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Temperature Sensitivity of the Penicillin-Induced Autolysis Mechanism in Nongrowing Cultures of *Escherichia coli*

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The effect of incubation temperature on the ampicillin-induced autolysis of nongrowing *Escherichia coli* was determined. The autolysis mechanisms in amino acid-deprived *relA* mutant cells treated with chloramphenicol were temperature sensitive. This temperature-sensitive autolysis was demonstrated in three independent ways: turbidimetric determinations, viable cell counts, and solubilization of radiolabeled peptidoglycan.

The biosynthesis of cell wall peptidoglycan in *Escherichia coli* is regulated by the stringent control mechanism (4). Amino acid deprivation of *relA*$^+$ strains results in the rapid accumulation of guanosine 5′-diphosphate 3′-diphosphate (ppGpp), the putative inhibitor of stringent control, and in the concomitant inhibition of peptidoglycan synthesis. In contrast, ppGpp does not accumulate during amino acid deprivation of *relA* mutants, and peptidoglycan synthesis consequently is not inhibited (i.e., is relaxed) under these conditions. An observation relevant to this work is that chloramphenicol and several other inhibitors of eubacterial ribosome function act as antagonists of the stringent control mechanism by inhibiting the synthesis of ppGpp in amino-acid-deprived *relA*$^+$ bacteria (1, 2). Thus, these agents cause relaxation of peptidoglycan synthesis during amino acid deprivation of *relA*$^+$ cells (5, 6).

We have recently demonstrated that under certain conditions, it is possible to kill nongrowing *E. coli* with β-lactam antibiotics and other inhibitors of peptidoglycan synthesis (5, 6). The results of these studies indicate that the *relA* gene is also involved in regulating the activities of the peptidoglycan hydrolase(s) responsible for the autolysis induced by the inhibitors of peptidoglycan synthesis. Thus, amino-acid-deprived *relA*$^+$ bacteria are tolerant to the lysis-inducing action of such agents, but amino-acid-deprived *relA* mutants are not (5, 6). Furthermore, the tolerance of the amino acid-deprived *relA*$^+$ cells to the lysis-inducing agents can be overcome by simultaneous treatment with chloramphenicol (5) or some other stringent control antagonist (6). We have continued to characterize the lysis mechanism in nongrowing bacteria and report here that autolysis is temperature sensitive.

The isogenic *E. coli* K-12 strains VC7 (*thi-1 lysA23 relA*$^+$) and VC8 (*thi-1 lysA23 relA2*) were from our collection. They were grown in M9 minimal medium with 0.5% glycerol and required growth factors as described previously (4, 5). Cultures were incubated in water bath shakers at the indicated temperatures, and growth was determined in a Klett-Summerson colorimeter with a blue filter. The general protocol for these experiments has been described previously (5, 6). Isoleucine deprivation was achieved by adding L-valine to the medium at 500 μg/ml. Ampicillin and chloramphenicol, both obtained from Sigma Chemical Co., St. Louis, Mo., were used at 40 and 100 μg/ml, respectively. In the experiments described in the legends to Fig. 1, 2, and 3, cultures were amino acid deprived and shifted to the indi-

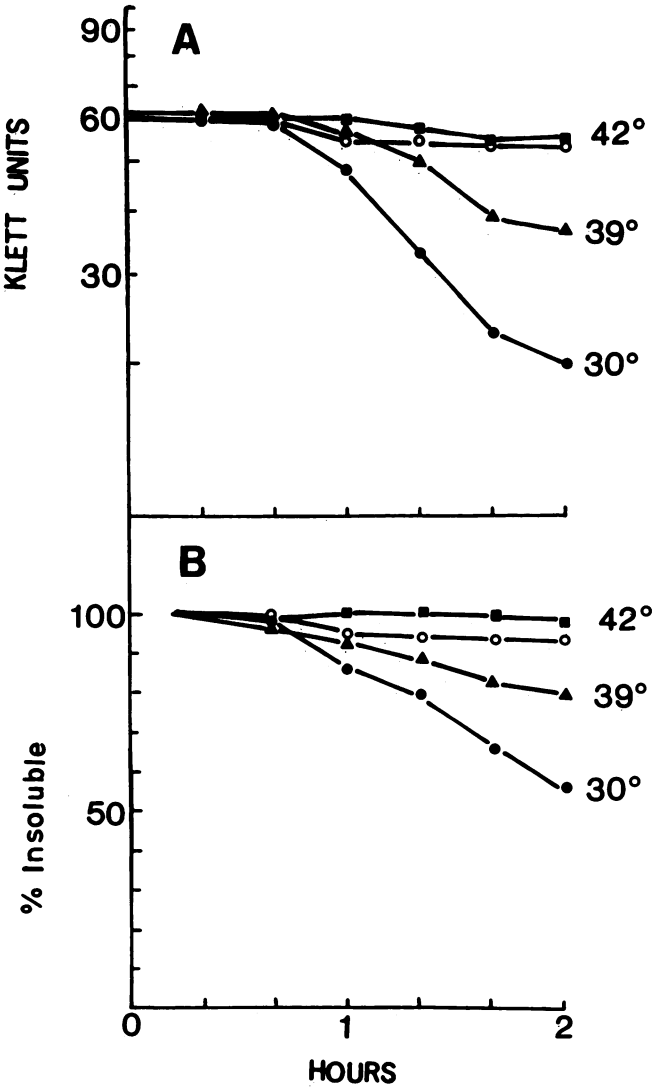

FIG. 1. Effect of incubation temperature on autolysis of isoleucine-deprived cells of strain VC7 *relA*$^+$ as determined by measuring optical densities (A) or solubilization of peptidoglycan prelabeled with [^{3}H]diaminopimelic acid (B). A culture grown at 30°C was divided into portions. One portion (○) was returned to 30°C with no further treatment. The other portions were incubated at the indicated temperatures with chloramphenicol and ampicillin.

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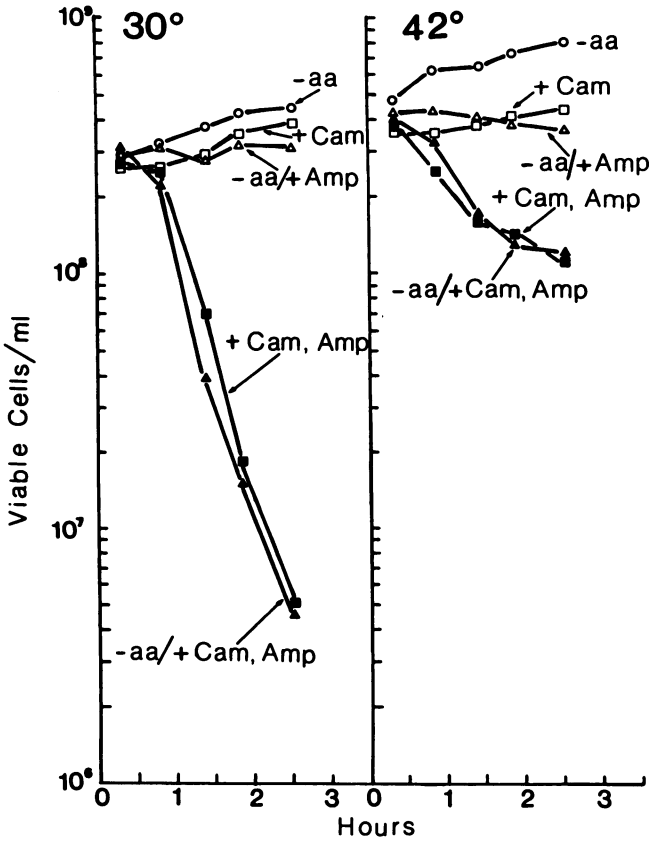

FIG. 2. Effect of temperature on viability of strain VC7 *relA*$^+$. A culture grown at 30°C was divided into duplicate portions; one set was incubated at 30°C, and the other was incubated at 42°C. One series in each set was isoleucine deprived with no further additions (O) or with either ampicillin (Amp) or ampicillin and chloramphenicol (Cam) added. Another series was treated with chloramphenicol either alone or with ampicillin; this series was not subjected to concomitant amino acid (aa) deprivation. Viable cell counts were determined by plating dilutions of the cultures on tryptic soy agar (Difco Laboratories, Detroit, Mich.).

cated temperatures at 0 min. Chloramphenicol and ampicillin were then added at 10 min.

Figure 1 shows the effects of incubation temperature on the autolysis of amino acid-deprived cultures of strain VC7 *relA*$^+$ which had been treated with a combination of ampicillin and chloramphenicol. Autolysis was determined by measuring either optical density or solubilization of peptidoglycan prelabeled as previously described (6) with [G-^{3}H]diaminopimelic acid (Amersham Canada Ltd., Oakville, Ontario, Canada). The following points are noteworthy. (i) As expected, cultures which were amino acid deprived only, i.e., were not simultaneously treated with chloramphenicol, were ampicilllin tolerant. (ii) In accordance with our recent reports (5, 6), the addition of chloramphenicol to amino acid-deprived cultures abolished the tolerance to ampicillin. (iii) However, the ampicillin-induced lysis of these cultures as measured by both methods exhibited a marked temperature dependence. Optimum lysis occurred at 30°C (the lowest temperature tested), and lysis was progressively inhibited at higher temperatures, with essentially complete inhibition occurring at 42°C. In this experiment, strain VC7 *relA*$^+$ was grown at 30°C and then subjected to the various treatments shown. It should be emphasized that identical results were obtained when the bacteria were pregrown at other temperatures, including 42°C. Furthermore, in agreement with previous results (6) and as confirmed below (see Fig. 2), cultures of VC7 *relA*$^+$ treated with chloramphenicol alone, i.e., without concomitant amino acid deprivation, were also sensitive to ampicillin-induced lysis. The lysis of these cultures as determined turbidimetrically showed the same temperature dependence as that of amino-acid-deprived cultures (data not shown).

The effect of temperature on the viability of VC7 *relA*$^+$ cultures treated with a combination of chloramphenicol and ampicillin was determined as described in the legend to Fig. 2. Amino-acid-deprived cultures treated with these agents at 30°C exhibited a 99% reduction in viability in 2.5 h. In contrast, the same treatment at 42°C resulted in only a 30% loss in viability. Cultures inhibited with chloramphenicol alone (i.e., without concomitant amino acid deprivation) were killed by ampicillin (Fig. 2). However, the degree of killing again was markedly reduced at 42°C. Together, these results suggest that the ampicillin-induced lytic activity responsible for loss in viability was temperature sensitive.

To determine whether the thermoinactivation of the lysis mechanism was reversible, portions of an amino acid-deprived culture of VC7 *relA*$^+$ treated with a combination of chloramphenicol and ampicillin at 42°C were shifted to 30°C at various times. The lysis potential decayed progressively as the incubation period at 42°C was increased, and reversal was not possible when the culture had been kept at 42°C for 60 min or longer (data not shown).

Figure 3 confirms previous observations (3, 5) indicating that amino acid-deprived *relA* mutants such as strain VC8 *relA* were sensitive to penicillin-induced lysis, i.e., without concomitant treatment with chloramphenicol. Furthermore, as shown for *relA*$^+$ bacteria, the lysis mechanism in nongrowing *relA* mutants was temperature sensitive, al-

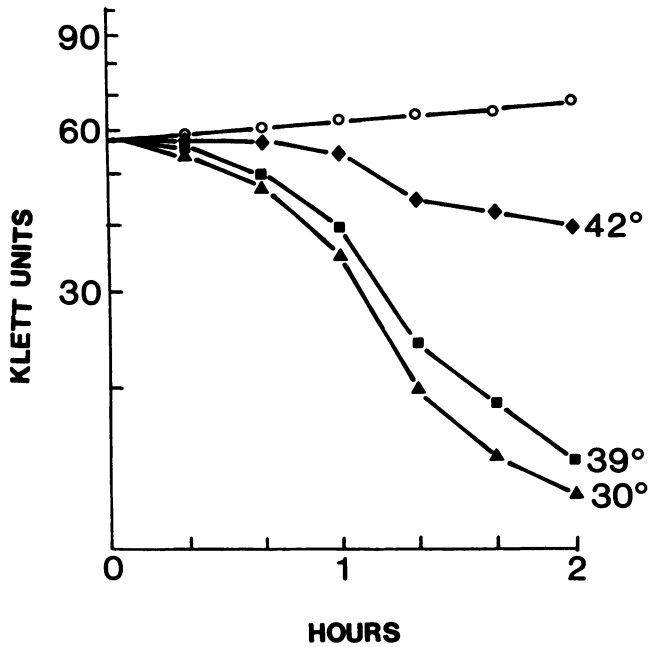

FIG. 3. Effect of temperature on autolysis of isoleucine-deprived cultures of strain VC8 *relA*. A culture grown at 30°C was divided into portions which were incubated at 30°C with no further treatment (O), or at the indicated temperatures with ampicillin.

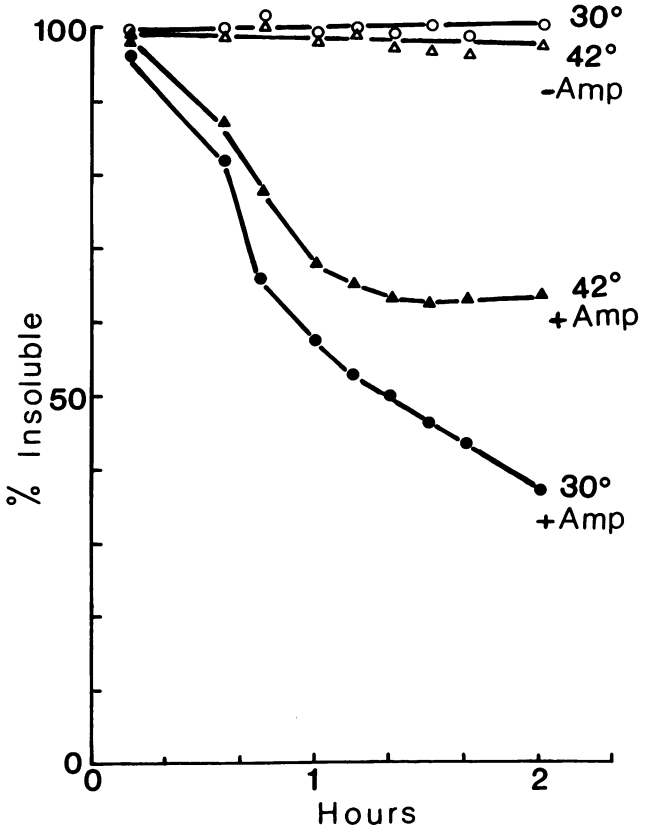

FIG. 4. Effect of temperature on ampicillin-induced solubilization of peptidoglycan prelabeled with [^{3}H]diaminopimelic acid in growing cells of strain VC7 *relA*$^+$. An exponential-phase culture of prelabeled bacteria at a density of 3×10^8 cells per ml was divided into four portions and incubated with and without ampicillin (Amp) at the indicated temperatures.

though the degree of temperature sensitivity appeared to be less pronounced. These results indicate that the temperature-sensitive lysis mechanism demonstrated for VC7 *relA*$^+$ was not chloramphenicol dependent.

It has been more difficult to conclusively demonstrate temperature sensitivity in the penicillin-induced lysis mechanism of actively growing bacteria. In the experiment described in the legend to Fig. 4, the effects of ampicillin treatment on the solubilization of peptidoglycan which was prelabeled with [^{3}H]diaminopimelic acid in strain VC7 *relA*$^+$ were determined at 30 and 42°C. There was clearly a reduction in the amount of peptidoglycan hydrolysis at 42°C, but there was also no doubt that a significant amount of hydrolysis had occurred under these conditions.

We cannot explain why the autolysis mechanism was more clearly temperature sensitive in nongrowing cells than in growing cells. Perhaps there was simply more active peptidoglycan hydrolase in growing cells (compare the rates of peptidoglycan hydrolysis in Fig. 1B and 4). In this connection, it is noteworthy that the degree to which the autolysis of nongrowing cells was temperature sensitive was dependent on the growth history of the culture before amino acid deprivation; i.e., autolysis of cells pregrown on a good carbon source such as glucose was apprently less temperature sensitive (data not shown) than autolysis of cells pregrown on poorer carbon sources such as glycerol. This may also indicate that the activities of the cellular autolytic system are directly related to growth rate and that there were simply more of its components to thermoinactivate in glucose-grown cells than in glycerol-grown cells. In any event, it is important that the temperature sensitivity of the peptidoglycan hydrolase activity in nongrowing cells could be measured in three independent ways, including by viable cell counts. This suggests that the activity was indeed relevant to the killing action of penicillin. Thus, nongrowing cells appear to provide a simpler system for studying the mechanism of penicillin-induced autolysis than do actively growing cells.

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