Amino acid deprivation results in the coordinate inhibition of a variety of metabolic activities in *Escherichia coli*. This phenomenon, known as the stringent response, probably represents a means of enhancing bacterial survival during periods of starvation (for a review, see reference 1). Amino acid-deprived *E. coli* cells rapidly accumulate guanosine 3',5'-bispyrophosphate (ppGpp), and this nucleotide is thought to mediate the stringent response. 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 the codon-specific binding of uncharged tRNA to the ribosome acceptor site. The stringent response can be prevented (i.e., relaxed) by inhibiting ppGpp synthesis. This may be accomplished by introducing a mutation in the *relA* gene or by treating amino acid-deprived bacteria with certain ribosome inhibitors, e.g., chloramphenicol, which apparently interfere with the activation of RelA.

The phospholipids (10) and cell wall peptidoglycan (2) are inhibited during the stringent response. Furthermore, amino acid-deprived *relA* bacteria exhibit tolerance to β-lactam antibiotic-induced lysis, indicating that the activities of peptidoglycan hydrolases are inhibited during the stringent response. We have demonstrated unequivocally that the inhibition of peptidoglycan metabolism is a direct consequence of elevated ppGpp levels and is not due to some other indirect effect of amino acid deprivation (7). We have also shown that peptidoglycan metabolism and β-lactam antibiotic-induced lysis are dependent on phospholipid synthesis (8). The inhibitory effects of ppGpp on peptidoglycan metabolism are therefore based directly on the inhibition of phospholipid synthesis by ppGpp.

Kusser and Ishiguro (3) reported that the lysis of amino acid-deprived *E. coli* induced by β-lactam antibiotics was temperature sensitive and was markedly inhibited at temperatures approaching 42°C. Powell and Young (5) subsequently demonstrated a positive correlation between the overexpression of heat shock genes and tolerance to some β-lactam antibiotics. They induced the heat shock response in *E. coli* growing at 30°C in Luria-Bertani medium by overexpressing the cloned *rpoH* gene (encoding the heat shock-specific σ subunit of RNA polymerase). They showed that bacteria were tolerant to lysis caused by β-lactam antibiotics under these conditions.

**Role of the heat shock response in temperature-dependent tolerance to β-lactam antibiotics.** Our first objective was to determine whether the temperature sensitivity of the ampicillin-induced lysis process previously observed in amino acid-deprived cells of the *E. coli* K-12 strain VC7 (3) was related to the induction of the heat shock response. For this purpose, we constructed isogenic derivatives of VC7, strains VC895 and VC896, carrying the *dnaJ259* and *dnaK756* mutations, respectively, by bacteriophage P1-mediated transduction with the closely linked *thr::Tn10* insertion used as a selective marker (6). Strain VC899 was a VC7 derivative carrying only *thr::Tn10*. The *dnaJ259* and *dnaK756* alleles were temperature-sensitive mutations (9). Therefore, although both VC895 and VC896 grew normally at 30°C, neither strain exhibited a normal heat shock response when subjected to a temperature upshift to the nonpermissive temperature of 42°C because of the thermoinactivation of DnaJ and DnaK, respectively. Exponential-phase cultures of strains VC895, VC896, and VC899, growing in M9 minimal medium at 30°C, were isoleucine deprived as previously described (2, 3). Each culture was divided into two equal portions. One portion was incubated at 30°C, and the second portion was subjected to a temperature upshift to 42°C. Ten minutes later, at the time corresponding to 0 min in Fig. 1, chloramphenicol (100 µg/ml) was added to relax the stringent response, and the cultures were treated with ampicillin (50 µg/ml). The control strain, VC899, exhibited normal lysis at 30°C as expected. Furthermore, it failed to lyse at 42°C, confirming the report of Kusser and Ishiguro (3), which indicated that the ampicillin-induced lysis of amino acid-deprived bacteria was temperature sensitive. The two isogenic heat shock-defective mutant strains VC895 and VC896 also lysed at 30°C when treated with ampicillin. Furthermore, neither strain lysed at 42°C. The results of additional experiments which are relevant here are as follows (data not shown). (i) The same results were obtained when viable cell counts were used to monitor ampicillin-induced killing. (ii) Treatment with other β-lactam antibiotics (benzylpenicillin [250 µg/ml], cephaloridine [60 µg/ml], and imipenem [2 µg/ml]) and the non-β-lactam antibiotic phosphomycin (150 µg/ml) gave the same results as treatment with ampicillin. All antibiotics were obtained from Sigma.
Chemical Co. (St. Louis, Mo.), except imipenem, which was a gift from Merck Sharp & Dohme (Rahway, N.J.). Collectively, these results clearly demonstrate that the inhibition of the lysis process in amino acid-deprived bacteria at 42°C could not be attributed to the heat shock response, because neither VC895 nor VC896 exhibited a normal heat shock response under these conditions. It is notable that the experimental conditions in the work of Powell and Young (5) were quite different from those employed in this study. Moreover, in retrospect, it was unlikely that the heat shock response was involved here since protein synthesis was inhibited in our model system, precluding the possibility of the induction of the heat shock response.

Reversibility of temperature-sensitive lysis. We tested the reversibility of the thermosensitive ampicillin-induced lysis process at 42°C as shown in Fig. 2. Cultures of strain VC7, grown at 30 and 42°C, were amino acid deprived and treated with a combination of chloramphenicol (to relax the stringent response) and ampicillin. The effect of this treatment was then determined by optical density measurements (Fig. 2A) and viable cell counts (Fig. 2B). Both methods gave the same results. The culture grown at 42°C exhibited tolerance to ampicillin-induced lysis when incubated further at 42°C (curve a), but lysis was inhibited when a portion of this culture was shifted down to 30°C (curve b). In comparison, the culture originally grown at 30°C lysed when treated at 30°C (curve c), but lysis was inhibited when a portion of this culture was shifted up to 42°C (curve d). The inhibitory effect of the upshift again demonstrates the temperature sensitivity of the lysis process. Moreover, the result of the downshift from 42°C to 30°C (curve b) suggests that the temperature sensitivity was reversible and that the reversal was not dependent on de novo protein synthesis. The fact that the lysis of the downshifted culture (curve b) was somewhat delayed and was not as pronounced as the lysis of the similarly treated culture grown originally at 30°C (curve c) suggests that the recovery of the lysis mechanism upon downshift was not complete. Similar results were obtained when cephaloridine, benzylpenicillin, or phosphonomycin was used instead of ampicillin (data not shown).

Effect of temperature on priming and lysis induction stages of ampicillin-induced lysis. The first demonstration of penicillin tolerance by Tomasz et al. (11) proved that β-lactam antibiotics kill bacteria through a two-step process. We (4) have devised an experimental model which permits the dissociation of these two steps in amino acid-deprived E. coli. In the first step, referred to here as the priming stage, amino acid-deprived cells are briefly incubated in the presence of a β-lactam antibiotic. We propose that the antibiotic interacts with its target penicillin-binding proteins during this initial incubation period. The second step, referred to as the lysis induction stage, is dependent on the completion of the priming stage but is otherwise independent of the antibiotic. To demonstrate lysis induction, the excess unbound β-lactam antibiotic is removed, and the β-lactam-primed cells are resuspended in fresh amino acid starvation medium (lacking β-lactam antibiotic). These primed cells are stable and will not undergo lysis, because, as noted above, β-lactam-induced lysis is inhibited by ppGpp. On the other hand, the lysis induction stage can be activated in the β-lactam-primed bacteria by relaxing the stringent response (i.e., by adding chloramphenicol). Since bacteria can be primed with inhibitors of peptidoglycan synthesis other than β-lactam antibiotics (e.g., phosphonomycin), it is likely that the priming stage, in more general terms, represents a period during which the terminal step in peptidoglycan synthesis is inhibited long enough to dissociate the activities of the peptidoglycan hydrolases.

We tested the effect of temperature on each of the two stages of the ampicillin-induced lysis process. The dissociation of the ampicillin-induced lysis process in amino acid-deprived...
bacteria into two stages was achieved by the method of Pisabarro et al. (4) with one modification. Isoleucine-deprived cells were incubated (i.e., primed) for 20 min with a 200-μg/ml (rather than 50 μg/ml as originally described) concentration of ampicillin. Figure 3 demonstrates the effect of a temperature upshift on the priming and lysis induction stages, as determined by measuring culture optical densities (Fig. 3A) and viable cell counts (Fig. 3B). Both methods gave identical results. A culture of strain VC7 was grown at 30°C, isoleucine deprived, and divided into two parts. One part was primed with ampicillin at 30°C. Upon removal of the excess ampicillin, the bacteria in the primed culture did not lyse (curve b) unless the stringent response was relaxed by treatment with chloramphenicol, i.e., to activate the lysis induction stage (curve a). As documented previously (9), these results indicate that the incubation period with ampicillin was sufficient to satisfy the requirements for priming at 30°C. The second part of the culture was primed at 42°C. Curves c and d are controls used to show that the bacteria in this culture did not lyse when they were further incubated at either 42 or 30°C, respectively, after the excess ampicillin had been removed from the priming mixture. However, the culture lysed when it was shifted down to 30°C and simultaneously treated with chloramphenicol to relax the stringent response (curve e). The results represented by curve e clearly indicate that the priming stage was not inhibited at 42°C and, when combined with curves c and d, suggest that the lysis induction stage was the temperature-sensitive event in the lysis process.

Two additional aspects of the experiment shown in Fig. 3 confirm that the lysis induction stage was temperature sensitive but the priming stage was not. (i) If the culture was primed at 30°C, lysis induction occurred when the stringent response was relaxed with chloramphenicol at 30°C, as already noted (curve b), but lysis induction was inhibited at 42°C (curve f). (ii) Likewise, if the culture was primed at 42°C, lysis induction could be demonstrated at 30°C, as already noted (curve e), but not at 42°C (curve g).

**Basis for temperature sensitivity.** We demonstrated that the relaxed syntheses of phospholipids and peptidoglycan were both unaffected by a temperature upshift from 30°C to 42°C (data not shown). These results are consistent with the observation that the priming stage was not temperature sensitive. They also indicate that the temperature sensitivity of lysis induction could not be attributed to the inhibition of phospholipid synthesis.

Strain VC7 and its derivatives exhibited normal thermal regulation of their fatty acid compositions (unpublished data). At 50°C, palmitoleic acid and cis-vaccenic acid accounted for over 75% of the fatty acid composition, whereas in cells grown at 42°C, palmitic acid became the predominant fatty acid, accounting for over 40% of the total. It was therefore likely that the abrupt temperature upshift employed in our experiments would result in an alteration in membrane fluidity, and this could possibly affect the activity of critical peptidoglycan hydrolase activities. However, this did not appear to be the basis for the observed temperature sensitivity of the lysis induction stage, because bacteria pregrown at 42°C, and therefore adapted to this growth temperature, still exhibited temperature-sensitive lysis induction (Fig. 2).

We consequently favor the idea that this phenomenon is based on the direct thermoinactivation of a key peptidoglycan hydrolase(s) which is inherently thermosensitive. In this connection, it is noteworthy that the in vitro activity of at least one *E. coli* peptidoglycan hydrolase, the membrane-bound lytic transglycosylase known as Mlt38, has been shown to be temperature sensitive at 42°C (12). Of course, we are aware of the difficulties in extrapolating in vitro results to the in vivo situation. It is notable that the peptidoglycan hydrolase activities in *E. coli* responsible for β-lactam-induced lysis have not yet been conclusively identified. If the hypothesis presented here is correct, amino acid-deprived bacteria may serve as a useful model for the identification of these peptidoglycan hydrolases because of their inherent temperature sensitivities.

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