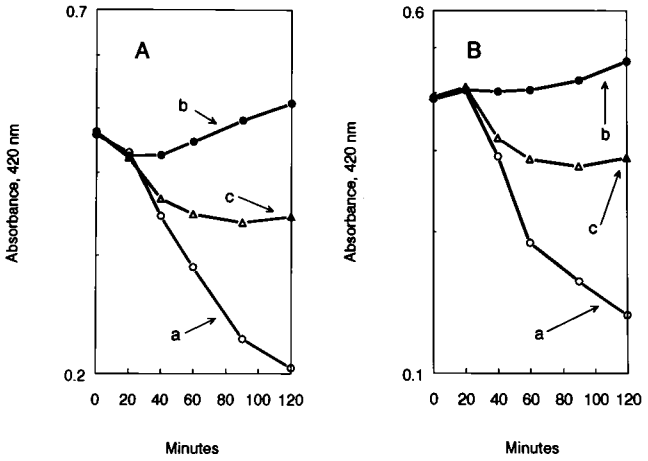

FIG. 4. Dependence of the lysis induction stage on phospholipid synthesis. (A) Cultures of strain VC8 (*relA*) were isoleucine deprived and treated with 50 μg of ampicillin per ml beginning at 0 min. Culture a received no further treatment, whereas cultures b and c were treated with cerulenin (50 μg/ml) at 20 and 30 min, respectively. (B) Isoleucine-deprived cultures of strain VC7 (*relA*$^+$) were treated with a combination of chloramphenicol (100 μg/ml to relax the stringent response) and phosphonomycin (150 μg/ml) beginning at 0 min. Culture a received no further treatment, whereas cultures b and c were treated with cerulenin (50 μg/ml) at 20 and 30 min, respectively.

performed with isoleucine-deprived cells of strain VC7 (*relA*$^+$) which were simultaneously treated with ampicillin and chloramphenicol to relax the stringent response. Therefore, phospholipid synthesis was required for lysis induction by ampicillin treatment.

We also determined whether phospholipid synthesis was required for lysis induced by non-β-lactam agents. At 0 min in Fig. 4B, strain VC7 (*relA*$^+$) was isoleucine deprived, relaxed with chloramphenicol, and treated with phosphonomycin (which, in the case peptidoglycan synthesis, inhibits the activity of UDP-*N*-acetylglucosamine enolpyruvyltransferase). Lysis was initiated shortly after 20 min (curve a). The addition of cerulenin at 20 min completely prevented lysis (curve b). Cerulenin had a significant protective effect even when added at 30 min, i.e., after lysis had been initiated (curve c). The same results were obtained with D-cycloserine. Furthermore, the same results were obtained when this experiment was performed with isoleucine-deprived cells of strain VC8 (*relA*) (data not shown). Therefore, lysis induced by non-β-lactam agents was also dependent on phospholipid synthesis.

DISCUSSION

The inhibition of autolysis by cerulenin treatment has been observed previously (17, 19), but the mechanism has not been determined. In their studies with *Bacillus subtilis*, Rogers and Thurman (19) used a subinhibitory concentration of cerulenin which only partially inhibited phospholipid synthesis and permitted exponential growth to continue. The protein and peptidoglycan contents of the cerulenin-treated cells were the same as those of untreated control cells, whereas the phospholipid content of the treated cells was reduced by 50%. Thus, it was suggested that the inhibitory effect of cerulenin on autolysin activity could be a reflection of a decrease in phospholipid-to-protein ratio of the membrane. We have shown here for the first time that the penicillin tolerance of amino acid-deprived *E. coli* is based on the dependence of both the peptidoglycan synthetase and peptidoglycan hydrolase activities on phospholipid synthesis.

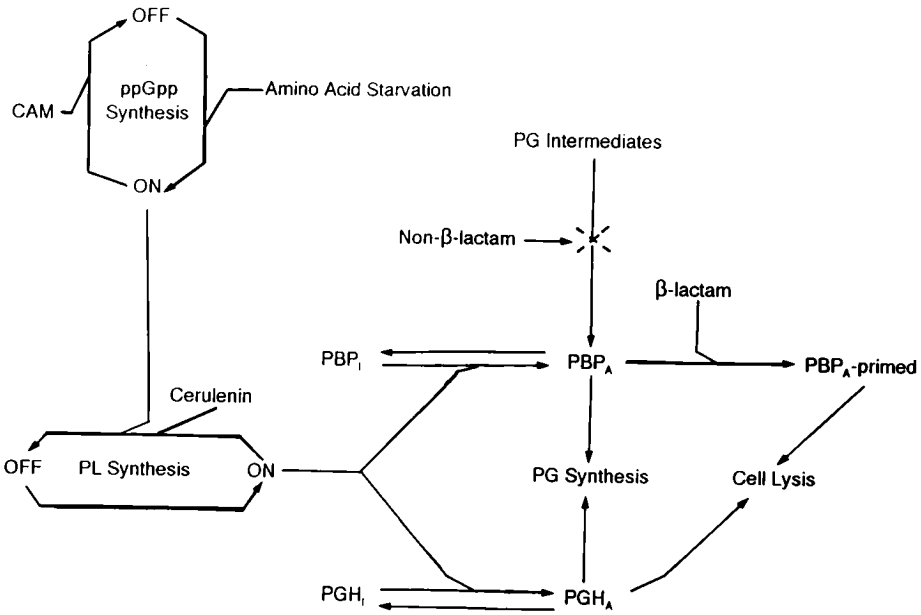

FIG. 5. Model for antibiotic-induced bacteriolysis of amino acid-deprived *E. coli*. The PBPs and peptidoglycan hydrolases (PGH) may exist in two alternate states, i.e., in inactive (PBP$_i$ and PGH$_i$) and active (PBP$_A$ and PGH$_A$) forms. The active forms of these enzymes are dependent on phospholipid (PL) synthesis. Normal peptidoglycan (PG) synthesis requires the coordinated activities of PBP$_A$ and PGH$_A$. Amino acid deprivation causes ppGpp accumulation and the subsequent inhibition of PL synthesis. Cerulenin treatment has the same effect. These events, in turn, inactivate both PBPs and PGHs. The PBP and PGH activities can be reactivated by relaxing the stringent response and restoring PL synthesis, e.g., by inhibiting ppGpp synthesis with chloramphenicol (CAM). The lethal activities of antibiotics which inhibit peptidoglycan synthesis are dependent on phospholipid synthesis and are therefore exhibited in amino acid-deprived cells only when the stringent response is relaxed. The interaction of β-lactam antibiotics with PBPs uncouples PG hydrolase activity to cause lysis, whereas non-β-lactam antibiotics achieve the same effect by interrupting the supply of peptidoglycan biosynthetic intermediates.

Figure 5 summarizes our current views on the regulation of peptidoglycan metabolism in *E. coli* by the stringent response. We have previously demonstrated a correlation between the inhibition of penicillin-sensitive peptidoglycan transpeptidation and the onset of the stringent response in amino acid-deprived *relA*$^+$ cells (13). Furthermore, the inhibition of transpeptidation is reversible, and peptidoglycan synthesis can be restored in the absence of de novo protein synthesis at any time during the initial 90 min of amino acid deprivation, e.g., by inhibiting ppGpp synthesis with chloramphenicol (15). We have demonstrated both in vivo (11) and in vitro (12) that peptidoglycan polymerization exhibits a strict requirement for ongoing phospholipid synthesis. Phospholipid synthesis is known to be inhibited during the stringent response (22). Therefore, Fig. 5 proposes that ppGpp inhibits peptidoglycan synthesis indirectly during the stringent response through its inhibitory action on phospholipid synthesis as previously reported (11).

It is generally thought that at least some peptidoglycan hydrolase activity is essential for normal peptidoglycan synthesis (e.g., see reference 7), but the exact nature of this requirement is far from understood. The model in Fig. 5 reflects the views of Weidel and Pelzer (27), who proposed that peptidoglycan synthesis involved the coordinated activities of the peptidoglycan synthetases and the peptidoglycan hydrolases. In *E. coli*, the bacteriolysis resulting from treatment with an inhibitor of peptidoglycan synthesis is peptidoglycan hydrolase mediated (reviewed in references 8 and 9). We have previously shown (18), and have confirmed here, that the role of the peptidoglycan hydrolases (i.e., the lysis induction stage) in β-lactam-induced killing can be experimentally distinguished from the role of the PBPs (i.e., the priming stage) in amino acid-deprived *E. coli*. In this case, the fact that the two stages can be dissociated indicates that the activities of the PBPs and the peptidoglycan hydrolases are regulated independently during the stringent response. Despite their independent regulation, the two activities appear to be tightly coupled. Although the kinetics of inactivation of the PBPs and peptidoglycan hydrolases during the stringent response cannot be measured accurately with the existing technology, we have previously estimated that both activities are inhibited at approximately the same time during the course of amino acid deprivation (18). Thus, it is possible to prime bacteria if β-lactam treatment and amino acid deprivation are initiated at the same time because there is still enough PBP activity (i.e., carried over from the growing state) during this period to achieve priming. We have estimated that a minimum of 20 min of penicillin treatment is essential to fulfill the priming requirement under these conditions. However, the fact that the second stage, lysis induction, does not occur under these circumstances indicates that this process is inhibited sometime during the first 20 min of amino acid deprivation. Furthermore, PBP activities appear to be substantially inactivated by 10 min after the start of amino acid deprivation because priming is impossible if started at this point or later. In summary, our best estimates indicate that both PBP and peptidoglycan hydrolase activities are inhibited by the stringent response within 10 to 20 min after the start of amino acid deprivation. The current study indicates that the activities of the PBPs and the peptidoglycan hydrolases were coupled through a common requirement for phospholipid synthesis and that the inhibition of phospholipid synthesis during the stringent response therefore resulted in the simultaneous inhibition of both activities. This is an important feature of the model shown in Fig. 5.

The observations summarized in Fig. 5 which are relevant to the phenomenon of penicillin tolerance are as follows. The characteristic penicillin tolerance of amino acid-deprived cells depends on when the penicillin treatment is initiated (18). If the β-lactam treatment is initiated after the PBPs have been inhibited by the stringent response (e.g., 10 min after the onset of amino acid deprivation [18]), the observed penicillin tolerance is due to a combination of two factors: (i) the failure to consummate priming, i.e., a deficiency in the component labeled PBP_A-primed in Fig. 5; and (ii) the inhibition of peptidoglycan hydrolase activities by the stringent response, i.e., the formation of the component labeled PGH_i. As already noted, the PBPs and the peptidoglycan hydrolases that are inactivated by the stringent response can be reactivated by inhibiting further ppGpp accumulation, e.g., with chloramphenicol, and penicillin tolerance can be reversed in this way. Quite a different situation exists if the penicillin treatment is initiated at the same time as amino acid deprivation because there is enough PBP activity during the early stages of amino acid deprivation to support the priming requirement for β-lactam-induced lysis (18). Consequently, penicillin tolerance in this case must be solely due to the RelA-dependent inactivation of the peptidoglycan hydrolase activities; indeed, as confirmed here, relaxation of the stringent response by chloramphenicol treatment causes lysis of such primed cells in the absence of exogenous β-lactam.

The inhibition of virtually any reaction in peptidoglycan biosynthesis appears to be sufficient to uncouple the activities of the peptidoglycan hydrolases. For example, non-β-lactam agents, such as D-cycloserine and phosphonomycin, cause lysis of growing bacteria by inhibiting the synthesis of key UDP-activated peptidoglycan precursors. Furthermore, amino acid-deprived *relA*$^+$ cells develop tolerance to lysis induced by these agents as they do to β-lactam agents, and inhibitors of the stringent response restore lysis competence. We have shown here that amino acid-deprived cells which were committed to lysis induction by non-β-lactam agents, through relaxation of the stringent response, could be rescued by treatment with cerulenin. We have used this observation to support our conclusion that phospholipid synthesis is necessary for the lysis induction process. Furthermore, these results indicate that tolerance to non-β-lactam agents could be attributed to the inhibition of peptidoglycan hydrolases by the stringent response. However, it is possible that non-β-lactam tolerance also occurs upon the inactivation of PBPs (i.e., the formation of PBP_i) by the stringent response. As depicted in Fig. 5, this event would block the biosynthetic pathway and would consequently prevent the detection of a deficiency of peptidoglycan precursors resulting from non-β-lactam treatment before the peptidoglycan hydrolase activities can be uncoupled. Further work is required to test the latter possibility.

The finding that the priming stage, which we believe represents the binding of β-lactam by PBPs, was dependent on phospholipid synthesis is consistent with our earlier observations indicating that phospholipid synthesis is required for peptidoglycan synthesis (11) and specifically for the transpeptidation activity (12). We have attempted to demonstrate the dependence of PBP activity on phospholipid synthesis more directly through in vivo labeling of PBPs with ^{125}I-labeled ampicillin by the method of Schwarz et al. (21). We have found that the inhibition of phospholipid synthesis by cerulenin treatment during amino acid deprivation did not affect the labeling efficiencies of the individual PBPs either in a *relA* mutant or in *relA*$^+$ cells relaxed with chloramphenicol (11). In each case, the normal full complement of PBPs was labeled, and a comparison of cells deficient in phospholipid synthesis and control cells (an equivalent amount of untreated growing bacteria) revealed no quantitative differences in their PBP labeling patterns. In

addition, we have confirmed Tuomanen's experiment (25), which indicated that the labeling efficiencies of the individual PBPs were not affected by the stringent response since the PBP labeling patterns of amino acid-deprived and normal *relA*$^+$ cells were identical. Presumably only the active forms of the PBPs could bind radiolabeled ampicillin in these experiments. Therefore, these results indicate that the majority of the PBPs remain active, in terms of β-lactam binding, under conditions whereby peptidoglycan synthesis is inhibited either by the stringent response or as a consequence of the inhibition of phospholipid synthesis by cerulenin; i.e., the majority of PBPs are not active in peptidoglycan synthesis and exhibit β-lactam-binding activities which are not dependent on phospholipid synthesis. Apparently, only a small fraction of the total PBP complement is involved in peptidoglycan synthesis, as noted by Tuomanen (25), and it is the activity of this small fraction which is differentially inhibited when phospholipid synthesis is inhibited. Höltje (7) has recently proposed a simple, highly feasible model for the growth of the *E. coli* peptidoglycan sacculus which couples the processes of peptidoglycan turnover and biosynthesis. A multienzyme complex composed of PBPs and peptidoglycan hydrolases is hypothesized to facilitate this process, and evidence for such a multienzyme complex has been recently presented by Romeis and Höltje (20). Our future investigations will be centered on testing a possible extension of Höltje's model. We are interested in the possibility of distinguishing the major and minor PBP fractions referred to here by showing that only the minor fraction is associated with Höltje's hypothetical multienzyme complex. According to this view, only the activities of the multienzyme complexes will be expected to exhibit a dependence on phospholipid synthesis.

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