ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1996, p. 899–903 Vol. 40, No. 4
0066-4804/96/$04.00+0

Effects of Inhibitors of Protein Synthesis on Lysis of *Escherichia coli* Induced by β-Lactam Antibiotics

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Received 8 September 1995/Returned for modification 2 December 1995/Accepted 18 January 1996

The role of protein synthesis in ampicillin-induced lysis of *Escherichia coli* was investigated. The inhibition of protein synthesis through amino acid deprivation resulted in the rapid development of ampicillin tolerance as a consequence of the stringent response, as previously reported. In contrast, inhibition of protein synthesis by use of ribosome inhibitors such as chloramphenicol did not readily confer ampicillin tolerance and, in fact, promoted the development of both stages of the ampicillin-induced lysis process, i.e., (i) an ampicillin-dependent stage which apparently involves the interaction of penicillin-binding proteins with ampicillin and (ii) an ampicillin-independent stage which may represent the events leading to the deregulation of peptidoglycan hydrolase activity. We propose that lysis was facilitated when protein synthesis was inhibited because the production of new penicillin-binding proteins to replace those which were ampicillin inhibited was prevented under these conditions.

Nongrowing bacteria are known to be insensitive to the bactericidal activities of β-lactam antibiotics (5). This penicillin tolerance is the basis of the procedures for the penicillin selection of auxotrophic mutants devised by Lederberg and Zinder (9) and by Davis (2). Furthermore, Tuomanen et al. (17) used glucose-limited chemostat cultures of *Escherichia coli* to demonstrate that the bactericidal activities of β-lactam antibiotics were directly related to the bacterial growth rate; i.e., an increase in generation time was associated with an increase in the degree of penicillin tolerance.

In *relA*$^+$ strains of *E. coli*, amino acid deprivation results in the global inhibition of a variety of metabolic activities in a phenomenon known as the stringent response (see reference 1 for a review), and there are reasons to believe that this event is directly responsible for the penicillin tolerance first observed by Lederberg and Zinder (9) and by Davis (2). The stringent response probably represents a global strategy to restrict energy consumption in an effort to promote survival during starvation. The mediator of this complex physiological phenomenon is believed to be a novel nucleotide, guanosine 3′,5′-bispyrophosphate (ppGpp), which rapidly accumulates in amino acid-deprived bacteria. 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 deprivation. The stringent response can be antagonized (i.e., relaxed) by inhibiting the synthesis of ppGpp. Two common strategies for accomplishing this have been to introduce a mutation in *relA* or to treat amino acid-deprived *relA*$^+$ bacteria with certain ribosome inhibitors, e.g., chloramphenicol, which appear to inhibit the activation of ppGpp synthetase I.

Membrane phospholipid synthesis (15), cell wall peptidoglycan synthesis (6), and penicillin-induced lysis (3, 7) are among the many metabolic activities which are inhibited by the stringent response. We have shown that the activities of the penicillin-binding proteins (PBPs) involved in peptidoglycan polymerization and the peptidoglycan hydrolases are dependent on ongoing phospholipid synthesis (14). Therefore, the inhibition of phospholipid synthesis by ppGpp results in the inhibition of the membrane-associated steps in peptidoglycan metabolism, and this is proposed to account for the aforementioned penicillin tolerance of amino acid-deprived *relA*$^+$ *E. coli* (12, 14). We have recently demonstrated a direct correlation between ppGpp accumulation and penicillin tolerance (13). The accumulation of ppGpp in the absence of amino acid deprivation was achieved by the controlled overexpression of the cloned *relA* gene. This resulted in the inhibition of phospholipid and peptidoglycan synthesis and in penicillin tolerance. Glycerol-3-phosphate acyltransferase, encoded by *plsB*, has been identified as the main site of ppGpp inhibition in phospholipid synthesis (4). The overexpression of the cloned *plsB* gene reversed the penicillin tolerance conferred by ppGpp overproduction (13).

The first demonstration of penicillin tolerance by Tomasz et al. (16) proved that β-lactam antibiotics kill bacteria through a two-step process. We (11) have devised an experimental model which permits the dissociation of these two steps. In the first step, which we refer to here as the priming stage, the β-lactam antibiotic interacts with its target PBPs. 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 β-lactam antibiotic. Lysis induction involves the deregulation of the peptidoglycan hydrolases.

This study concerns the role of protein synthesis in ampicillin-induced lysis of *E. coli*. We verify that inhibition of protein synthesis by amino acid deprivation of *relA*$^+$ bacteria brought on the rapid development of ampicillin tolerance as a consequence of the stringent response. In contrast, the inhibition of protein synthesis by chloramphenicol and other ribosome inhibitors did not confer rapid ampicillin tolerance. In fact, ribosome inhibitors promoted both the priming and the lysis induction stages.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* K-12 strain VC7 (*thi-1 lysA23 rpsL109*) and its isogenic *relA* derivative, strain VC8 (*thi-1 lysA rpsL109 relA2*), were from our laboratory collection. The bacteria were grown in M9 minimal medium containing 0.2% glucose and the required growth factors at 37°C in a water bath shaker as described previously (6). Isoleucine deprivation was achieved by the addition of L-valine to the growth medium at 500 μg/ml. Ampicillin was used at either 50 or 200 μg/ml as specified in Results. The MIC of

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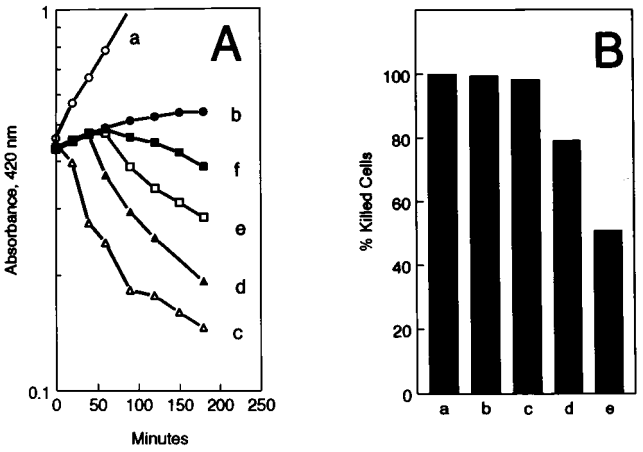

FIG. 1. (A) Effect of chloramphenicol treatment on ampicillin-induced lysis. A portion of a normal exponential-phase culture of strain VC7 (curve a) was treated with chloramphenicol at 100 μg/ml beginning at 0 min (curve b). Subcultures of the chloramphenicol-treated culture were treated with ampicillin at 50 μg/ml at 0 (curve c), 20 (curve d), 40 (curve e), and 60 (curve f) min. (B) Effect of chloramphenicol on the bactericidal activity of ampicillin. In the experiment described for panel A, viable cell counts in each of the subcultures were determined before the addition of ampicillin and after 2 h of treatment, and the percentages of cells killed are shown for the chloramphenicol-treated subcultures which were treated with ampicillin at 0 (bar b), 20 (bar c), 40 (bar d), and 60 (bar e) min (Note that the letters used here to designate the various subcultures are different from those used in panel A). For comparison, bar a represents a culture which was treated with ampicillin alone (i.e., in the absence of chlorampohenicol) for 2 h.

ampicillin for both bacterial strains was 5 μg/ml. The ribosome inhibitors chloramphenicol, gentamicin, and tetracycline were used at concentrations of 100, 50, and 20 μg/ml, respectively. Norfloxacin was used at 8 μg/ml. Viable cell counts were determined by plating serial dilutions of culture samples in triplicate on tryptic soy agar (Difco Laboratories). Counts were done after 16 h of incubation at 37°C.

Two-stage ampicillin-induced lysis procedure. The method for dissociating the priming and lysis induction stages, originally described by Pisabarro et al. (11), was modified for the purposes of this study. The main modification was the use of exponential-phase cultures for the priming stage rather than amino acid-deprived cultures. Cultures were grown for three to four doublings to a density of about 4×10^8 cells per ml. These cultures were primed with 200 μg of ampicillin per ml rather than 40 μg/ml as originally described (11). We found that priming was more efficient at this higher concentration of ampicillin. After 20 min of treatment, the unbound ampicillin was removed by centrifugation at 18,000 × *g* for 1 min; the cells were washed once in sterile saline before resuspension in fresh medium (7). As reported previously (7, 11), this method was judged to be adequate for the removal of unbound ampicillin because cell growth resumed after only a brief lag after a sample of the treated bacteria was resuspended in fresh medium without ampicillin.

Antibiotics. All antibiotics were purchased from Sigma Chemical Co. (St. Louis, Mo.), except norfloxacin, which was obtained from Merck Sharp & Dohme Research Laboratories (West Point, Pa.).

RESULTS

Fig. 1 illustrates the effect that chloramphenicol treatment had on ampicillin-induced bacteriolysis of strain VC7. Chloramphenicol (100 μg/ml) was added to a portion of an exponential-phase culture of VC7 beginning at 0 min. This resulted in an immediate inhibition of growth (compare curves a and b in Fig. 1A). A series of chloramphenicol-treated subcultures were treated with ampicillin (50 μg/ml) at the indicated times. As reported previously (8), treatment with chloramphenicol for periods of up to 20 min did not inhibit ampicillin-induced lysis (Fig. 1A, curves c and d). Moreover, significant lysis was observed even when ampicillin was added 40 or 60 min after the start of the chloramphenicol treatment (Fig. 1A, curves e and f). This is noteworthy in view of the generally accepted notion that nongrowing bacteria are tolerant to β-lactam antibiotics. Ampicillin treatment initiated 90 min after the addition of chloramphenicol did not result in lysis (data not shown). The effects of chloramphenicol treatment on the bactericidal activities of ampicillin in this experiment were determined by measuring the percentage of cells killed after 2 h of ampicillin treatment. The results are summarized in Fig. 1B. Pretreatment of *E. coli* with chloramphenicol for periods of up to 20 min had little effect on the efficacy of killing by ampicillin, and over 99% of the population lost viability under these conditions (compare samples b and c with sample a, which represents a culture which was not treated with chloramphenicol). The killing efficacies were reduced to 80% (sample d) and 50% (sample e) when the chloramphenicol pretreatment times were increased to 40 and 60 min, respectively. Treatment with chloramphenicol alone did not result in loss of viability (data not shown). Therefore, ampicillin remained significantly bactericidal even when bacterial growth had been inhibited by chloramphenicol treatment for up to 60 min. On the other hand, bacteria which were subjected to chloramphenicol treatment for 90 min were ampicillin tolerant; they did not lyse, as noted above, and less than 1% of the cells lost viability (data not shown).

The inhibition of protein synthesis and the concomitant stringent response induced by amino acid deprivation result in the rapid onset of ampicillin tolerance, as previously reported (3, 7). Therefore, the relative ineffectiveness of ribosome inhibitors in countering ampicillin-induced lysis as shown in Fig. 1 was considered unusual. The following experiments addressed this issue. They show that chloramphenicol (and other ribosome inhibitors) and amino acid deprivation affected β-lactam-induced bacteriolysis of *relA*$^+$ bacteria in different ways.

The system for dissociating the priming and lysis induction stages was originally developed by using amino acid-deprived bacteria (11). We have now modified this procedure by achieving the priming stage in normal growing bacteria. The remainder of our strategy was essentially the same as in the original procedure; i.e., after priming with ampicillin, the development of the lysis induction stage was inhibited by inducing the stringent response, and the lysis induction stage was subsequently activated by relaxing the stringent response with chloramphenicol. In our initial experiments, samples from an exponential-phase culture of strain VC7 (*relA*$^+$) were treated with 200 μg of ampicillin per ml (50 times the MIC) for various times to determine the minimum time of treatment required for the priming of growing bacteria. After the treatment, the unbound ampicillin was removed as described previously (7), and the cells were resuspended in fresh complete M9 minimal medium. The results of 10- and 15-min treatments with ampicillin are shown in Fig. 2A and B, respectively. Bacteria which were treated with ampicillin for 10 min recovered from the treatment and resumed growth about 10 min after resuspension in fresh M9 medium (Fig. 2A, curve a). In contrast, bacteria were committed to lysis after 15 min of ampicillin treatment, and they began to lyse within 10 min after resuspension in fresh medium (Fig. 2B, curve a). Isoleucine deprivation, achieved by the addition of L-valine at 500 μg/ml, prevented the lysis of bacteria which were treated with ampicillin for 15 min (Fig. 2B, curve b). This inhibition of lysis was attributed to the induction of the stringent response; for example, lysis was restored if the amino acid-deprived bacteria were simultaneously treated with chloramphenicol to inhibit the stringent response (Fig. 2B, curve c). It is important to note that the lysis in this case occurred in the absence of exogenous ampicillin. This result, along with the fact that the ampicillin-treated bacteria were committed to lysis (Fig. 2B, curve a), indicates that the 15-min

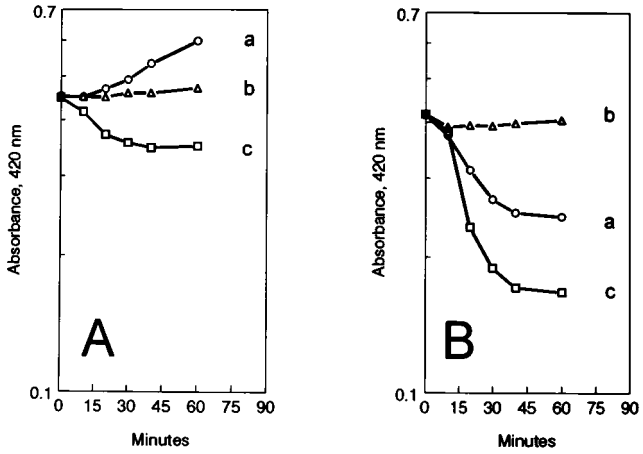

FIG. 2. Establishment of conditions for a modified two-stage ampicillin-induced lysis protocol. Exponential-phase cultures of strain VC7 were treated with ampicillin at 200 μg/ml for 10 min (A) or 15 min (B). The unbound ampicillin was then removed from each culture, and the bacteria were resuspended in fresh M9 medium. Each culture was divided into three portions. One of the subcultures received no further treatment (curves a). The other two subcultures received L-valine at 500 μg/ml (curves b) or L-valine and chloramphenicol at 500 and 100 μg/ml, respectively (curves c).

treatment with ampicillin was more than sufficient to fulfill the requirements for the priming stage in normal growing bacteria and that the stringent response inhibited the lysis induction stage; i.e., the priming and lysis induction stages were successfully dissociated by this modified protocol. It is also relevant to note the efficiency of the stringent response in inducing ampicillin tolerance: the lysis induction process was inhibited within the first 10 min of amino acid deprivation (e.g., compare curves b and c in Fig. 2B). With respect to the bacteria subjected to a 10-min treatment with ampicillin, a comparison of curves a and b in Fig. 2A shows that isoleucine deprivation inhibited the growth recovery process. Furthermore, a combination of chloramphenicol and amino acid deprivation resulted in only a small, but significant and reproducible, amount of lysis (Fig. 2A, curve c). Therefore, a 10-min period of ampicillin treatment was clearly insufficient to achieve full priming. It is also apparent that amino acid deprivation by itself (Fig. 2A, curve b) not only inhibited the growth recovery process, as already noted, but also inhibited the small degree of lysis induction. The inhibition of lysis induction was again attributed to the stringent response. On the other hand, the lysis represented by curve c in Fig. 2A was curious; it appeared to be related to the inhibition of protein synthesis by chloramphenicol, because if protein synthesis was not inhibited, growth resumed without any signs of lysis (Fig. 2A, curve a). This possibility is considered below.

Further experiments were designed to optimize this modified two-stage ampicillin-induced lysis protocol for the purpose of studying the relationship between protein synthesis and ampicillin-induced lysis. By varying the time of treatment, the maximum period of treatment with 200 μg of ampicillin per ml from which the bacteria could still recover was determined to be 13 min. This is demonstrated with strain VC7 and its isogenic *relA* derivative, strain VC8, in Figs. 3A and B, respectively. Both strains recovered from the 13-min ampicillin treatment and grew when resuspended in ampicillin-free medium (Fig. 3, curves a). In strain VC7, isoleucine deprivation inhibited the growth recovery process (Fig. 3A, curve c), but the combination of isoleucine deprivation and chloramphenicol treatment resulted in lysis (data not shown). This situation is analogous to that described in Fig. 2A; the lysis observed here is unusual because the bacteria were clearly not irreversibly primed by the 13-min ampicillin treatment employed (i.e., they were capable of recovering from the treatment). Moreover, the addition of chloramphenicol alone (i.e., without concomitant amino acid deprivation) also resulted in lysis (Fig. 3A, curve b). Interestingly, isoleucine deprivation of strain VC8 (*relA*) resulted in lysis (Fig. 3B, curve c) rather than in inhibition of growth as was the case with strain VC7 (Fig. 3A, curve c). Chloramphenicol treatment had the same effect (Fig. 3B, curve b). Other ribosome inhibitors, e.g., gentamicin and tetracycline, also induced lysis of both strains after they had been subjected to the 13-min ampicillin treatment (data not shown). On the other hand, growth inhibitors which had no direct effect on protein synthesis did not induce lysis. For example, the addition of norfloxacin at 8 μg/ml, rather than chloramphenicol, to the ampicillin-treated cultures resulted in the arrest of growth recovery but not in lysis (data not shown). Under these conditions, norfloxacin did not cause the accumulation of ppGpp (14) and had no effect on the rate of protein synthesis for at least 15 min. Therefore, the observed lysis was specifically related to the inhibition of protein synthesis and not simply to the inhibition of growth. In summary, we interpret these results as follows. The ampicillin treatment employed here was sublethal, and both strains, VC7 and VC8, were able to recover from the treatment. However, if protein synthesis was inhibited during the recovery process, lysis was induced except in situations in which the process also provoked the stringent response, i.e., during amino acid deprivation of *relA*$^+$ bacteria.

The experiment whose results are shown in Fig. 4 was designed to determine the effect of the presence of chloramphenicol on the efficiency of the ampicillin priming activity. A culture of strain VC7 was divided into two parts which were then primed with ampicillin. This was achieved by the addition of a combination of chloramphenicol (100 μg/ml) and ampicillin (200 μg/ml) to one of the subcultures and of ampicillin (200 μg/ml) alone to the second subculture. The antibiotics were removed by centrifugation after 13 min of treatment, and the

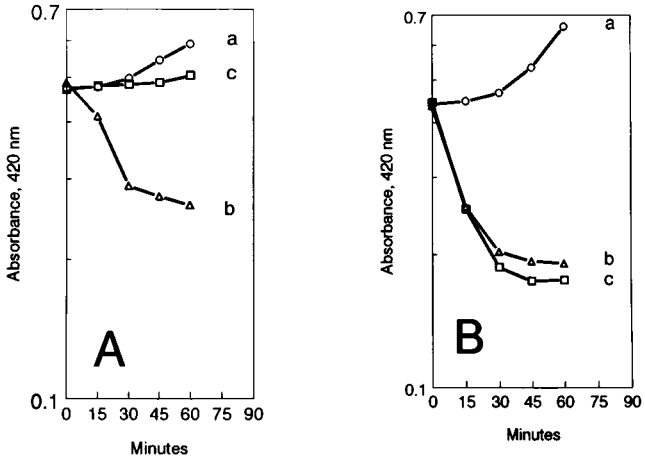

FIG. 3. Effect of chloramphenicol on the lysis induction stage. Exponential-phase cultures of strains VC7 (A) and VC8 (B) were primed with 200 μg of ampicillin per ml for 13 min. The unbound ampicillin was removed, and the bacteria were resuspended in fresh M9 medium. Each culture was divided into three parts. One subculture received no further treatment (curves a). The other subcultures received either 100 μg of chloramphenicol per ml (curves b) or 500 μg of L-valine per ml (curves c).

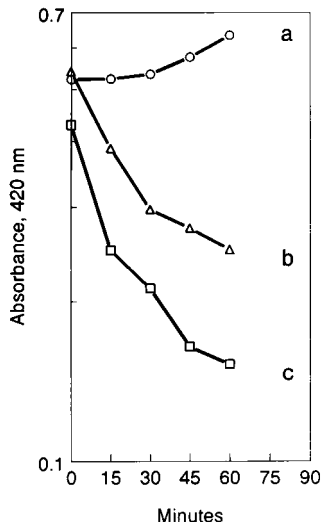

FIG. 4. Effect of chloramphenicol on the priming stage. An exponential-phase culture of strain VC7 was divided into two portions. Both subcultures were primed with 200 μg of ampicillin per ml. In one of the subcultures, 100 μg of chloramphenicol per ml was added along with the ampicillin. After 13 min of incubation, the unbound ampicillin (and chloramphenicol, in one case) was removed from each subculture, and the bacteria were resuspended in fresh M9 medium. A portion of the subculture which was primed with ampicillin alone received no further treatment (curve a), whereas a second portion was treated with 100 μg of chloramphenicol per ml (curve b). The subculture which was primed with ampicillin in the presence of chloramphenicol received no further treatment (curve c).

cells were resuspended in fresh M9 medium. In agreement with data shown in Fig. 3A (curves a and b), the subculture treated with ampicillin alone eventually recovered and started to grow after the removal of the ampicillin (Fig. 4, curve a), but the addition of chloramphenicol resulted in the induction of lysis (Fig. 4, curve b). In contrast to the subculture treated solely with ampicillin, the subculture which was treated with ampicillin in the presence of chloramphenicol underwent lysis after resuspension in fresh medium (Fig. 4, curve c). Therefore, the presence of chloramphenicol clearly enhanced the efficacy of the ampicillin priming activity. The same results were obtained if strain VC7 was treated with ampicillin in the presence of gentamicin or tetracycline rather than chloramphenicol in the priming stage of this experiment (data not shown). Furthermore, when the *relA* mutant, strain VC8, was used in this experiment, the enhanced effect on the ampicillin priming reaction was observed when the ampicillin treatment was combined with isoleucine deprivation instead of treatment with a ribosome inhibitor (data not shown). These latter results indicate that the ampicillin priming stage is enhanced when protein synthesis is inhibited provided that the stringent response is not provoked in the process.

DISCUSSION

We have confirmed and extended a previous report (8) indicating that the treatment of *E. coli* with ribosome inhibitors did not readily result in tolerance to ampicillin. It is shown here that the inhibition of ribosome function for up to 60 min still did not confer complete protection against ampicillin-induced lysis. We consider this noteworthy in view of the widely accepted notion that nongrowing bacteria are tolerant to β-lactam antibiotics. In fact, the overall conclusion derived from this work is that the inhibition of protein synthesis actually promoted ampicillin-induced lysis. The only exception was when this also resulted in provoking the stringent response, i.e., by amino acid deprivation of *relA*$^+$ bacteria. Incidentally, we have further documented the efficiency of the stringent response in the establishment of ampicillin tolerance: the lysis induction stage of primed *relA*$^+$ bacteria was inhibited within 10 min of amino acid deprivation (Fig. 2A).

Two new findings are important in the development of a working hypothesis. They both relate to observations made with bacteria which were subjected to only partial priming with ampicillin, i.e., a 13-min treatment with 200 μg/ml. Such bacteria eventually recovered from the ampicillin treatment and resumed growth when the antibiotic was removed. The first observation was that the bacteria became fully primed if protein synthesis was inhibited during the partial priming process (Fig. 4). The second observation was that the inhibition of protein synthesis during the growth recovery process of partially primed bacteria resulted in lysis induction (Fig. 3). Therefore, the inhibition of protein synthesis promoted the development of both the priming and lysis induction stages. We therefore propose that the inhibition of protein synthesis by ribosome inhibitors would short-circuit the process by which old, spent (or acylated) PBPs are replaced by new PBPs. This should make the priming process (and, in turn, the lysis induction process) more efficient because the critical subset of inactivated PBPs cannot be replaced by new, active PBPs. Furthermore, it seems likely that new PBPs are required to establish the growth recovery process in bacteria subjected to partial priming. Thus, chloramphenicol may prevent this growth recovery and induce lysis by inhibiting the production of new PBPs. Finally, it should be noted that the mechanism of the stringent response is quite different. In this case, not only is the synthesis of new PBPs inhibited but the activities of all existing PBPs involved in peptidoglycan synthesis are also inhibited.

The observations reported here seemingly contradict certain previous reports (see, e.g., reference 10) that demonstrate protection from penicillin-induced lysis by chloramphenicol treatment. The reasons for these apparent discrepancies are uncertain, but we think that they are likely due to differences in experimental conditions, specifically in growth media. The experiments reported here were performed with minimal medium, whereas those in the other study referred to (10) were performed with complex media. We have already noted that the degree of lysis induced by ampicillin in the presence of chloramphenicol was markedly reduced in complex medium compared with minimal medium (8). We currently do not have an explanation for this, and further studies are warranted. It is possible that protein synthesis is not as critical in penicillin-induced lysis of bacteria grown in complex media because either the size of the critical PBP pool is larger or the PBPs exhibit a longer functional half-life under these conditions.

ACKNOWLEDGMENTS

This work was supported by an Operating Grant from the Natural Sciences and Engineering Research Council of Canada to E.E.I. D.G.R. was the recipient of The Ray Hadfield Memorial Fellowship.

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