Dependence of peptidoglycan metabolism on phospholipid synthesis during growth of Escherichia coli

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The role of phospholipid synthesis in peptidoglycan metabolism during growth of Escherichia coli was determined. The inhibition of phospholipid synthesis, achieved by inhibiting fatty acid synthesis with cerulenin or by glycerol deprivation of gpsA mutant strains, resulted in the concomitant inhibition of peptidoglycan synthesis. These effects on peptidoglycan synthesis were relatively specific in that the treatments did not cause a general inhibition of macromolecular synthesis. Furthermore, the inhibition of phospholipid synthesis also resulted in the rapid development of penicillin tolerance. It was unlikely that penicillin tolerance in these cases were simply due to the inhibition of growth caused by cerulenin treatment or glycerol deprivation because treatments with more effective growth inhibitors, e.g. chloramphenicol or norfloxacin, did not confer penicillin tolerance. Penicillin tolerance was shown to be a direct consequence of the inhibition of phospholipid synthesis and not due to the possible accumulation of guanosine-3',5'-bispyrophosphate (ppGpp), the starvation stress signal molecule known to be responsible for the development of penicillin tolerance in amino-acid-deprived bacteria. Therefore, peptidoglycan metabolism is coupled to phospholipid synthesis during growth of E. coli, and this may represent an important means to ensure the coordination of cell envelope synthesis in growing bacteria.

Keywords: penicillin tolerance, peptidoglycan, phospholipid, Escherichia coli, β-lactam antibiotics

INTRODUCTION

Peptidoglycan is the covalently closed bag-shaped macromolecular component of eubacterial cell walls. The synthesis of peptidoglycan is widely believed to require not only the peptidoglycan polymerase activities of the high-molecular-mass penicillin-binding proteins (PBPs) but also the activities of certain peptidoglycan hydrolases (for reviews, see Høltje & Tuomanen, 1991; Høltje, 1995). Very little has been established with regard to the mechanism by which PBPs and peptidoglycan hydrolases participate in the synthesis of peptidoglycan. The peptidoglycan hydrolase activities also play a decisive role in the killing action of inhibitors of peptidoglycan synthesis such as the β-lactam antibiotics. Treatment of bacteria with such agents apparently uncouples the peptidoglycan hydrolase activities normally involved in the biosynthetic process, and their hydrolytic action on the existing peptidoglycan is responsible for the characteristic cell lysis which occurs.

Nongrowing bacteria are tolerant to the killing action of β-lactam antibiotics. In the case of amino-acid-deprived Escherichia coli, penicillin tolerance has been attributed to the phenomenon known as the stringent response (Kusser & Ishiguro, 1985). Amino-acid-deprived E. coli cells rapidly accumulate the novel nucleotide guanosine 3',5'-bispyrophosphate (ppGpp), which is believed to mediate the stringent response by globally inhibiting a diverse array of metabolic processes. Therefore, the stringent response probably represents a means of enhancing bacterial survival during periods of amino acid deprivation (for a review, see Cashel & Rudd, 1987). The

Abbreviations: DAP, diaminopimelic acid; PBP, penicillin-binding protein.
Synthesis of ppGpp during the stringent response is catalysed by ppGpp synthetase I, a ribosome-associated enzyme encoded by the relA gene, which is activated during amino acid deprivation. The stringent response can be eliminated (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. Under certain conditions of nutritional stress, e.g., energy source starvation, a RelA-independent accumulation of ppGpp is observed. This process is catalysed by ppGpp synthetase II, which has been identified as the product of the spoT gene (Hernandez & Bremer, 1991; Xiao et al., 1991). Of direct relevance to this study is the observation that SpoT catalyses the accumulation of ppGpp when fatty acid synthesis is inhibited in E. coli (Seytzadich et al., 1993).

Sokawa et al. (1968) first demonstrated that phospholipid synthesis is inhibited during the stringent response. Heath et al. (1994) have subsequently proven that ppGpp directly mediates this process by showing that the overproduction of ppGpp in normal growing E. coli, achieved through the controlled overexpression of the cloned relA gene, resulted in the inhibition of phospholipid synthesis. Furthermore, the observed inhibition of phospholipid synthesis was relieved by the overexpression of the plsB gene, which encodes sn-glycerol-3-phosphate acyltransferase, and this indicates that PlsB is the enzyme inhibited by ppGpp.

The synthesis of peptidoglycan (Ishiguro & Ramey, 1976) and penicillin-induced lysis (Kusser & Ishiguro, 1985) are also inhibited during the stringent response. The following observations indicate that phospholipid synthesis plays a pivotal role in regulating peptidoglycan metabolism and β-lactam-induced lysis in amino-acid-deprived cells. (i) The terminal stage of peptidoglycan synthesis, apparently corresponding to the activities of the PBPs, has been shown to be obligately dependent on phospholipid synthesis when the stringent response is relaxed in amino-acid-deprived bacteria; i.e., the inhibition of relaxed phospholipid synthesis in amino-acid-deprived cells results in the concomitant inhibition of relaxed peptidoglycan synthesis (Ishiguro, 1983). (ii) Rodionov et al. (1995) have recently demonstrated that the lysis of relaxed amino-acid-deprived bacteria induced by treatment with β-lactam antibiotics is also dependent on phospholipid synthesis; their results suggest that both PBP and peptidoglycan hydrolase activities exhibit this dependence. (iii) The overproduction of ppGpp, resulting from the overexpression of the relA gene in otherwise normally growing bacteria, causes the inhibition of both peptidoglycan and phospholipid synthesis and results in the development of penicillin tolerance (Rodionov & Ishiguro, 1995). Furthermore, the overexpression of PlsB under these conditions eliminates the penicillin tolerance conferred by ppGpp accumulation. In summary, studies to date indicate that ppGpp accumulation during the stringent response directly inhibits PlsB, and this, in turn, results in the coordinate inhibition of PBP and peptidoglycan hydrolase activities, and, consequently, in penicillin tolerance.

The objective of the present study was to determine whether the observations made on amino-acid-deprived E. coli could be extended to normal growing bacteria. We demonstrate here that peptidoglycan metabolism is indeed coupled to concomitant phospholipid synthesis during growth of E. coli.

**METHODS**

**Bacterial strains.** All bacteria were derivatives of E. coli K-12. Strain VC7 (thi-1 lysoA23 rpsL109) was from our laboratory collection. Strain VC7001 is a derivative of VC7 carrying relA251::kan and spoT707::cat and is therefore unable to synthesize ppGpp (Rodionov & Ishiguro, 1995). Strains VC66 (thi-1 lysoA23 rpsL109 gusA zib-22::Tna10) and VC58 (thi-1 lysoA23 rpsL109 gusA zib-22::Tna10 relA2) were constructed in this laboratory. The gusA allele carried by these strains was derived from strain WN20, which was the gift of the late W. D. Nunn; this mutation resulted in an auxotrophic requirement for either glycerol-3-phosphate or glycerol. In all experiments, 0.05% glycerol was used to satisfy the nutritional requirement imposed by the gusA mutation.

**Growth conditions.** Bacteria were grown in M9 minimal medium containing required growth factors in a 37 °C waterbath shaker as described previously (Ishiguro & Ramey, 1976). The only exceptions were experiments involving radiolabelling with 32P, which were carried out in Bell's (1974) modified M56LP low-phosphate medium. Glucose at 0.2% was used as the carbon source in all experiments except the one described in Fig. 2, in which the carbon source was 0.4% glycerol (Rodionov et al., 1995). Strain VC7001 was grown in M9 medium containing 0.1% Casamino acids (Difco). Culture turbidity was determined with a Beckman DU-64 spectrophotometer at 420 nm.

**Antibiotic treatment.** The general methods for antibiotic treatment have been described previously (Kusser & Ishiguro, 1985; Rodionov et al., 1995). Unless indicated otherwise, antibiotics were used at concentrations equal to about 10 times their minimum inhibitory concentrations (MICs). The MICs for the agents used in this study were 15 μg ml⁻¹ for cephaloridine, 5 μg ml⁻¹ for ampicillin, 6 μg ml⁻¹ for cephalothin, 0.2 μg ml⁻¹ for imipenem, 10 μg ml⁻¹ for cefsulodin, and 15 μg ml⁻¹ for phosphomycin. The inhibitory effect of cephaloridine was reversed by supplementing the growth medium with a mixture of potassium citrate and potassium palmatate (100 μg ml⁻¹ of each, final concentration) as described by Rodionov et al. (1995).

**Assay of peptidoglycan synthesis.** Bacteria were grown for two doublings in M9-glucose minimal medium to a density of 2 × 10⁸ cells ml⁻¹, meso-2,6-Diaminopimelic acid ([3H]DAP; 10 μCi μg⁻¹) was added to a final concentration of 0.2 μg ml⁻¹, and incubation was continued for an additional 45 min. Peptidoglycan synthesis was then assayed by measuring the incorporation of [3H]DAP into cold trichloroacetic acid insoluble fractions as described by Ishiguro & Ramey (1976).

**Assay of phospholipid synthesis.** Bacteria were grown for two doublings in M56LP low-phosphate medium to a density of 2 × 10⁸ cells ml⁻¹, 32P (5 μCi ml⁻¹, 185 kBq ml⁻¹) was added, and the culture was incubated for an additional 45 min before incorporation of 32P into the phospholipid fraction was measured. Phospholipids were extracted as described by Bligh & Dyer (1959). The amounts of radioactivity in 200 μl aliquots
of the chloroform fractions were quantified by liquid scintillation counting.

**Quantification of ppGpp.** The ppGpp fraction in cold formic acid extracts of 32P-labelled bacteria was quantified after separation by thin-layer chromatography on polyethyleneimine cellulose F plates developed in 1.5 M KH2PO4 as described by Rodionov & Ishiguro (1995).

**Assays for lysis.** Antibiotic-induced lysis was routinely determined by measuring OD650. In addition, all experiments described were repeated using viable cell counts as a measure of lysis as described by Rodionov & Ishiguro (1995). In one experiment, bacteria were prelabelled with [3H]DAP and bacteriolysis was then determined by assaying the solubilization of radiolabelled peptidoglycan; this procedure has been described by Kusser & Ishiguro (1986).

**Liquid scintillation counting.** Radioactive samples were counted in Ready Flow III liquid scintillation cocktail (Beckman) with a Beckman LS 8100 liquid scintillation counter.

**Materials.** All antibiotics were purchased from Sigma except for imipenem and norfloxacin, which were obtained from Merck, Sharp & Dohme. 32P (carrier-free, 10 mCi ml⁻¹; 370 MBq ml⁻¹) was obtained from Amersham. meso-2,6-Diamin0[2,6-~H]pimelic acid (30 Ci mmol⁻¹; 1.1 GBq mmol⁻¹) was purchased from American Radiolabeled Chemicals.

**RESULTS**

**Dependence of peptidoglycan synthesis on phospholipid synthesis during growth**

Fig. 1 shows the effects of different concentrations of cerulenin on growth, phospholipid synthesis and peptidoglycan synthesis in strain VC7. As expected, cerulenin inhibited growth (Fig. 1a) and phospholipid synthesis (Fig. 1b). However, cerulenin treatment also resulted in the inhibition of peptidoglycan synthesis (Fig. 1c). In all cases, the degrees of inhibition exhibited a direct dependence on the concentration of cerulenin employed.

The inhibition of peptidoglycan synthesis was considered significant because cerulenin was not a general inhibitor of macromolecular synthesis. For example, cerulenin did not inhibit either protein or DNA synthesis over the time course of these experiments when used at a concentration as high as 100 µg ml⁻¹, i.e. 6.5 times the MIC. RNA synthesis was inhibited by 20% in the presence of 100 µg cerulenin ml⁻¹ but only after 40 min of treatment, and lower concentrations had no inhibitory effects. These results are in general agreement with those of Goldberg et al. (1973).

In additional experiments, peptidoglycan synthesis was determined in glycerol-limited cultures of the gpsA mutant strains, VC56 and VC58. The gpsA gene encodes the biosynthetic m-glycerol-3-phosphate dehydrogenase, and these strains were therefore dependent on an exogenous supply of glycerol (or glycerol 3-phosphate) for growth. Furthermore, phospholipid synthesis was arrested in these strains when the supply of glycerol was exhausted. In both cases, glycerol deprivation also resulted in the inhibition of peptidoglycan synthesis (data not shown). Collectively, the results of the experiments described above indicate that peptidoglycan synthesis in growing bacteria was dependent on concomitant phospholipid synthesis.

**Induction of β-lactam tolerance by cerulenin treatment of growing bacteria**

The effect of cerulenin treatment on the susceptibility of strain VC7 to ampicillin-induced bacteriolysis was tested. As shown in Fig. 2, lysis was initiated within 40 min when VC7 growing in glycerol-minimal medium was treated with ampicillin at 50 µg ml⁻¹. The addition of cerulenin at 50 µg ml⁻¹ at the same time as ampicillin resulted in the development of lysis tolerance. Furthermore, the combination of cerulenin and ampicillin inhibited growth
Fig. 2. Inhibitory effect of cerulenin on the ampicillin-induced lysis of strain VC7. At 0 min, portions of an exponential-phase culture growing in glycerol-minimal medium were treated with cerulenin (○), ampicillin (▲), a combination of cerulenin and ampicillin (△), and a combination of cerulenin and ampicillin along with a mixture of oleate and palmitate (□). An untreated control (●) is shown for comparison.

sooner than cerulenin treatment alone, suggesting that cerulenin was not interfering with the interaction of ampicillin and its PBP targets. Finally, the addition of a mixture of oleate and palmitate not only reversed the growth-inhibitory activity of cerulenin but it also reversed the penicillin tolerance caused by cerulenin treatment. The same results were obtained when penicillin tolerance in this experiment was determined by viable cell counts rather than optical density measurements. Furthermore, in experiments using bacteria which were prelabelled with [3H]DAP, treatment with either cerulenin alone or cerulenin combined with ampicillin failed to cause measurable solubilization of the radiolabelled peptidoglycan. Therefore, these experiments collectively indicate that cerulenin treatment results in development of tolerance to ampicillin-induced lysis. Finally, it should be noted that cerulenin also induced tolerance to lysis by benzylpenicillin, imipenem, cefsulodin, cephalexin and phosphomycin.

**Penicillin tolerance was caused by inhibition of phospholipid synthesis**

Fig. 3 shows that glycerol deprivation of the gbsA mutant, strain VC58, also resulted in ampicillin tolerance. Therefore, although cerulenin is an inhibitor of fatty acid synthesis, it is clear that its ability to cause penicillin tolerance was based on the inhibition of phospholipid synthesis resulting from fatty acid deprivation.

The possibility that the observed penicillin tolerance was due simply to the inhibition of growth caused by cerulenin treatment or glycerol deprivation was considered. The experiments described in Fig. 4 make this unlikely. Treatment of strain VC7 with either chloramphenicol (100 μg ml⁻¹; Fig. 4a) or norfloxacin (10 μg ml⁻¹; Fig. 4b) resulted in almost immediate cessation of growth. In both cases, ampicillin still caused lysis in spite of the inhibition of growth. In fact, lysis induction by ampicillin was initiated earlier in the presence of chloramphenicol or norfloxacin; the basis for this is not known. The same results were obtained at concentrations closer to the MICs for these agents, e.g. 10 μg ml⁻¹ for chloramphenicol and 1 μg ml⁻¹ for norfloxacin.

**Penicillin tolerance was not due to ppGpp accumulation**

As noted above, Seyfzadeh et al. (1993) showed that fatty acid deprivation induced the accumulation of ppGpp. They further showed that ppGpp did not accumulate during glycerol deprivation of a gbsA mutant and the effect was therefore not due to inhibition of phospholipid synthesis. We have already shown (Fig. 3) that glycerol deprivation of the gbsA mutant, strain VC58, resulted in ampicillin tolerance. Therefore, the ampicillin tolerance observed in our studies was not due to ppGpp accumulation induced by fatty acid deprivation resulting
treated with 100 pg ml⁻¹ chloramphenicol, 50 pg ml⁻¹ for ampicillin, and 10 pg ml⁻¹ for norfloxacin.

In the absence of the agents used were lOOpg ml⁻¹ for norfloxacin. The other subcultures were treated with norfloxacin (A), and a combination of ampicillin and chloramphenicol (O). The concentrations of the agents used were 100 pg ml⁻¹ for chloramphenicol, 50 μg ml⁻¹ for ampicillin, and 10 μg ml⁻¹ for norfloxacin.

First, lysis of untreated control cultures in our experiments was initiated before there were any detectable changes in the growth rates of the treated cultures (Figs 2 and 3). Second, agents which caused abrupt inhibition of growth, e.g. chloramphenicol and norfloxacin (Fig. 4), did not cause penicillin tolerance, indicating that inhibition of phospholipid synthesis during steady growth, but how this is accomplished is not known (Cronan & Rock, 1987).

Fig. 4. Effects of ribosome and DNA gyrase inhibitors on ampicillin-induced lysis of strain VC7. Exponential-phase cultures were divided into four parts. In (a), one subculture received no further treatment (O). The other subcultures were treated with 100 μg ml⁻¹ chloramphenicol (△), ampicillin (●), and a combination of ampicillin and chloramphenicol (▲). In (b), one subculture served as an untreated control (O). The other subcultures were treated with norfloxacin (△), ampicillin (●), and a combination of norfloxacin and ampicillin (▲). The concentrations of the agents used were 100 μg ml⁻¹ for chloramphenicol, 50 μg ml⁻¹ for ampicillin, and 10 μg ml⁻¹ for norfloxacin.

Fig. 5. Effect of cerulenin treatment on ampicillin-induced lysis of strain VC7001 (relA spoT). Portions of an exponential phase culture were treated with ampicillin in the presence (●) and absence (O) of 50 μg cerulenin ml⁻¹.

from the cerulenin treatment. To further establish this point, two additional experiments were performed. In the first of these experiments, the ppGpp levels during cerulenin treatment were quantified. The peak accumulation was determined to be 450 pmol (mg cell dry weight)⁻¹. In comparison, the basal level in normal exponential-phase bacteria was 40 pmol ppGpp (mg cell dry weight)⁻¹. Therefore, cerulenin treatment caused over a 10-fold accumulation of ppGpp but, significantly, this was still far less than the minimum amount required to establish ampicillin tolerance, which was previously determined to be 870 pmol (mg cell dry weight)⁻¹ (Rodionov & Ishiguro, 1995). In the second experiment, we constructed a relA spoT derivative of VC7, strain VC7001. This strain was truly ppGpp-deficient; i.e. ppGpp was undetectable in amino-acid-deprived bacteria and in cerulenin-treated bacteria. Fig. 5 shows that cerulenin treatment of strain VC7001 resulted in ampicillin tolerance. Together, these results indicate that the observed ampicillin tolerance was directly related to the inhibition of phospholipid synthesis and not to ppGpp accumulation.

DISCUSSION

We have shown here that peptidoglycan metabolism was coupled to phospholipid synthesis during growth of E. coli. This phenomenon is therefore not a unique aspect of amino-acid-deprived bacteria. The observed dependence of peptidoglycan synthesis on phospholipid synthesis may represent an important means of coordinating cell envelope synthesis in growing bacteria. On the other hand, membrane protein synthesis may not be similarly coupled to phospholipid synthesis since the protein to phospholipid ratios in both the inner and outer membranes were found to increase by about 60% when phospholipid synthesis was inhibited in E. coli (McIntyre & Bell, 1975). Nevertheless, bacteria still generally maintain a constant protein to phospholipid ratio during steady growth, but how this is accomplished is not known (Cronan & Rock, 1987).

Leduc et al. (1982) previously showed that E. coli cells pretreated with cerulenin for 30 min were tolerant to penicillin-induced lysis. They concluded that lysis exhibited a dependence on fatty acids but did not elaborate on the nature of this dependence. In this regard, it is widely accepted that nongrowing or slow-growing bacteria are penicillin-tolerant, and it was possible that the observed penicillin tolerance was simply due to the inhibition of growth brought about by fatty acid deprivation. For example, Tuomanen et al. (1986) have demonstrated in experiments involving chemostat-grown cultures of E. coli that the rate of killing by β-lactam antibiotics is directly related to the growth rate of the bacteria. However, several observations suggest that the penicillin tolerance observed in the experiments reported here was not due to the inhibition of growth resulting from either cerulenin treatment or glycerol deprivation. First, lysis of untreated control cultures in our experiments was initiated before there were any detectable changes in the growth rates of the treated cultures (Figs 2 and 3). Second, agents which caused abrupt inhibition of growth, e.g. chloramphenicol and norfloxacin (Fig. 4), did not cause penicillin tolerance, indicating that in-
hibition of growth by itself was not sufficient to prevent penicillin-induced lysis under our experimental conditions. Third, we have previously demonstrated that the penicillin tolerance of amino-acid-deprived E. coli is based on the inhibition of phospholipid synthesis, and the dependence of peptidoglycan metabolism on phospholipid synthesis (Rodionov et al., 1995; Rodionov & Ishiguro, 1995). We therefore conclude that the penicillin tolerance reported here in growing bacteria was a direct consequence of the inhibition of phospholipid synthesis. It should be noted that we have also unequivocally ruled out the possibility that the inhibitory effect of cerulenin on the inhibition of phospholipid synthesis, consequence of the inhibition of phospholipid synthesis.

Ishiguro, 1995). We therefore conclude that the penicillin on the inhibition of phospholipid synthesis, and the phospholipid synthesis (Ishiguro, 1983) have suggested that the membrane-bound enzymic reactions in peptidoglycan metabolism are dependent on phospholipid synthesis (Romeis & Hötijte, 1994). It is possible that some aspect of either the assembly or the function of the putative multienzyme complex may be dependent on phospholipid synthesis.

The pattern of cell envelope synthesis during the cell cycle of E. coli has been the subject of numerous reports (reviewed by Cronan & Rock, 1987; Cooper, 1991). Although they are not all in agreement, some provide circumstantial evidence suggesting a link between membrane phospholipid synthesis and peptidoglycan metabolism. For example, several reports indicate that the rate of phospholipid synthesis increases abruptly at the onset of septum formation and remains high throughout the process of cell division (Carty & Ingram, 1981; Hakenbeck & Messer, 1977a; Pierucci, 1979). An increase in the rate of peptidoglycan synthesis has also been shown to occur during this period (Hoffman et al., 1972). Furthermore, the activities of several peptidoglycan hydrolases undergo cell-cycle-dependent fluctuations (Beck & Park, 1976; Hakenbeck & Messer, 1977b; Mirelman et al., 1978). Gally et al. (1993) have also studied the synthesis of peptidoglycan and membrane during the cell division cycle of E. coli and Salmonella typhimurium, and it is important to note that they did not observe cell cycle fluctuations in peptidoglycan and phospholipid synthesis.

Gally et al. (1993) concluded that cell surface synthesis in E. coli and S. typhimurium is regulated primarily, and possibly solely, by mass increase, and they proposed that the peptidoglycan layer enlarges in response to the accumulation of cell mass. Furthermore, they suggested that the cell membrane grows passively in response to the enlargement of the peptidoglycan layer. Our conclusion is just the opposite, namely that peptidoglycan expansion is dependent on membrane phospholipid synthesis. These differences of opinion will need to be addressed in future studies.

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