Regulation of β-Lactam-Induced Lysis in *Escherichia coli*

by

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ABSTRACT

The penicillin tolerance of amino acid-deprived *relA*$^+$ *Escherichia coli* is attributed to the stringent response. 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. Both the priming and the lysis induction stages of β-lactam-induced lysis were shown to require phospholipid synthesis. It has been known for some time that phospholipid synthesis is inhibited by the stringent reponse. These results indicate that the inhibition of peptidoglycan synthesis and the induction of penicillin tolerance during the stringent response are both secondary consequences of the inhibition of phospholipid synthesis.

Direct experimental evidence is presented for the first time indicating that the penicillin tolerance of amino acid-deprived *E. coli* was directly attributable to action of guanosine 3',5'-bispyrophosphate (ppGpp) and not to some other effect of amino acid deprivation. The overproduction of ppGpp resulted in the inhibition of peptidoglycan and phospholipid synthesis and in penicillin tolerance. Penicillin tolerance and the inhibition of peptidoglycan synthesis were both suppressed when ppGpp accumulation was prevented by treatment of the bacteria with chloramphenicol, an inhibitor of ppGpp synthetase I activation. Glycerol-3-phosphate acyltransferase, the product of *plsB* gene, was recently identified as the main site of ppGpp inhibition in phospholipid synthesis. The overexpression of the cloned *plsB* gene reversed the penicillin tolerance conferred by ppGpp accumulation. This also indicates that the membrane-associated events in peptidoglycan metabolism were dependent on ongoing phospholipid synthesis. Interestingly, treatment with β-lactam antibiotics by itself induced *relA*-dependent ppGpp accumulation, but the maximum levels attained were insufficient to confer penicillin tolerance.

It was also demonstrated that penicillin tolerance was induced when phospholipid

synthesis was inhibited in normal growing *E. coli*. This penicillin tolerance was not the result a simple inhibition of growth or a decrease in the membrane levels of individual phospholipids (e.g., acidic phospholipids), but rather the direct result of the inhibition of net phospholipid synthesis.

A number of factors that interfere with β-lactam-induced lysis were investigated. (i) It was demonstrated that de-energization of the *E. coli* cytoplasmic membrane resulted in penicillin tolerance due to the inhibition of both the priming and the lysis induction stages. (ii) Inhibition of protein synthesis in the absence of the stringent response promoted both the priming and the lysis induction stages resulting in a faster onset of β-lactam-induced lysis. (iii) The temperature sensitivity of β-lactam-induced lysis in amino acid-deprived *E. coli* was re-investigated. Penicillin tolerance resulting from a temperature up-shift was not due to the induction of the heat-shock response, as previously reported, but from a reversible inhibition of unidentified thermosensitive enzyme(s) involved in the lysis induction stage.

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LIST OF ABBREVIATIONS.

Amp^R	ampicillin-resistant
Amp^S	ampicillin-sensitive
BIO-AMP	biotinylated ampicillin
BSA	bovine serum albumin
CCCP	cyanide *m*-chlorophenylhydrazone
DAP	*meso*-diaminopimelic acid
DMSO	dimethyl sulfoxide
EGSC	*E. coli* genetic stock centre
GlcNAc	*N*-acetylglucosamine
GCL	glycosyl carrier lipid
G3P	glycerol-3 phosphate
HMW	high molecular weight
IPTG	isopropyl-β-D-thiogalactoside
Kan^R	kanamycin-resistant
KDa	kilodalton
LB	Luria broth
LMW	low molecular weight
LPS	lipopolysaccaride
MIC	minimum inhibitory concentration
Mlt	membrane-bound lytic transglycosylase
MurNAc	*N*-acetylmuramic acid
PBP	penicillin-binding protein
PG	peptidoglycan
PG-3-P	phosphatidylglycerol-3-phosphate
ppGpp	guanosine 3',5'-bispyrophosphate
PPO	2,5-diphenyloxazol
SDS	sodium dodecyl sulfate
Slt	soluble lytic transglycosylase

Spc^R	spectinomycin-resistant
Str^R	streptomycin-resistant
TCA	trichloroacetic acid
TPP^+	[^{3}H]-tetraphenylphosphonium$^+$
TSA	tryptic soy agar
TSB	tryptic soy broth

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Chapter I: Introduction

I. The cell wall of *E. coli*

Like other gram negative bacteria, *E. coli* possesses an outer and an inner (cytoplasmic) membrane (Fig. 1). The outer membrane contains phospholipids, lipopolysaccarides (LPS) and a set of characteristic proteins (Nikaido & Vaara, 1987). The cytoplasmic membrane is a phospholipid bilayer permeable only to water, gases, and small hydrophobic molecules such as medium chain fatty acids (Cronan *et al.*, 1987). The cytoplasmic membrane contains a number of proteins involved in bioenergetic and biosynthetic reactions and in transport of specific solutes. The cytoplasmic membrane of gram negative bacteria is protected by the other two components of cell walls: the outer membrane, which provides protection from the surface-active agents present in the natural environment (e.g., bile salts) and from host defence mechanisms (such as complement) and by the peptidoglycan (PG) layer located in the periplasmic space. PG layer counteracts the cytoplasmic turgor pressure and determines the characteristic shape of bacteria (Park, 1987).

The outer membrane is an asymmetric bilayer. Phospholipids occur exclusively in the inner leaflet of the outer membrane whereas the outer leaflet contains the rigid hydrophobic portion of the LPS that is much less fluid than a typical phospholipid membrane (Labischinski *et al.*, 1985). This property explains the high resistance of wild type *E. coli* strains to hydrophobic antibiotics, detergents and dyes. Several outer membrane proteins (porins) form transmembrane channels that allow diffusion of small hydrophillic molecules into the cell while restricting the entry of large or hydrophobic compounds (Nikaido & Vaara, 1987).

PG can be isolated as a porous macromolecule of characteristic rod shape (Weidel & Pelzer, 1964). Physical contacts between PG and the cytoplasmic membrane have never been documented. However, fusions between the inner and the outer membranes have been reported (Bayer, 1979). These so-called Bayer adhesion zones are proposed to represent the sites of export of the outer membrane proteins and LPS, as well as the sites for insertion of newly synthesised PG chains to the preexisting PG layer (Park & Burman, 1987). There is no information on the state of PG in these sites.

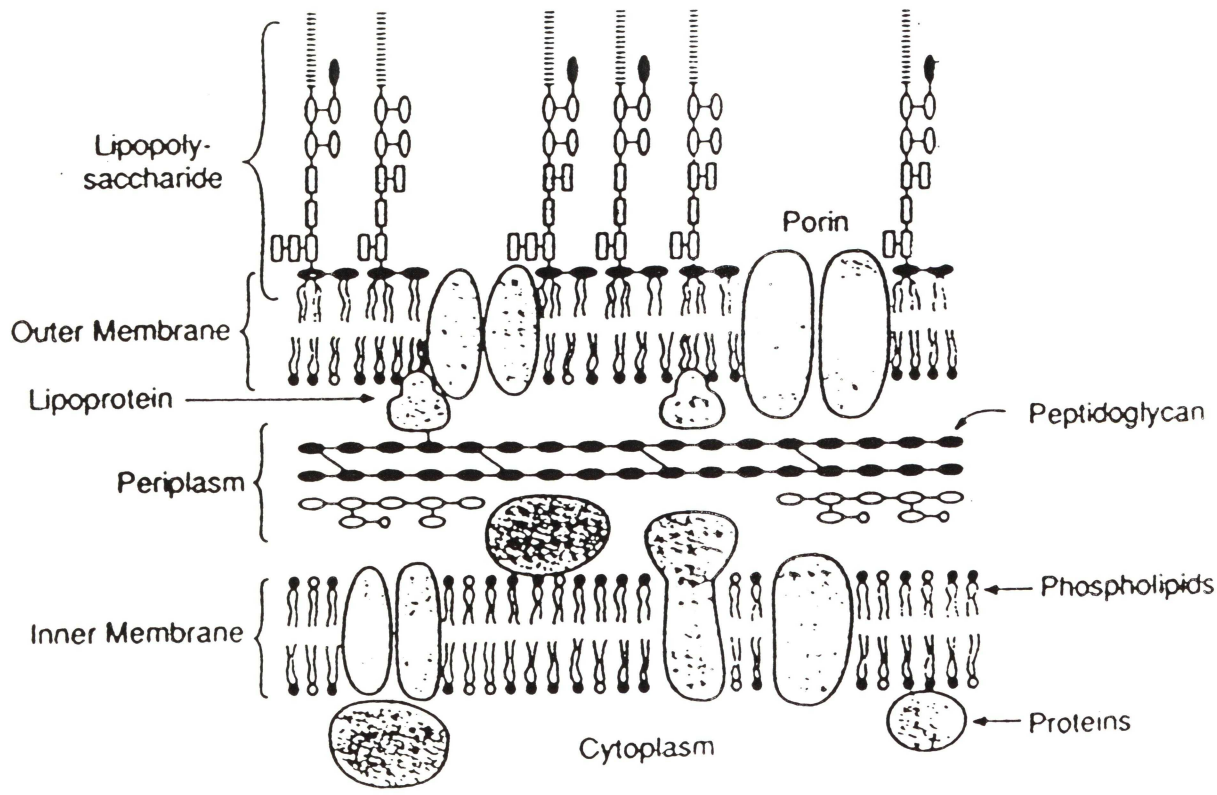

Figure 1. Schematic structure of *E. coli* cell wall. See the text for explanations. Adapted from Raetz & Dowhan (1990).

The outer membrane is firmly anchored to PG layer by the PG lipoprotein molecules. About one-third of PG lipoprotein molecules occur in a form that is bound covalently to PG layer through their C-terminal lysine residues. Their N-terminal cysteine residues are modified with three fatty acid residues so they can insert in the outer membrane (Braun, 1975). The PG lipoprotein-deficient mutants are viable, but tend to release outer membrane vesicles and periplasmic enzymes into the growth medium (Hirota *et al.*, 1977). In addition, some outer membrane proteins, e.g., porins OmpC and OmpF, are tightly associated with PG through non-covalent interactions (Rosenbush, 1974). It is estimated that over 400,000 contacts are made between PG layer and the outer membrane (Park, 1987).

Cell walls grow constantly during the cell cycle. Since PG layer determines the cell shape and counteracts the turgor pressure, the integrity of PG layer is of vital importance to the cell. A fault in PG synthesis resulting in a nick in PG layer brings about rupture of the cytoplasmic membrane and cell lysis. This happens, for example, in the course of β-lactam antibiotic treatment (Höltje & Schwarz, 1985). Therefore, PG synthesis must be tightly controlled during the cell growth and division.

II. Structure of *E. coli* PG

PG of *E. coli* is a polymer that consists of two amino sugars and four amino acids. The basic repeating unit of PG is shown in Fig. 2. *N*-acetylglucosamine (GlcNAc) is linked to *N*-acetylmuramic acid (MurNAc) by 1→4 glycosidic bond. The carboxyl group of each muramic acid residue forms a peptide bond with a short peptide. A common structure of this peptide is L-alanyl-D-isoglutamyl-L-*meso*-diaminopimelyl-D-alanine; however, a small percentage of the *E. coli* peptides either lack D-alanine or contain an extra C-terminal D-alanine. The extra C-terminal alanine residue comes from a precursor of PG biosynthesis. This basic PG unit is found in almost all gram negative bacteria and some gram positive rods (Scheifer & Kandler, 1972).

An average mature PG strand consists of about 30 basic repeating units linked by the 1→4 glycosidic bond, with a non-reducing 1,6-anhydro MurNAc being present at the reducing end of each strand (Glauner & Höltje, 1990). The adjacent strands are cross-

linked to each other through their peptide chains. It was estimated, that between 45% and 60% of PG basic units are involved in cross-links depending on the age of PG and experimental conditions (de Pedro & Schwarz, 1981; Glauner & Höltje, 1990). Normally, these cross-links are between the carboxyl group of D-alanine in position 4 of one peptide and the free amino group of DAP residue from a peptide on an adjacent PG strand. However, about one-tenth of the cross-links were shown to occur between two DAP molecules: the number of such DAP-DAP dimers increases as the cells reach stationary phase (Glauner *et al.*, 1988). In addition, lipoprotein anchors the outer membrane to the PG layer; it is known to bind to approximately 10% of PG basic repeating units (Burman & Park, 1983). There is no obvious consensus site on PG to which lipoprotein molecules bind, as the distribution of the PG-bound lipoprotein among the different peptides after PG degradation shows no specificity for a certain structure (Glauner *et al.*, 1988). The cross-linking continues in the PG strands that are already inserted in the PG layer, and the newly synthesised PG is less cross-linked than the mature PG (de Pedro & Schwarz, 1981). Overall, the two-dimensional structure of PG is often likened to a net (Fig. 2).

The three dimensional structure of *E. coli* PG has not been resolved. Earlier observations suggested that PG layer of gram negative bacteria consisted of a single monomolecular PG sheet (Braun *et al.*, 1973). PG growth was thus proposed to occur by inserting new glycan strands between the existing ones (Burman & Park, 1984). Such growth, however, would not account for PG turnover which was estimated to be about 50% per generation (Goodell, 1985). Neutron small-angle scattering studies indicated that the average thickness of PG was 1.5 PG monolayers (Labischinski *et al.*, 1991). PG composition studies also suggested that PG layer was thicker than a single monolayer. For example, Glauner & Höltje (1990) reported that *E. coli* has enough PG to form about three monolayers. Thus, it has been suggested that new PG has to be made before the old PG would be degraded in an inside-to-outside replacement process (Glauner & Höltje, 1990). A modified model postulated the existence of "smart enzymes" that would recognize the presence of new PG underneath the stress-bearing single layer and insert two new PG strands at the expense of one old strand (Koch, 1990). Another model postulated the existence of a multienzyme complex with both synthetic and hydrolytic

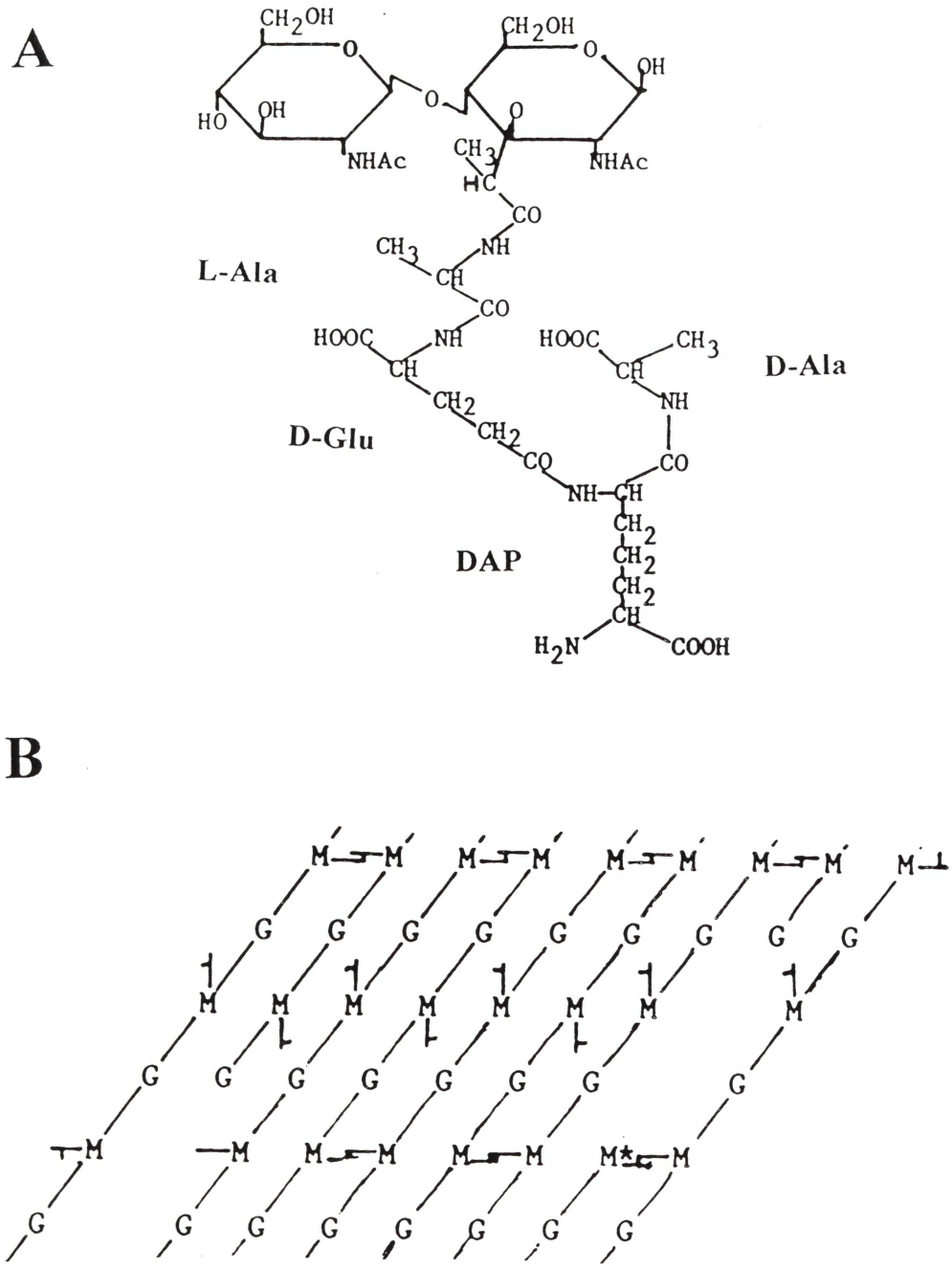

Figure 2. Structure of PG. **A**, structure of the basic unit of PG from *E. coli* and other bacteria: Ac, acetyl. **B**, net-like two-dimensional structure of PG: M, *N*-acetylmuramic acid; G, *N*-acetylglucosamine; M*, 1,6-anhydro-*N*-acetylmuramic acid; ⊢, peptide links. Adapted form Park (1987).

activities. This multienzyme complex was proposed to substitute one old glycan strand for a newly synthesized trimer (the "three-for-one" growth model; Höltje, 1993). Both models suggest that PG growth in *E. coli* follows the inside-to-outside pattern and the new PG patch is created underneath the old stress-bearing layer before the latter can be recycled.

In summary, PG layer (also known as PG sacculus) is a thin porous macromolecule of heterologous subunits held together by various peptide cross-links. The stress-bearing layer of PG sacculus may be a monolayer: thus, a precise regulation of PG turnover is necessary to ensure its integrity. This control is likely to be ensured by a continuous synthesis of PG patches underneath the existing monolayer.

III. Biosynthesis of PG

PG biosynthesis takes place in three cellular compartments and thus is subdivided into three stages (Fig. 3; Höltje & Schwarz, 1985). The first stage takes place in cytoplasm and involves the synthesis of UDP-GlcNAc and UDP-MurNAc-pentapeptide. The peptide sequence of UDP-MurNAc-pentapeptide is L-alanyl-D-isoglutamyl-L-*meso*-diaminopimelyl-D-alanyl-D-alanine: it varies from the peptide shown in Fig. 2 by the presence of an extra C-terminal alanine residue.

The synthesis of UDP-GlcNAc is not relevant to this Dissertation and thus is not shown in Fig. 3. UDP-MurNAc is formed from UDP-GlcNAc in a two-step reaction involving the addition of phosphoenolpyruvate and its subsequent reduction. Phosphoenolpyruvate addition is irreversibly inhibited by an antibiotic phosphonomycin (Cassidy & Kahan, 1973). Next, UDP-MurNAc-pentapeptide is formed by the sequential addition of L-alanine, D-glutamic acid, *meso*-DAP, and D-alanyl-D-alanine in a number of ATP-dependent reactions catalyzed by specific ligases (Höltje & Schwarz, 1985). These reactions are also irrelevant to this Dissertation and will not be discussed. It must be noted, however, that all these enzymes have been identified and intensive studies are underway to investigate their mechanisms of action in order to create new antimicrobial agents (e.g., Bugg & Walsh, 1992; Matsuhashi *et al.*, 1994). D-cycloserine, a structural analogue of alanine, blocks the final stage of UDP-MurNAc synthesis by inhibiting the

conversion of L-alanine to D-alanine and the formation of D-alanyl-D-alanine (Lambert & Neuhaus, 1973).

The second and the third stages of PG synthesis are membrane-associated. In order to transport PG precursors from the cytoplasm to the periplasm they are covalently bound to a membrane glycosyl carrier lipid (GCL), also known as undecaprenol or bactoprenol (Umbreit & Strominger, 1972). UDP-MurNAc-pentapeptide transferase (the product of the *mraY* gene) catalyzes the formation of GCL-P-P-MurNAc-pentapeptide from the UDP-MurNAc-pentapeptide (Geis & Plapp, 1978; Ikeda *et al.*, 1991), whereas UDP-GlcNAc transferase (the product of the *murG* gene) catalyses transglycosylation of its substrate to GCL-P-P-MurNAc-pentapeptide to form a lipid-linked disaccharide-pentapeptide, the direct precursor of PG (Mengin-Lecreulx *et al.*, 1991; Ikeda *et al.*, 1992). Recent evidence suggests, that the product of *murG* gene is peripherally associated with the inner face of the cytoplasmic membrane, and, therefore, the direct precursor of PG is completely assembled before it traverses the cytoplasmic membrane (Bupp & van Heijenoort, 1993). The exact mechanism of its transport from the cytoplasmic to the periplasmic side of the inner membrane is, however, still unknown.

The last stage of PG biosynthesis is carried out in the periplasm. It has been demonstrated that the nascent PG is covalently linked to both PG sacculus and bactoprenol (Glauner & Höltje, 1990). Thus, PG precursors are cross-linked to the pre-existing PG almost immediately upon translocation. A number of penicillin-sensitive enzymes, also known as PBPs (for penicillin-binding proteins) take part in the terminal stages of PG biosynthesis.

IV. The Penicillin-Binding Proteins

At least eight distinct integral membrane PBPs were identified in *E. coli* using radiolabelled β-lactams (Spratt, 1977). Their genes have been cloned and sequenced. The PBPs function in cell wall elongation, bacterial shape determination, and septation. They are subdivided into the high and the low molecular weight (HMW and LMW) PBPs. The HMW-PBPs include PBP1A, PBP1B, PBP2, and PBP3, whereas the LMW-PBPs include PBP4-7. The PBPs are minor constituents of the cell membrane: their total

number was estimated to be about 3,000 molecules per cell in *E. coli* (Spratt, 1977).

The PBPs belong to the protein family of penicillin-interactive serine enzymes, which also includes the β-lactamases. Despite their different biological functions, these enzymes share certain features, such as the SXXK box (X stands for any amino acid) which contains the active site serine residue, and the S(Y)XN and the K(H/R)T(S)G motifs, (reviewed in Ghuysen, 1991). So far the crystal structure has not been solved for any of the *E. coli* PBPs; however, the three-dimensional data is available for a number of β-lactamases, as well as for some PBPs from gram-positive bacteria (e.g., Ghuysen, 1991; Strunadka *et al.*, 1992; Charlier *et al.*, 1993). The available data demonstrate, that the three repeating motifs come together to form an active site. The catalytic mechanism is thought to be similar to that of other active-site serine proteases (e.g., chymotrypsin), involving successive acylation and deacylation steps (Jamin *et al.*, 1993). The mechanism of inhibition of the PBPs by β-lactams is shown in Fig. 4. It is believed, that the β-lactam ring resembles acyl-D-alanyl-D-alanine and thus forms a stable covalent bond with the serine residue of the PBP active site (Tipper & Strominger, 1965).

The HMW-PBPs are integral transmembrane type II proteins and have a membrane-spanning segment at their N-termini (Broome-Smith *et al.*, 1985). The HMW-PBPs carry out two reactions: they elongate glycan chains (transglycosylation) and cross-link the peptide chains (transpeptidation). Transglycosylation creates a 1→4 glycosidic bond between the GlcNAc and MurNAc of two different disaccharide units. Transpeptidation reaction uses the energy of the D-alanyl-D-alanine bond of the donor pentapeptide to create a peptide bond between the position 4 of the donor peptide and DAP in position 3 of the acceptor peptide (Fig. 2). The D-alanyl-D-alanine bond in the donor peptide is broken to form the tetrapeptide. The acceptor peptide may be the tri, tetra, or pentapeptide (Höltje & Schwarz, 1985). As shown in Fig. 4, an intermediate step in the transpeptidation reaction catalyzed by the HMW-PBPs involves the formation of an ester bond between the active site serine residue and the acyl moiety of D-alanine residue of the donor pentapeptide.

PBP1A is a 94 KDa protein encoded by the *ponA* (*mrcA*) gene (Broome-Smith *et al.*, 1985). Purified PBP1A possesses both the transpeptidase and transglycosylase

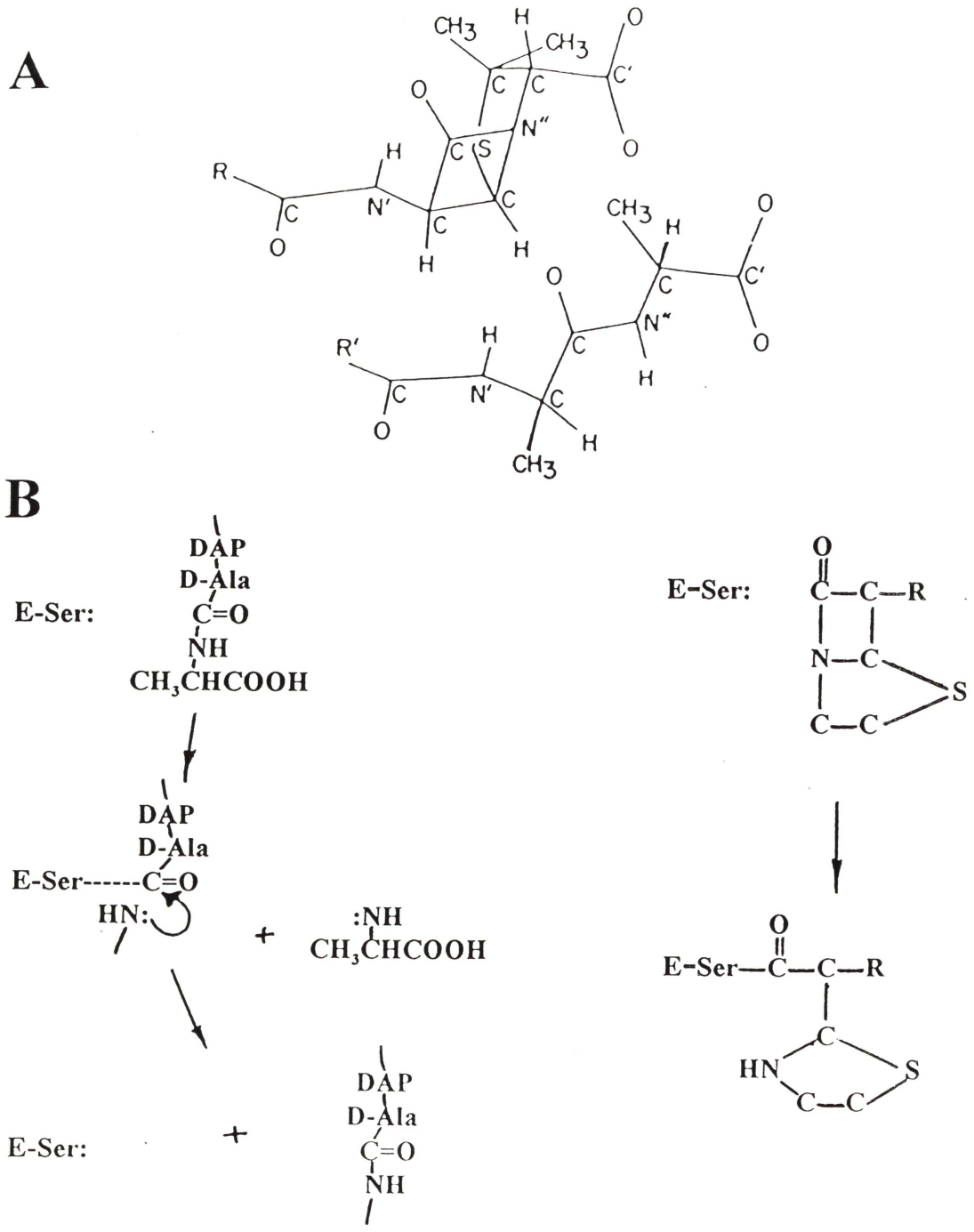

Figure 4. Transpeptidation reaction and its inhibition by β-lactam antibiotics. **A**, the resemblance between β-lactams (above) and D-alanyl-D-alanine (below). **B**, transpeptidation reaction (left) and its inhibition by a β-lactam (right). Adapted from Tipper & Strominger (1965) .

activities and catalyzes the formation of a highly cross-linked PG from the lipid-linked disaccharide precursor (Tomioka *et al.*, 1982). Importantly, in the presence of a low concentration of β-lactams, PBP1A produced uncrosslinked PG strand and the transpeptidase activity correlated with the binding affinity of this protein for β-lactams (Tomioka *et al.*, 1982). The penicillin-binding residue is Ser-465 in the C-terminal domain (Broome-Smith *et al.*, 1985). The transglycosylase activities of PBP1s is inhibited by the antibiotic moenomycin, but normally not by β-lactams (van Heijenoort *et al.*, 1978). However, the transglycosylase activity of PBP1A was also sensitive to some β-lactams (Tomioka *et al.*, 1982). The basis for this phenomenon is not understood. PBP1A is thought to be involved in cell elongation; however, the absence of PBP1A is not lethal for cells, presumably, due to overlapping functions of PBP1A and PBP1B (Spratt *et al.*, 1977).

PBP1B is encoded by the *ponB* (*mrcB*) gene. This gene has an open reading frame with two different start codons and a common terminator codon (Broome-Smith *et al.*, 1985). The larger frame encodes the 94 KDa PBP1Bα and the smaller frame encodes the 89 KDa PBP1Bγ. Ser-570 of the α-peptide is the penicillin-binding residue (Nicholas *et al.*, 1985). Purified PBP1Bs possess both penicillin-sensitive transpeptidase activity and moenomycin-sensitive transglycosylase activities (Nakagawa *et al.*, 1984). PBP1Bs are important for cell wall elongation: a mutation in *ponB* gene caused thermosensitive lysis in a low salt medium (Tamaki *et al.*, 1977). Deletion of both PBP1A and PBP1B is lethal to the cell (Yousif *et al.*, 1985).

The 71 KDa PBP2 is the product of the *pbpA* (*mrdA*) gene (Asoh *et al.*, 1986). It requires another integral membrane protein, RodA, for its transpeptidase activity *in vitro* (Ishino *et al.*, 1982). Initially, the transglycosylase activity has also been identified in the PBP2/RodA preparations (Ishino *et al.*, 1982). The more resent data, however, suggest, that PBP2 may only possess the transpeptidase activity (Höltje, 1995). PBP2 is the only PBP that binds the amidinopenicillin, mecillinam, and this results in the formation of spherical cells and a block in cell division. PBP2 mutants also grow as spherical cells that can no longer divide, unless the production of the stress nucleotide ppGpp is induced (Joseleau-Petit *et al.*, 1994). The PBP2/RodA complex is thus thought to function in an early step of cell elongation, possibly in the production of an initiation

PG piece in the growth zone which would later be extended by the PBP1 proteins (Spratt, 1975). A suppressor mutation of a thermosensitive PBP1B mutant led to a simultaneous overproduction of PBP1A and PBP2, suggesting a correlative function of these two enzymes (Tamaki *et al.*, 1977). The penicillin-binding residue is Ser-330 in the C-terminal domain (Takasuga *et al.*, 1988).

Mutations in the *pbpB* (*ftsI*) gene encoding the 64 KDa PBP3 cause filamentation of cells, suggesting the involvement of PBP3 in septum formation (Spratt, 1975; Nakamura *et al.*, 1983). Purified PBP3 posesses transpeptidase activity (Höltje, 1995). Ser-307 was identified as the penicillin-binding residue (Nicholas *et al.*, 1985). A deletion of a cluster of genes involved in cell division, *mreBCD*, lead to overproduction of PBP1B and PBP3 implying a joint control over their production and a possible interaction between these proteins (Wachi *et al.*, 1989). It was suggested that PBP3 might require a recently identified membrane protein that is involved in cell division, FtsW, for its proper functioning in septum formation on the basis of sequence homology between RodA and FtsW proteins (Ikeda *et al.*, 1989). Other studies suggested possible interactions between PBP3 and other proteins involved in cell division, FtsA (Tormo *et al.*, 1986) and FtsZ (Ayala *et al.*, 1988).

The LMW-PBPs possess β-lactam-sensitive DD-peptidase activities and are involved in secondary cross-linking and PG modification. Thus, they are often classified as PG hydrolases (Fig. 5; Höltje & Tuomanen, 1991). The LMW-PBPs have a cleavable signal peptide that transports them to periplasm, where they associate with the inner membrane by means of their C-terminal peptide segment (Joris *et al.*, 1988). This segment (about 20 amino acid residues) folds into an amphiphilic α-helix, which interacts with the membrane (reviewed in Gittins *et al.*, 1994). Theoretical calculations show that the whole helix can not be inserted into the phospholipid bilayer unless it forms a complex with other membrane proteins (Gittins *et al.*, 1994). Recent evidence suggests that electrostatic interactions between the amphiphilic helix and the charged head groups of the acidic phospholipids are not critical for the proper contact (Harris *et al.*, 1995), supporting the hypothesis of possible protein-protein contacts as the means of anchoring of the LMW-PBPs to the membrane.

The PBPs 4, 5, and 6 are products of the *dacB*, *dacA*, and *dacC* genes,

respectively. Mutations in any one of these genes are not lethal to the cells (Matsuhashi *et al.*, 1994). The LMW-PBPs are thought to function in maturation and reorganization of PG. PBP4 (49 KDa) has an endopeptidase, as well as DD-carboxypeptidase activity (Korat & Keck, 1988; Korat *et al.*, 1991). The 42 KDa PBP5 and 40 KDa PBP6 were originally characterized as DD-alanine carboxypeptidases (Ananuma & Strominger, 1980), the activity of PBP6 being about fourfold lower than that of PBP5. It was suggested that PBP5 and PBP6 were involved in septation creating tripeptide chains in old PG layer that are known to be the preferential acceptors of transpeptidation reaction catalyzed by PBP3 (Begg *et al.*, 1990). A recent study, however, failed to detect the DD-carboxypeptidase activity in purified preparation of PBP6 using a number of substrates that were readily hydrolysed by PBP5 (van der Linden *et al.*, 1992). Since the levels of PBP6 (but not PBP5) increase during the stationary phase (Buchanan & Sowell, 1982), it was suggested that PBP5 and PBP6 might act on slightly different substrates, PBP5 being the predominant DD-carboxypeptidase during bacterial growth and PBP6 being active in the stationary-phase bacteria at a time when no new PG side chains are present (van der Linden *et al.*, 1992).

The gene for PBP7 (*pbpG*) has recently been identified (Henderson *et al.*, 1995). The role of PBP7 in cell division is currently under investigation. It functions *in vitro* as DD-endopeptidase, which only accepts insoluble high-molecular-mass PG sacculi as a substrate, but fails to react with soluble low-molecular-mass PG subunits (Romeis & Höltje, 1994a). Inactivation of the *pbpG* gene is not lethal for cells (Henderson *et al.*, 1995). The 32 KDa PBP7 is proteolytically cleaved by the OmpT protease when cell membranes are disrupted yielding an artificial 29 KDa penicillin-binding fragment that is referred to as PBP8 (Henderson *et al.*, 1994). Importantly, PBP7 preferentially binds imipenem, a highly potent β-lactam that can reportedly lyse nongrowing cells (Tuomanen & Schwarz, 1987).

It has been demonstrated that not all PBPs present in the cell may be involved in active PG synthesis. Tuomanen (1986) presented evidence that only a minor fraction of newly made PBPs (less than 10% of their total number) were necessary to support normal growth and division in *E. coli*. Older PBPs were dispensable for cell wall growth, but still retained their capacity to bind β-lactams. It was thus suggested that the newly made

enzymes appeared in the growth zones and later moved out of them, becoming disconnected from new cell wall synthesis and the autolytic pathway (Tuomanen, 1986). These old PBPs may still retain the function of secondary PG modification, but this function would clearly be non-essential for the cell survival. Since these old PBPs retain the capability to bind penicillin, they could act as a "β-lactam sink", protecting the active PBPs which may be less accessible for the antibiotic. The lethality of β-lactam antibiotics for bacteria may thus be seen at the level of the acylation of newly synthesized PBPs that are responsible for the ongoing PG synthesis (Tuomanen, 1986).

In summary, the final stages in PG biosynthesis are carried out by a group of inner membrane or PG-associated PBPs. Different PBPs are involved in the initiation of PG elongation, elongation itself, and in septation. Only a minor fraction of PBPs may act in ongoing PG synthesis, leaving the rest of PBPs some role in secondary PG modification.

V. Penicillin-Insensitive PG Hydrolases

Expansion of the cell wall during cell growth, and septum formation during cell division, requires enzymes that cleave glycosidic and peptide bonds in PG (Weidel & Pelzer, 1964). These enzymes are constantly active in the cell, as up to 50% of PG per generation is recycled (Goodell, 1985). Four DD-peptidases are the LMW PBPs; however, there are also a number of PG hydrolases in *E. coli* that do not interact with β-lactams. Some PG hydrolases can cleave PG *in vitro* and are therefore termed autolysins. These autolysins represent potential suicide enzymes that are involved in lysis resulting from inhibition of PG synthesis, e.g., by treatment with β-lactam antibiotics.

A number of penicillin-insensitive PG hydrolases have been purified from *E. coli*. For most of them, the genes have been cloned (Höltje & Tuomanen, 1991; Engel *et al.*, 1992; Ursinus & Höltje, 1993). The target bonds cleaved by these enzymes are shown in Fig. 5.

The peptide moiety is cleaved from the PG disaccharide by the action of the 39 KDa periplasmic *N*-acetylmuramyl L-alanine amidase. The *E. coli* enzyme does not act on intact cell wall PG but uses disaccharide-pentapeptides as substrates (van Heijenoort

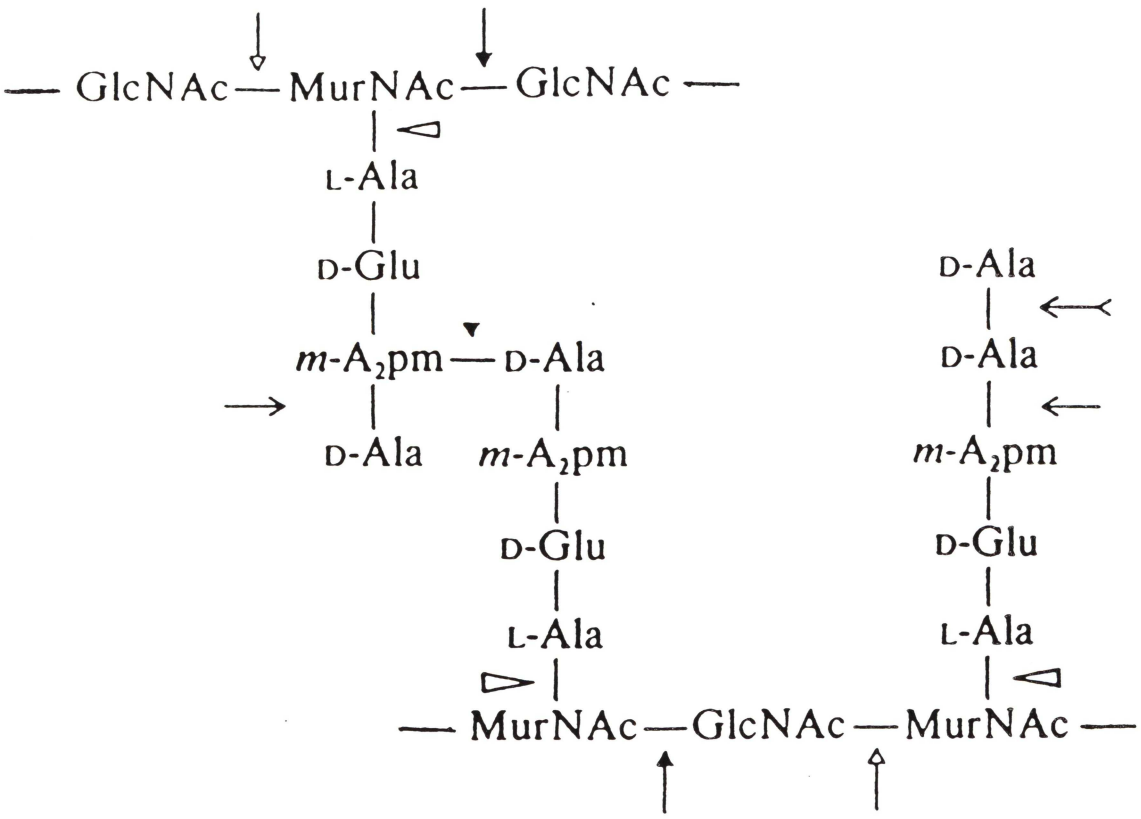

Figure 5. Cleavage of bonds in PG sacculus by different PG hydrolases. GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; *m*-A_2pm, *m*-diaminopimelic acid. Murein hydrolases: -▸, lytic transglycosylase; -▹, -*N*-acetylglucosaminidase; ▽, *N*-acetylmuramyl-L-alanine-amidase; ▾, D,D-endopeptidase; ⟩→, D,D-carboxypeptidase; →, L,D-carboxypeptidase. Adapted from Höltje & Tuomanen (1991).

et al., 1975). Its gene, *amiA*, has been mapped at 51 minutes on the *E. coli* chromosome (Tomioka *et al.*, 1983). Recently, another *N*-acetylmuramyl L-alanine amidase have been identified which takes part in the recycling of PG. This amidase is a product of the *ampD* gene (Lindberg *et al.*, 1987) and functions in the cytoplasm. Its substrates are 1,6-anhydro muropeptides which are produced in the periplasm by the action of lytic transglycosylases and transported to the cytoplasm to be recycled (Höltje *et al.*, 1994; Jacobs *et al.*, 1995).

The β-*N*-acetylglucosaminidase also does not have autolytic activities. This 36 KDa enzyme has been purified (Yem & Wu, 1976). Strains that carry mutations in this enzyme grow and divide normally, but no conclusion can be made with regard to their possible function because the mutations are leaky (Yem & Wu, 1976a; Höltje & Tuomanen, 1991).

Penicillin-insensitive soluble D,D-endopeptidase is the 30 KDa of the *mepA* gene (Keck *et al.*, 1990). MepA is an autolysin. Since there are a number of DAP-DAP cross-links in PG sacculus of *E. coli*, there is probably a specific L,D-endopeptidase, but it has not yet been identified. Also, three enzymes possessing L,D-carboxypeptidase activity have been purified and characterized in *E. coli* but their genes have not been yet cloned (Beck & Park, 1977; Metz *et al.*, 1986; Ursinus *et al.*, 1992).

Finally, three lytic transglycosylases have been identified in *E. coli*. All of these enzymes utilize PG as their substrates. All three are exo-enzymes that progressively degrade PG. The reaction mechanism of these enzymes is rather unusual: the 1→4 glycosidic bond cleavage is followed by an intramolecular transglycosylation yielding a 1,6-anhydro muramic acid (Höltje *et al.*, 1975). The physiological significance of this energy-conserving intramolecular transglycosylation is not yet understood. One soluble (Slt) and two membrane-bound (Mlt) lytic transglycosylases have been purified.

The structural gene for Slt70 (molecular weight, 70 KDa) has been cloned (*sltY*; Betzner & Keck, 1989). Slt70 is the predominant lytic transglycosylase of *E. coli*. It possesses a signal peptide for periplasmic export (Engel *et al.*, 1991). Interestingly, its 250-fold overexpression was achieved *in vivo* without lysing the overproducing cells (Engel *et al.*, 1991). The overproduction of Slt70, however, did not markedly changed its periplasmic concentration and a major fraction of the enzyme was found in the

cytoplasm, suggesting that its export to the periplasm was somehow regulated (Höltje & Tuomanen, 1991). Cells can grow normally when the *slt70* gene is deleted from the chromosome and its function is, therefore, uncertain. The enzyme has been crystallized: its structure resembles that of lysozyme (Thunnissen *et al.*, 1994).

One membrane-bound lytic transglycosylase activity was purified as a 38 KDa enzyme tightly associated with the membrane fraction (Ursinus & Höltje, 1994). Further characterization of Mlt38 should provide more insight into the mechanisms of PG biosynthesis and degradation. Its gene, *mltA*, has recently been identified (Höltje, 1995). The second membrane-bound lytic transglycosylase, MltB, the product of the *mltB* gene, is an outer membrane lipoprotein (Ehlert *et al.*, 1995; Dijkstra *et al.*, 1995). This enzyme becomes active after it is proteolytically degraded to a 35 kDa soluble protein. This 35 kDa protein has been initially purified and referred to as Slt35 (Engel *et al.*, 1992).

A number of unidentified PG hydrolases may also be present in the cell. A recent investigation of renatured *E. coli* inner membrane proteins after separating them on a polyacrylamide gel suggested that there may be as many as 17 autolytic enzymes (Bernadsky *et al.*, 1994). Although it is difficult to access the real number of autolysins (for example, some enzymes may be processed by proteases during the membrane purification step and create artifacts), the data of Bernadsky *et al.* (1994) have stimulated a search for more PG autolysins.

The idea of a complex between the PBPs and some PG hydrolases is quite attractive. According to the "three-for-one" PG growth model suggested by Höltje (1993), a multienzyme complex takes part in a coordinated insertion of three new PG strands at the expense of one old one. Such multienzyme complex should possess both synthetic and hydrolytic activities (Höltje, 1993). Recently, some evidence was obtained to support this model: *in vitro* studies revealed physical associations between PBP3, PBP7 and Slt70 (Romeis & Höltje, 1994). This complex was proposed to function in septation (Romeis & Höltje, 1994). Earlier cross-linking studies from the same group implied that there may also be a physical association between PBPs 1, 3, and 5 (Said & Höltje, 1983). It would be interesting to see, if other PBPs and PG hydrolases are able to interact with each other.

In summary, a number of penicillin-insensitive PG hydrolase activities have been

detected in *E. coli* and little is known about their function.

VI. Mechanism of Killing of *E. coli* Induced by Inhibitors of PG Synthesis

Inhibition of virtually any step of PG biosynthesis results in bacteriolysis and cell death. Thirty years ago, Weidel and Pelzer (1964) proposed that PG growth required a coordinated network of PG synthetases and PG hydrolases. The lethal action of β-lactam antibiotics is based on their abilities to deregulate or uncouple the activities of penicillin-insensitive PG hydrolases (referred to hereafter as PG hydrolases) in some way that is not understood (for reviews, see Höltje & Schwarz, 1985; Höltje & Tuomanen, 1991; and Tomasz, 1979). Non-β-lactams, such as D-cycloserine and phosphonomycin, inhibit the synthesis of key UDP-activated PG precursors (Lambert & Neuhaus, 1973; Cassidy & Kahan, 1973), which also uncouples PG hydrolases.

The first experimental evidence for the involvement of PG hydrolases in the cell death induced by cell wall-active agents came from the studies on an autolysis-defective mutant of *Streptococcus pneumoniae* (Tomasz *et al.*, 1970). Penicillin inhibited growth of this mutant, but the characteristic lysis and cell death did not occur. These results indicated that there were two distinct stages in penicillin-induced death: the first stage involved inhibition of the PBPs by penicillin; the second stage involved the uncoupling of PG hydrolases from their normal roles in PG synthesis. Therefore, the killing action of penicillin was dependent on PG hydrolase-mediated bacteriolysis (Tomasz *et al.*, 1970; Tomasz, 1979). The term "antibiotic tolerance" was coined to describe the phenotype of Tomasz's autolysis-defective mutant.

Antibiotic tolerance can now be subdivided into genotypic and phenotypic phenomena (Tuomanen *et al.*, 1986). By definition, genotypic tolerance is a property of bacterial mutants that can evade the killing effect of antibiotics but not their growth-inhibitory effect, and this is usually due to alteration of certain key enzymes that are involved, directly or indirectly, in bacteriolysis, like the mutant described by Tomasz *et al.* (1970). Growth of such mutants is still inhibited by the minimum inhibitory concentration of the drug, but no lysis is observed and the cell population loses its viability much slower than the control wild type population. Phenotypic tolerance, on

the other hand, is a property of wild type strains that is usually a response to changes in growth conditions, for example, a decrease in growth rate (Tuomanen *et al.*, 1986). Phenotypically tolerant bacteria may not be completely immune to the bactericidal effects of antibiotics: however, the loss of viability in such strains is greatly compromised in comparison to the normal growing cells. Phenotypic tolerance represents an important clinical problem.

Tuomanen *et al.* (1986a) grew *E. coli* cells in a chemostat to demonstrate that the rate of killing of bacteria by β-lactam antibiotics was proportional to the rate of bacterial growth. This has been suspected for a long time. For example, penicillin selection procedures designed by Lederberg and Zinder (1948) and Davis (1949) to enrich cultures for auxotrophic mutants are based on the ability of slow growing mutants to avoid killing by penicillin. It is now known, that the penicillin tolerance exhibited by amino acid-deprived *E. coli* can be attributed to the stringent response (Kusser and Ishiguro, 1985).

VII. The Stringent Response

Amino acid deprivation in *E. coli* results in the coordinate inhibition of a variety of metabolic activities which are not required by nongrowing bacteria. This phenomenon, known as the stringent response, probably represents a means of promoting survival of bacteria during periods of starvation (reviewed in Cashel & Rudd, 1987). The stringent response is thought to be mediated by guanosine 3',5'-bispyrophosphate (ppGpp), an unusual nucleotide that is rapidly accumulated in amino acid-deprived *E. coli*.

The synthesis of ppGpp in response to amino acid deprivation is catalyzed by the ribosome-associated product of the *relA* gene, ppGpp synthetase I (Fill & Friesen, 1968; Cochran & Byrne, 1974). Amino acid deprivation results in the accumulation of unacylated tRNA and in the codon-specified binding of this unacylated tRNA to the acceptor site of the ribosome. The subsequent ejection of the tRNA from this site is proposed to cause a conformational activation of RelA, and this results in the synthesis of a single molecule of ppGpp. Under the conditions of severe amino acid starvation, the stalled ribosomes undergo repeated rounds of unacylated tRNA binding and ejection,

and this results in a rapid increase in the intracellular ppGpp pools. Thus, the severity of the stringent response in amino acid-starved cells is determined by the ratio of acylated to unacylated tRNA. Strains that carry mutations in the *relA* gene are no longer capable of synthesizing ppGpp during amino acid starvation: such mutants are referred to as relaxed. RelA-dependent ppGpp synthesis can also be relaxed phenotypically by protein synthesis inhibitors, such as chloramphenicol (Gallant *et al.*, 1974).

When amino acid starvation is reversed, ppGpp is rapidly degraded by the action of *spoT*-encoded ppGpp hydrolase, a cytoplasmic enzyme (Laffler & Gallant, 1974; Heinemeyer *et al.*, 1978; Gentry & Cashel, 1995). It has been demonstrated recently that SpoT also possessed the ppGpp synthetase II activity (Hernandes & Bremer, 1991; Xiao *et al.*, 1991). A *spoT*-dependent accumulation of ppGpp is observed during carbon source limitations (Metzger *et al.*, 1989), during starvation for inorganic phosphate (Spira *et al.*, 1995), or during the inhibition of fatty acid synthesis (Seyfzadeh *et al.*, 1993). It has been proposed that the function of ppGpp synthetase II was to sense changes in growth rate and coordinately inhibit the energy-consuming processes (Hernandez & Bremer, 1991, Sarubbi *et al.*, 1988). It is not known what switches SpoT from the hydrolytic to the synthetic mode. A strain that carries deletions in both *relA* and *spoT* genes produces no detectible ppGpp levels (Xiao *et al.*, 1991), suggesting, that there is no third ppGpp synthetase.

It has been proposed that ppGpp mediates the global inhibition of metabolic processes not required by starving cells, including RNA and protein synthesis (Cashel & Rudd, 1987). Of particular interest is inhibition of PG synthesis (Ramey & Ishiguro, 1978) and phospholipid synthesis (Sokawa *et al.*, 1968; Heath *et al.*, 1994).

VIII. Phospholipid Synthesis

The predominant glycerophospholipids in *E. coli* (referred to hereafter as phospholipids) are phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin. The synthesis of phospholipids can be separated into two stages. First, long-chain fatty acids are synthesized in the cytoplasm by a group of soluble enzymes (reviewed in Magnuson *et al.*, 1993). The synthesis of phosphatidic acid and the subsequent steps are carried out

by membrane-associated enzymes (Fig. 6; reviewed in Cronan & Rock, 1987). This involves the acylation of glycerol-3 phosphate (G3P) to form phosphatidic acid which, in turn, is modified by the addition of a head group to yield mature phospholipids. The following aspects of phospholipid biosynthesis are relevant to this Dissertation.

The cytosolic G3P synthetase, encoded by the *gpsA* gene, reduces dihydroacetone phosphate to G3P (Cronan & Bell, 1974). This is the major enzyme involved in the synthesis of G3P in the absence of exogenous glycerol. Therefore, *gpsA* mutants require exogenous glycerol or G3P to support phospholipid synthesis and cell growth. When glycerol is present in the growth media, it can be phosphorylated by glycerol kinase to yield G3P (Lin, 1976). G3P synthetase is subject to feedback inhibition by G3P to ensure the tight regulation of intracellular G3P levels (Edgar & Bell, 1978).

The acylation of G3P at position 1 is catalyzed by 1-acyl-G3P-acyltransferase (PlsB), the product of the *plsB* gene (Bell, 1974). This is the first committed step in membrane phospholipid biosynthesis. Next, phosphatidic acid is formed by acylation of 1-acyl-G3P at the position 2. Phosphatidic acid is turned into CDP-diacylglycerol which is used to synthesize the final products. A *plsB* mutation has been described that resulted in an enzyme with a decreased affinity for G3P in an *in vitro* PlsB assay (Bell, 1974). Since G3P inhibits its own production, its intracellular levels are not sufficient to support phospholipid synthesis by their mutated PlsB. Thus, bacteria carrying this mutation require exogenous G3P or glycerol for growth, and glycerol deprivation results in a rapid inhibition of phospholipid synthesis (Bell, 1974).

Phosphatidylglycerol-3-phosphate (PG-3-P) synthetase, the product of *pgsA* gene, catalyses the conversion of CDP-diacylglycerol to PG-3-P. This is the first committed step in the biosynthesis of acidic phospholipids. The *pgsA* gene is absolutely essential for cell viability, indicating that acidic phospholipids are indispensable (Raetz & Dowhan, 1990). The concentration of acidic phospholipids in the membrane can, however, be genetically manipulated. Dowhan and colleagues (Heacock & Dowhan, 1989; Kusters *et al.*, 1991) reported a construction of a *pgsA* allele in which the gene is fused to the chromosomal copy of *lacOP*. The levels of *pgsA* expression in this strain can be regulated by the controlled addition of isopropyl-β-D-thiogalactoside (IPTG) to the medium, resulting in an up to 10-fold variation in the concentration of the membrane

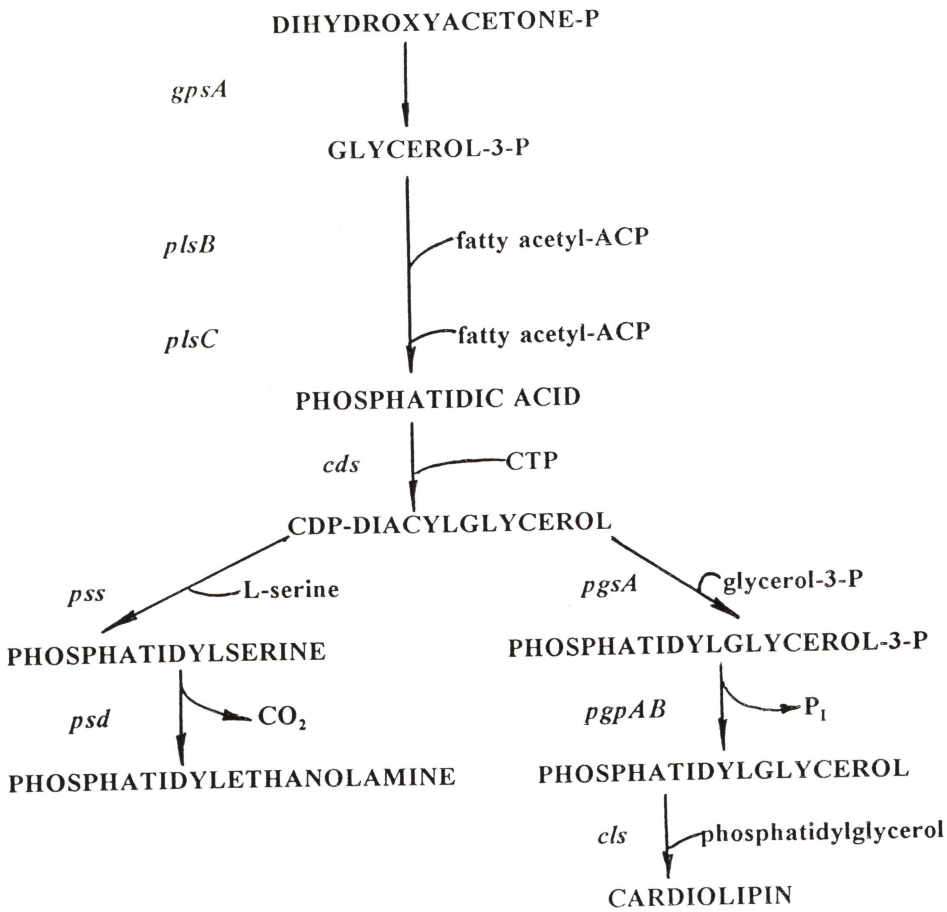

Figure 6. Biosynthesis of phospholipids in *E. coli* (grown on carbon source other than glycerol). *gpsA*, glycerol-3-P dehydrogenase (G3P synthetase); *plsB*, glycerol-3-P acyltransferase; *plsC*, 1-acyl-glycerol-3-P acyltransferase; *cds*, CDP-diglyceride synthase; *pgsA*, phosphatidylglycerol-3-P (PG-3-P) synthase; *pgpAB*, PG-3-P phosphatase; *cls*, cardiolipin synthase; *pss*, phosphatidylserine synthase; *psd*, phosphatidylserine decarboxylase. Adapted from Cronan and Rock (1987).

acidic phospholipids (Heacock & Dowhan, 1989).

Although earlier studies suggested that several enzymes involved in phospholipid synthesis were inhibited by ppGpp *in vitro* (reviewed in Cashel & Rudd, 1987), recent *in vivo* studies indicate that there is only a single key target (Heath *et al.*, 1994). Heath *et al.* (1994) have shown that the activity of PlsB is inhibited when intracellular concentrations of ppGpp are elevated. Both fatty acid and phospholipid syntheses were inhibited under these conditions. Furthermore, the overexpression of PlsB in the presence of high levels of ppGpp restored both fatty acid and phospholipid synthesis. It is notable that Heath *et al.* (1994) used growing cells carrying plasmids containing *relA* gene cloned under the control of *tac* promoter (Svitil *et al.*, 1992), which permitted them to quantitatively manipulate intracellular ppGpp levels by the addition of varying amounts of IPTG to the medium. Therefore, since amino acid starvation was not employed in those experiments, the inhibition of PlsB was very likely a direct consequence of ppGpp action and was not due to unrelated metabolic perturbation related to amino acid deprivation. The detailed mechanism of inhibition of PlsB by ppGpp remains to be determined.

IX. Regulation of PG Metabolism by the Stringent Response

The stringent response inhibits PG synthesis at two key sites. One site is an unidentified early step in the synthesis of UDP-MurNAc-pentapeptide (Ishiguro & Ramey, 1978). The second site corresponds to the terminal transpeptidation reaction itself (Ramey & Ishiguro, 1978). Thus, the stringent response effectively abolishes both the terminal stage of PG biosynthesis and unnecessary accumulation of PG precursors.

Several lines of evidence suggest that there might be a relationship between inhibition of phospholipid and PG syntheses in the course of stringent response in *E. coli*. In amino acid-deprived relaxed cells both phospholipid and PG continue at the same rate as in growing cells. When phospholipid synthesis was blocked in such cells by cerulenin treatment, PG synthesis was concomitantly inhibited (Ishiguro, 1983). The addition of exogenous fatty acids restored both phospholipid and PG syntheses in cerulenin-treated bacteria. When phospholipid synthesis was inhibited by glycerol deprivation of a *gpsA*

mutant, PG synthesis was also inhibited; both inhibitory effects were reversed upon restoration of glycerol supply (Ishiguro, 1983). Moreover, the experiments performed in ether-treated *E. coli* demonstrated that phospholipid synthesis was absolutely required for the transpeptidase and carboxypeptidase activities (Ishiguro, 1993).

As already noted, Tomasz *et al.* (1970) demonstrated that the bactericidal action of penicillin is a two step phenomenon involving inhibition of PBPs in the first step and the deregulation of the PG hydrolases in the second step. Pisabarro *et al.* (1990) have devised an experimental method, hereafter referred to as the two stage lysis procedure, which permits the distinction of these two steps in *E. coli*. To achieve the first step, referred to as the priming stage, bacteria were incubated with penicillin long enough to permit PBP-binding. The unbound penicillin was then removed. The induction of the stringent response at this time inhibited the second step, designated the lysis induction stage. The subsequent relaxation of the stringent response by chloramphenicol treatment, resulted in lysis induction. Therefore, priming was penicillin-dependent but lysis induction was not. This observation indicated that the activities of the PG hydrolases responsible for penicillin-induced cell death were negatively regulated by the stringent response. Further experiments by Pisabarro *et al.* (1990) demonstrated that the priming stage was also inhibited during the stringent response. Therefore, penicillin tolerance of amino acid-deprived *E. coli* may be attributable to the inhibition of either the PBP or the PG hydrolase activities.

The mechanism by which the stringent response regulates PG metabolism and causes penicillin tolerance is not known. There are at least two possibilities. First, it has been proposed that PG structure is altered during amino acid deprivation resulting in a form that is PG hydrolase-resistant (Goodell & Tomasz, 1980; Tuomanen & Tomasz, 1985; Tuomanen *et al.*, 1988). The second possibility is that ppGpp directly or indirectly inhibits the activities of PBPs and PG hydrolases. In this regard, the evidence indicating that the PBP biosynthetic activity was dependent on phospholipid synthesis has already been discussed. With respect to PG hydrolase activities, Betzner *et al.* (1990) demonstrated that the stringent response negatively controlled autolytic activity of overexpressed Slt70 *in vivo*. However, they found no evidence for a direct interaction between Slt70 and ppGpp and thus suggested that ppGpp must create some unfavourable

conditions that keep Slt70 inactive. Harkness *et al.* (1983) demonstrated the negative regulation of carboxypeptidase activity by the stringent response in ether-permeabilized *E. coli*. Ether-permeabilized cells retain normal cellular controls involved in regulation of PG synthesis but become permeable to the low molecular weight compounds such as ppGpp (Mirelman *et al.*, 1976). Due to this permeability a direct inhibitory effect of ppGpp on transpeptidase and carboxypeptidase activities was also ruled out.

X. Purpose of this Dissertation

Preliminary data from this laboratory (W. Kusser, unpublished) indicate that inhibition of phospholipid synthesis in amino acid-deprived cells resulted in penicillin tolerance, suggesting that phospholipid synthesis may be involved in the regulation of PG hydrolase activity. Since the stringent response independently regulates PBPs and PG hydrolases (Pisabarro *et al.*, 1990), it was further proposed that both the PBP and PG hydrolase activities may be regulated through the regulation of phospholipid synthesis (Ishiguro *et al.*, 1994).

The objectives of this Dissertation were to investigate the dependence of the PBP and PG hydrolase activities on the ongoing phospholipid synthesis using the two stage lysis procedure (Pisabarro *et al.*, 1990) and the proposed connection between ppGpp accumulation during the stringent response and inhibition of phospholipid and PG syntheses and, consequently, acquisition of penicillin tolerance by the amino acid-deprived cells. Another objective was to further characterize the two stage lysis procedure of Pisabarro *et al.* (1990) and determine the mode of action of a number of factors that interfere with the β-lactam-induced lysis, such as energization of the cytoplasmic membrane, growth temperature, and protein synthesis.

Chapter 2: Materials and Methods

I. Plasmids

Plasmids pALS10 and pALS14 (Amp^R) carrying the complete *relA* gene and its inactive truncated derivative, respectively, under the control of *tac* promoter were obtained from J. Zyskind (Svitil *et al.*, 1992). Plasmid pHP45Ω carrying the Ω interposon, which encodes spectinomycin and streptomycin resistance, was obtained from M. Krish (Prentki & Krish, 1984). Plasmids pRJ10 and pRJ12 (Kan^R) encoding the complete *plsB* gene and its inactive deletion derivative, respectively, were obtained from C. O. Rock (Heath *et al.*, 1994). Strains bearing pRJ10 overexpress *plsB* gene product 3 to 4-fold.

Since the experiments described in this Dissertation dealt with penicillin tolerance, it was necessary to inactivate β-lactamase gene encoded on pALS10 and pALS14. These plasmids contain a unique *PvuI* site within their β-lactamase gene. Both plasmids were restricted with *PvuI* and the cohesive ends were blunted by the action of Klenow enzyme. The Ω interposon was cut from pHP45Ω with *SmaI* and ligated into the blunted *PvuI* sites of pALS10 and pALS14. The ligation mixture was transformed into DH5α (*thi hsdR17 recA1 relA1 endA1 gyrA96* ϕ80d*lac* ZΔM15) competent cells by electroporation and Str^RSpc^R colonies selected. The nature of the plasmids from positive clones was verified by the restriction analysis and by ppGpp accumulation assay. Thus, plasmids pDR24 and pDR25 were created as $Str^RSpc^RAmp^S$ derivatives of pALS10 and pALS14, respectively.

II. Bacterial Strains

All *E. coli* strains used in this study were K-12 derivatives. Strain VC7 (*thi1 lysA23 rpsL109*) and its isogenic *relA2* derivative, VC8, were from the laboratory collection (Kusser & Ishiguro, 1987). Strain CF1693 (*ΔrelA251::kan ΔspoT207::cat*) was obtained from M. Cashel (Xiao *et al.*, 1991). Strain HDL11 (*pgsA30::kan Φ(lacOP-pgsA$^+$) lacY*::Tn*9 lacZ' recA srl*::Tn*10 lpp2*) was obtained from W. Dowhan (Kusters *et al.*, 1991). Strain VC58 was a *gpsA* derivative of VC8 constructed in this laboratory

(Ishiguro, unpublished). Strain BBfe2 (*plsB glpD glpk phoA)* was a derivative of BB26-36 (Bell, 1974) and was obtained from the late W. Nunn. Strains MF634 (*dnaJ259*) and GR756 (*dnaK*756) were obtained from the *E. coli* genetic stock centre.

Several strains were constructed in the course of this study. Strains VC891 and VC892 were derivatives of VC7 carrying plasmids pDR24 and pDR25, respectively. Strains VC7004 and VC7005 were derivatives of strain VC891 carrying plasmids pRJ10 and pRJ12, respectively. These strains were constructed by electroporation of plasmid DNA into competent recipient cells and further selection for the appropriate drug-resistant phenotype. The presence of the correct plasmids in each strain was verified by purifying the plasmid DNA and separating it by agarose gel electrophoresis (0.8% agarose gel).

Strain VC7000 (*ΔrelA*) was constructed by transducing the *ΔrelA 251::kan* allele from strain CF1693 into strain VC7. Strain VC7001 (*ΔrelA ΔspoT*) was constructed by transducing the *ΔspoT207::cat* allele from strain CF1693 into strain VC7000. Strains VC895 (*dnaJ259*) and VC896 (*dnaK756*) were constructed by transducing the *dnaJ259* from strain MF634 and *dnaK756* from strain GR756, respectively, into strain VC7. During the first step, a Tn*10*::*thr* insertion was transduced into strains MF634 and GR756, and during the second step, mutant *dnaJ* and *dnaK* genes linked to the Tn*10*::*thr* insertion were transduced into strain VC7. VC899 was a control strain created by transducing the Tn*10*::*thr* insertion into strain VC7. P1*vir*-mediated general transduction was performed according to Miller (1972).

III. Growth Conditions

For experiments involving bacteriolysis, bacteria were grown in M9 medium supplemented with the required growth factors (Miller, 1972). For the experiments involving strains VC7001, VC7004, and VC7005, 0.1% Casamino Acids (Difco Laboratories, Detroit, Mich.) were also added to the medium. The carbon source was normally 0.2% glucose except for some temperature upshift experiments and the experiments involving the addition of cerulenin, in which 0.4% glycerol was used (Goldberg *et al.*, 1973). Strains VC58 and BBfe2 were grown with 0.2% glucose and 0.05% glycerol to satisfy the glycerol requirement. Strain HDL11 was always grown in

Luria broth (LB, Difco Laboratories). In the experiments involving radiolabelling with ^{32}P, bacteria were grown in low-phosphate M56LP medium (Bell, 1973). For all the experiments involving strain construction or plasmid modification, bacteria were grown in tryptic soy broth (TSB, Difco Laboratories) and plated on its solidified version, containing 1% agar (TSA).

Cultures in liquid media were incubated in waterbath shakers at the indicated temperatures. When necessary, antibiotics were added to the media to select for the cells that contained a plasmid or a transposon insertion at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; tetracycline, 20 μg/ml; chloramphenicol, 50 μg/ml; streptomycin, 100 μg/ml; and spectinomycin, 20 μg/ml. Growth and lysis were monitored at 420 nm with a Beckman DU-64 spectrophotometer.

The stringent response was normally induced by adding L-valine to the medium at 500 μg/ml to achieve isoleucine deprivation. In the experiments involving strain HDL11, the stringent response was induced by the addition of serine hydroxylamate at 500 μg/ml. Expression of the *relA* or truncated *relA* genes on plasmids pDR24 and pDR25, respectively, was induced with IPTG. The stringent response was normally relaxed by the addition of chloramphenicol at 100 μg/ml, except in the strain HDL11, where gentamicin (50 μg/ml) had to be used due to the presence of Tn*9* transposon insertion.

Temperature sensitivity was determined by replica plating individual colonies on two TSA plates. One set of plates was incubated at 30^0C and the other at 42^0C. Putative temperature sensitive clones were grown and plating efficiency tests were performed. Serial dilutions of cultures in question were plated in triplicates on two sets of TSA plates which were incubated at 30^0C and at 42^0C. Plate counts were determined after 24 hours of incubation at the indicated temperature.

For the experiments involving glycerol deprivation of *gpsA* or *plsB* mutants glycerol dependence of cell growth was verified by plating about 10^5 viable cells on TSA plates with and without glycerol.

IV. Treatment with β-Lactam Antibiotics and Other Inhibitors

For the experiments involving bacterial lysis, β-lactam antibiotics were added at concentrations equal to approximately 10 times their minimum inhibitory concentrations (MICs). Cerulenin was used at 50 μg/ml. Other agents were added at the concentrations specified in the text.

MICs were determined after incubating VC7 cells (about 10^5 viable cells per ml) with serial dilution of reagents for 24 hours at 37^0C in M9 medium with all required growth supplements without shaking. MICs for the reagents used in this study were 5 μg/ml for ampicillin, 50 μg/ml for benzylpenicillin, 0.2 μg/ml for imipenem, 6 μg/ml for cephaloridine, 10 μg/ml for cefsulodine, 10 μg/ml for D-cycloserine, 15 μg/ml for phosphonomycin, 1.25 mM for sodium azide, 0.6 mM for potassium cyanide, 25 μM for cyanide *m*-chlorophenylhydrazone (CCCP), 0.125 μg/ml for norfloxacin, 2.5 μg/ml for rifampicin, and 10 μg/ml for cerulenin.

The dissociation of ampicillin-induced lysis into two stages in amino acid-deprived cells was achieved by the modified method of Pisabarro *et al.* (1990). Briefly, exponential phase cultures were starved for isoleucine and primed for 20 minutes with 200 μg/ml of ampicillin. The excess of unbound ampicillin was removed by a rapid centrifugation at 12,000 rpm. The primed cells were then resuspended in fresh isoleucine deprivation medium and the lysis induction was monitored as A_{420}. Priming of growing cells was also achieved by treating them with 200 μg/ml of ampicillin for the specified periods of time. After that, the unbound ampicillin was removed by centrifugation and the cells were resuspended in fresh growth medium.

Cell dry weights were determined turbidimetrically on the basis of a standard curve. Viable cell counts were determined by plating serial dilutions of culture samples in triplicate on TSA. The plates were counted after 16 hours of incubation at 37^0C.

V. Liquid Scintillation Counting

Radioactive samples were counted in Ready Flow III liquid scintillation cocktail (Beckman Instruments Inc., Mississauga, Ontario) with a Beckman LS 8100 liquid

scintillation counter.

VI. Assay of PG Synthesis

PG synthesis was assayed by measuring the incorporation of [2,6-^{3}H]-*m*-DAP into trichloroacetic acid (TCA)-insoluble fraction according to the procedure of Ishiguro and Ramey (1976). Briefly, cultures were grown in M9 for at least three doublings to a density of 4 x 10^{8} cells per ml. At this point, [2,6-^{3}H]-*m*-DAP was added to a final concentration of 0.2 μg/ml (10 μCi/ml). The culture was incubated in the presence of the label for 20 minutes before the zero point measurement. At the specified times, 100 μl samples of the cultures were applied to Whatmann 3MM filter paper disks. The disks were immersed in ice-cold 5% TCA for 30 minutes, and washed once in ice-cold 5% TCA and twice in ice-cold 95% ethanol. Disks were dried and subjected to liquid scintillation counting.

VII. Assay of Stable RNA Synthesis

Stable RNA synthesis was assayed by the procedure identical to that described for PG synthesis. RNA was labelled with [^{14}C]-uracil (final concentration, 1μg/ml, 0.5 μCi/ml).

VIII. Assay of Phospholipid Synthesis

Bacteria were grown for three doublings in M56LP low-phosphate medium to a density of 2 x 10^{8} cells per ml. At this point $^{32}P_{i}$ (5 μCi/ml) was added and cells were incubated for an additional 15 minutes prior to zero point measurement. At the designated times, samples were removed and phospholipids were extracted with chloroform-methanol by the method of Bligh and Dyer (1959). Samples (0.8 ml) were mixed with 3 ml of chloroform-methanol (1:2) and incubated on ice for 10 minutes. Then, 1 ml of chloroform was added to the mixture, followed by 1 ml of ice-cold water. The phases were mixed thoroughly and separated by centrifuging at 400 rpm for 10 minutes. The amounts of radioactivity in 200 μl aliquots of the chloroform fractions

were determined by liquid scintillation counting.

IX. Assay of Levels of Individual Phospholipids in Strain HDL11

HDL11 was grown in LB in the presence of various concentrations of IPTG for at least 8 generations as described by Heacock and Dowhan (1989). Phospholipids were labelled with $^{32}P_i$ (20 μCi/ml) for the last three generations and extracted as described in the previous section. The chloroform fractions was concentrated under a stream of nitrogen. Several nmol of phospholipid (about 15, 000 cpm) from each fraction were mixed with a mixture of *E. coli* phospholipid standards. The mixtures were applied to a silica gel G thin-layer chromatography plate (Eastman Kodak, Rochester, N.Y.) and the individual phospholipids were separated in chloroform-methanol-water (65:25:4) as described by Ames (1968). The positions of phospholipid standards were visualised by developing the plate in iodine vapours. Spots corresponding to the standards were scraped off the plate and the amounts of radioactivity in the spots were determined by liquid scintillation counting. The total amount of radioactivity in the spots corresponding to phosphatidylglycerol, phosphatidylethanolamine, and cardiolipin in each sample was assumed to be 100%.

X. Quantification of ppGpp

Intracellular levels of ppGpp were quantified according to a modified procedure of Bochner and Ames (1982). Cultures grown in M56LP low-phosphate medium were labelled with $^{32}P_i$ (40 μCi/ml) for at least three doublings prior to sampling. Samples (200 μl) of the cultures were extracted with 20 μl of 11M formic acid on ice for 30 minutes. The samples were centrifuged to remove cell debris and 10- to 20-μl aliquots of the extracts were applied to polyethyleneimine cellulose F thin-layer chromatography plates (Merk, Germany). Chromatograms were developed in 1.5 M KH_2PO_4. The separated nucleotides were visualized by autoradiography on Royal "X-Omat" film (Kodak). The ppGpp spots were scraped off and quantified by liquid scintillation counting. For each ppGpp sample, the radioactivity in an equal amount of material from

the area between the ppGpp and GTP spots was subtracted as a background value. The values reported were the means of at least three experiments. The values were expressed as pmol of ppGpp per mg of dry cell weight. Dry cell weight was determined turbidimetrically on the basis of a standard curve.

XI. Determination of Proton-Motive Force

Intracellular volume was determined by the centrifugation method of Rottenberg (1979) with $^{3}H_2O$ (5 μCi/ml) as the permeant and [^{14}C]-sucrose (2 μCi/ml, 5 μM) as the nonpermeant. After 2 minutes of incubation with intensive stirring at room temperature, triplicate 1 ml samples of concentrated cell suspensions (4 x 10^9 cells per ml, in M9 medium) were centrifuged for 1 minute and 100 μl aliquots of supernatant and all of the pellet (resuspended in 100 μl of M9) were subjected to liquid scintillation counting simultaneously for [^{3}H] and [^{14}C] windows. The amount of [^{14}C] spillover into the [^{3}H] window was 11%.

ΔpH across the inner membrane was determined according to Rottenberg (1979). Concentrated cell suspension (4 x 10^9 cells per ml, in M9 medium) was incubated with [^{14}C]-benzoic acid (2 μCi/ml, 5 μM) and $^{3}H_2O$ (5 μCi/ml) for 15 minutes at room temperature with intensive stirring. Triplicate samples (1 ml) were centrifuged for 30 seconds, and 100 μl aliquots of supernatant and all of the pellet (resuspended in 100 μl of M9) were counted.

Membrane potential ($\Delta\psi$) was determined as described by Marino *et al.* (1985). Briefly, concentrated cell suspensions (2 x 10^9 cell per ml) were incubated with 2 μCi/4 μM of [^{3}H]-tetraphenylphosphonium^{+} (TPP^{+}) for 15 minutes with stirring at room temperature in the Tris/Mes/choline/KCl buffer (5 mM Tris, 5 mM 4-morpholino-ethanesulphonic acid (Mes), 150 mM choline chloride, and 0.2 mM KCl), pH 7.0. Triplicate 200 μl samples were removed and rapidly filtered on Millipore filters (type HA 0.45 μm) pre-soaked in TPP^{+}. Filters were washed once with 5 ml of buffer, dried, and counted. Formaldehyde- and CCCP-treated cells were used to determine nonspecific binding. The concentration of TPP in the supernatant was determined by centrifuging parallel samples at the same time and counting 100 μl of the supernatant. Membrane

potential was determined on the basis of the Nernst equation. Proton motive force (Δp) was determined on the basis of the following equation: Δp (mV) = $\Delta\psi$ - 59ΔpH (Rottenberg, 1979).

XII. Labelling of the PBPs with [^{3}H]-Benzylpenicillin

The method of Broome-Smith and Spratt (1982) was used to assay PBP binding in whole cells. Bacteria were grown to the density of 4 x 10^8 cells per ml and incubated with various agents (as indicated in the text) for 20 minutes. After that, duplicate 1 ml samples were removed and the cells were pelleted by brief centrifugation (all samples were adjusted to the same cell density). Pellets were resuspended in 20 µl of 50 mM sodium phosphate buffer (pH 7) and subjected to two freeze-thaw cycles. Each sample was exposed to 5 µg/ml of [^{3}H]-benzylpenicillin (final concentration, 150 µCi/ml) and to the same agents as before pelleting. Cells were incubated at 37^{0}C for 15 minutes. The reaction was stopped by the addition of 50 µg/ml of unlabelled benzylpenicillin. To each sample 25 µl of 20% sodium lauroyl sarcosinate was added and the samples were incubated for an additional 10 minutes at 37^{0}C to solubilize the inner membrane. The samples were then boiled for 5 minutes in electrophoresis loading buffer consisting of 2.5% glycerol, 5% β-mercaptoethanol, and 1% sodium dodecyl sulfate (SDS) in 1 M Tris (pH 6.8). Proteins were separated by electrophoresis on SDS-polyacrylamide gels (6% stacking gel and 11% separating gel).

To achieve better sensitivity for X-ray film detection, gels were treated with 2,5-diphenyloxazol (PPO) by the method of Bonner and Laskey (1974). Briefly, gels were soaked in dimethyl sulfoxide (DMSO) for 1 hour followed by 3 hour exposure to 22% PPO in DMSO (w/v). Gels were then washed in water for 1 hour and dried under vacuum. The bands corresponding to PBPs that bound [^{3}H]-benzylpenicillin were visualised by autoradiography of Royal "X-Omat" film (Kodak) at -70^{0}C for one month. Band intensities were compared with a Molecular Dynamics personal densitometer (Canberra Packard, Canada).

XIII. Labelling of the PBPs with Biotinylated Ampicillin

Biotinylation of ampicillin and labelling of PBPs was carried out according to a modified procedure of Dargis and Malouin (1994). Briefly, an *N*-hydroxysuccinimide ester of biotin (NHS-LC-biotin, Pierce Chemical, Rockford, Ill.) was linked to the primary amine group of ampicillin. Ampicillin was incubated with fivefold molar excess of NHS-LC-biotin in an Eppendorf tube. The reaction was performed in 0.1 M sodium phosphate buffer (pH 7.2) with continuous gentle agitation for 30 minutes at room temperature. The reaction was stopped by addition of an appropriate amount of Affi-Gel 102 (Bio-Rad Laboratories, Richmond, Calif.) and an additional 30 minute of incubation. Biotinylated ampicillin (BIO-AMP) was separated from the unreacted immobilised ligand by centrifugation in an Eppendorf centrifuge. BIO-AMP was always freshly prepared for use.

Growing bacteria were treated with the various agents (as indicated in the text) for 20 to 30 minutes and exposed to 0.5 µg/ml of BIO-AMP for 20 minutes in 37^{0}C waterbath shaker. Labelled cultures were adjusted to the same cell density and duplicate or triplicate samples (1 ml) were spun down in an Eppendorf centrifuge to pellet the cells. Pellets were resuspended in 20 µl of 50 mM sodium phosphate buffer (pH 7) and incubated with 25 µl of 20% sodium lauroyl sarcosinate for 10 minutes at 37^{0}C prior to boiling in electrophoresis loading buffer (see previous section). Proteins were separated by electrophoresis on SDS-polyacrylamide gels (6% stacking layer, 11% separating layer).

After electrophoresis, proteins were transferred to a Trans-Blot nitrocellulose membrane (Bio-Rad Laboratories) for 30 minutes at 100 V in the Bio-Rad Mini-PROTEAN II transfer cell (Bio-Rad Laboratories) in transfer buffer (20 mM Tris, 192 mM glycine (pH 8.3), 4% methanol (v/v). After blotting, the membrane was incubated overnight in blocking solution (10% calf serum, 1% BSA, 0.5% Tween-20 in TTBS (pH 7.4) at 4^{0}C. TTBS contained 0.1% Tween-20 in 20 mM Tris-137 mM NaCl. The next day the membrane was rinsed four times (5 minutes each) at room temperature in TTBS and incubated for an hour in a streptavidine-peroxidase conjugate (Boehringer Mannheim Canada, Dorval, Quebec; 1:10,000 dilution, 0.05 U/ml in TTBS (pH 7.4) containing 10% calf serum, 10 % glycerol, 0.5% Tween-20, and 1 M D-glucose) with shaking. The

unbound conjugate was removed by several washes in TTBS: 15 minutes at 60°C, 15 minutes at 37°C, 5 minutes at 37°C, and three 5 minute washes at room temperature.

The peroxidase-streptavidine-BIO-AMP-PBP complexes were detected with Renaissance Western blotting detection reagents (DuPont). The membrane was flooded with substrates for 1 minute at room temperature and the excess reagent was drained. The membrane was covered with Saran Wrap and immediately exposed to a Reflection hyperfilm (DuPont) for 10 seconds to 2 minutes before film development. Band intensities were quantified with a Molecular Dynamics personal densitometer (Canberra Packard).

XIV. Determination of Intracellular ATP Levels

ATP pools were measured using a luciferase assay by a modified procedure of Cole *et al.* (1967). Samples (1 ml) were added to 0.25 ml of 30% $HClO_3$ (w/v) and incubated on ice for 10 minutes. After incubation, the extract was shaken and neutralized with 1.5 ml of 1 M KOH and the precipitate was allowed to settle. ATP assay was carried out immediately after the neutralization step. Luciferin and luciferase were from Boehringer Manheim and were used according to specifications of the manufacturer. The luciferin-luciferase reagent (3.5 mM of luciferin and 140,000 units per ml of luciferase in 20 mM Tris pH 7.75, containing 0.2 mM EDTA, 0.05 dithiothretiol, and 0.5% BSA) was prepared the day before the assay and kept in the dark at room temperature. 0.95 ml of the reagent was pipetted into a clean plastic tube and the background was determined. Background did not exceed 10^4 counts per minute (sample counts were in the order of 10^8 cpm). 50 µl of the sample extract was added to the 0.95 ml of the reagent and the counted immediately in a Beckman LS 8100 liquid scintillation counter (coincidence off). The amount of counts in the first 10 seconds was proportional to the concentration of ATP in the sample.

XV. Materials

Restriction endonucleases, T4 DNA ligase, and IPTG were from Boehringer

Mannheim Canada (Laval, Quebec). [^{32}P]-orthophosphate (carrier free, 10 mCi per ml) was obtained from Amersham Canada (Oakville, Ontario). [^{3}H]-DAP (30 Ci per mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). All other materials (unless specified in the text) were obtained from Sigma Chemical Co. (St. Louis, MO) except norfloxacin which was provided by Merck Sharp & Dohme Research Laboratories (West Point, PA), and moenomycin and imipenem which were gifts from Hoechst Aktiengesellshaft (Frankfurt am Main, Germany) and Merk Sharp & Dohme (Rahway, N. J.), respectively.

Chapter 3: Phospholipid Synthesis and β-Lactam-Induced Lysis in *E. coli*

I. Overview of Chapter Contents

The results presented in this Chapter lead to the following conclusions. (i) Ongoing phospholipid synthesis is required for lysis of amino acid-deprived bacteria. (ii) There is a direct correlation between ppGpp accumulation and the inhibition of phospholipid synthesis and PG metabolism and the acquisition of penicillin tolerance by *E. coli*. (iii) Inhibition of phospholipid synthesis confers penicillin tolerance in growing bacteria. (iv) Changes in the levels of acidic phospholipids in bacterial membranes do not affect PG synthesis or β-lactam-induced lysis, suggesting that these phenomena are not controlled at the level of specific groups of individual phospholipids.

II. β-Lactam-Induced Lysis of Amino Acid-Deprived *E. coli* Requires Phospholipid Synthesis

The role of phospholipid synthesis in β-lactam-induced lysis of amino acid-deprived *E. coli* was investigated. Isoleucine-deprived cultures of strains VC7 (*relA*$^+$) and VC8 (*relA*) were treated with ampicillin. As shown in Fig. 7A, 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. 7B, 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. 7A, 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. 7B. In both strains, the inhibitory effect of cerulenin on lysis was antagonized by the addition of exogenous oleate and palmitate (curves d and c in Figs. 7A and 7B, respectively). Similar results were obtained when viable cell counts were monitored instead of optical density (not shown).

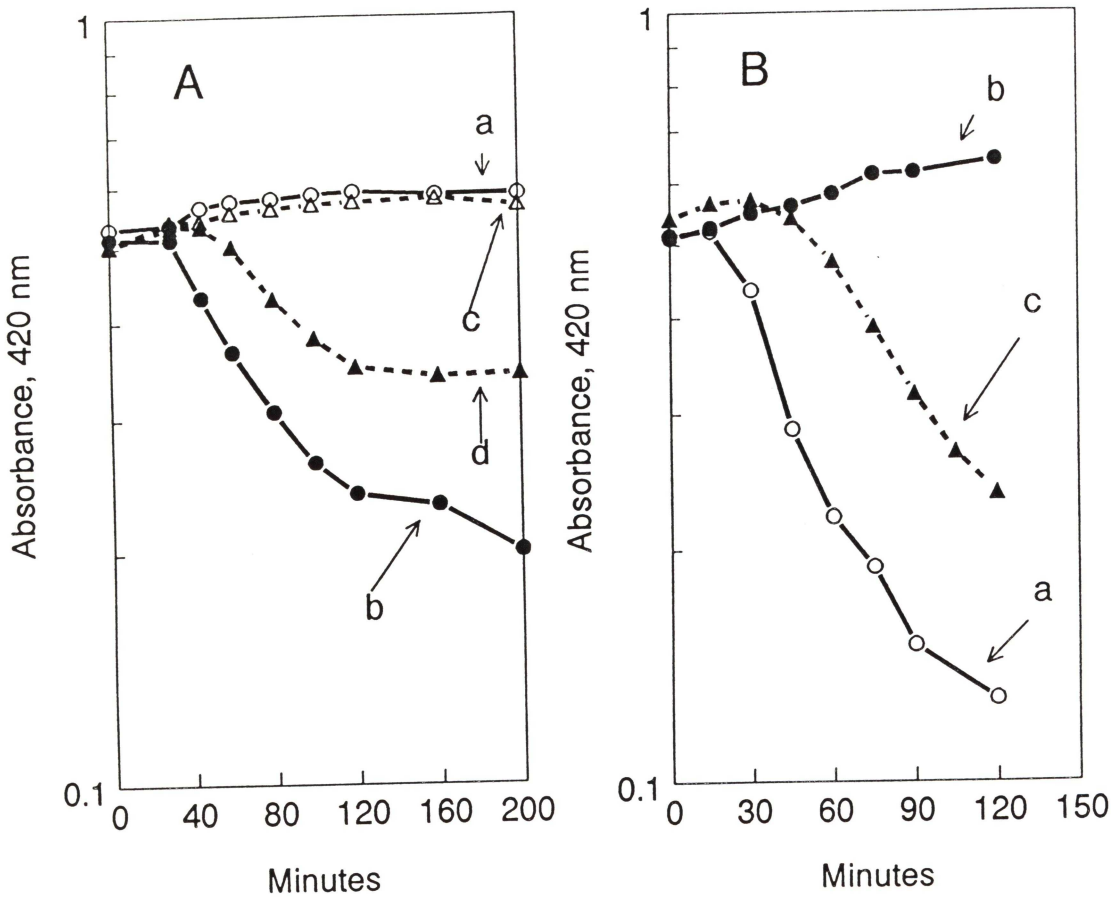

Figure 7. Inhibition of ampicillin-induced lysis of amino acid-deprived bacteria by cerulenin. **Panel A.** A series of exponential phase cultures of strain VC7 were subjected to isoleucine deprivation at 0 minutes. Cultures b-d also received 100 μg/ml of chloramphenicol at 0 minutes to relax the stringent response. In addition to chloramphenicol, cultures c and d received cerulenin (50 μg/ml) and cerulenin (50 μg/ml) combined with a mixture of oleate and palmitate (100 μg/ml of each), respectively. All cultures were treated with ampicillin (50 μg/ml) starting at 5 minutes. **Panel B.** A series of exponential phase cultures of strain VC8 (*relA*) were subjected to isoleucine deprivation at 0 minutes. Cultures b and c received cerulenin (50 μg/ml) alone and cerulenin (50 μg/ml) combined with a mixture of oleate and palmitate (100 μg/ml each), respectively. At 5 minutes, all cultures were treated with ampicillin (50 μg/ml).

Cerulenin blocks fatty acid synthesis by inhibiting 3-ketoacyl-acyl-carrier protein synthetases I and II (D'Angolo *et al.*, 1973; Vance *et al.* 1973). Therefore, the inhibitory effects of cerulenin on lysis shown in Figs. 7A and 7B 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 where a direct block in phospholipid synthesis was imposed. For this purpose, strain VC58, a derivative of VC8 (*relA*) with a mutation in *gpsA*, was used. Strain VC58, therefore, requires glycerol or G3P for growth and phospholipid synthesis, and phospholipid synthesis is blocked 30-40 minutes after the onset of glycerol deprivation (Ishiguro, 1983). Fig. 8 shows the effect of ampicillin on cultures of VC58 which were subjected to isoleucine starvation in the presence and in the absence of glycerol. Isoleucine-starved cells which were also deprived of glycerol were lysis-tolerant (curve a). However, ampicillin-induced lysis occurred when the glycerol requirement was satisfied (curve b). Similar results were obtained with isoleucine-deprived cultures of strain BBfe2 (*plsB*) which also depends upon the exogenous glycerol for phospholipid synthesis and growth (not shown); in this case, chloramphenicol was added at the beginning of amino acid deprivation to relax the stringent response. These results indicate that the ampicillin-induced lysis of amino acid-deprived *E. coli* was dependent on ongoing phospholipid synthesis.

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 (not shown). 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 (not shown). In these experiments, all agents were used at concentrations equal to 10 times their minimum inhibitory concentrations. Therefore, it would appear that phospholipid synthesis is a general requirement for lysis induced by any antimicrobial agent which interferes with PG synthesis.

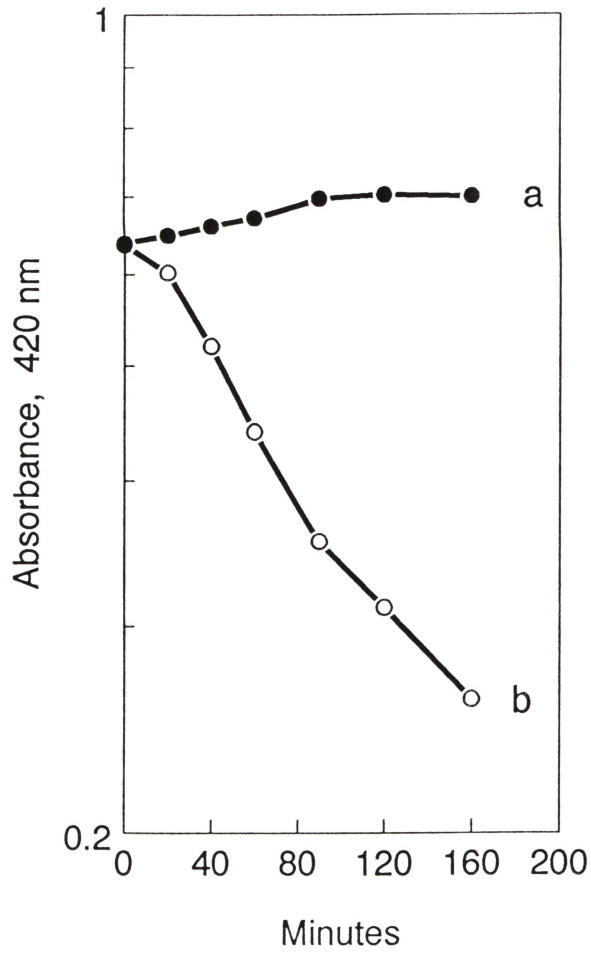

Figure 8. Inhibitory effect of glycerol deprivation on ampicillin-induced lysis of amino-acid-deprived strain VC58 (*relA gpsA*). An exponential phase culture of strain VC58 was deprived of glycerol at -40 minutes. At 0 minutes, the culture was starved for isoleucine and divided into two parts. Both parts were treated with ampicillin (50 μg/ml) in the absence (a) or presence (b) of exogenous glycerol.

II. 1. Dependence of the Priming Stage on Phospholipid Synthesis

As noted above, 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 (Pisabarro *et al.*, 1990). Fig. 9 describes 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 containing a mixture of oleate and palmitate 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. 9A. 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 divided into three cultures. Lysis was not observed in cells which did not receive further treatment (not shown). Furthermore, attempts to induce lysis of these cells by chloramphenicol treatment were unsuccessful (curve c). However, such cells were still able to lyse upon treatment with a combination of chloramphenicol and ampicillin, demonstrating that the presence of cerulenin did not irreversibly block the lysis induction (curve d). Importantly, cells became prone to lysis by the combination of chloramphenicol and ampicillin in the absence of net protein synthesis. Similar results were obtained when the lysis induction was monitored with viable cell counts (not shown).

In the second experiment to test whether phospholipid synthesis was required for the priming stage, strain VC8 (*relA*) was used. In this case, three parallel isoleucine-deprived cultures were prepared and treated with ampicillin either in 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 minutes, the cells in all three cultures were washed and resuspended in fresh isoleucine starvation medium. As shown in Fig. 9B, 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). Therefore, cerulenin treatment inhibited the ampicillin priming stage.

An attempt was made to directly demonstrate the dependence of PBP activity on phospholipid synthesis. The PBPs of amino acid-deprived relaxed cells of strain VC7 were labelled with either [^{3}H]-benzylpenicillin (Broome-Smith & Spratt 1982) or biotinylated ampicillin (Dargis & Maloin, 1994) in the presence or absence of cerulenin. As shown in Fig. 10, all of the PBPs were labelled in both sets of cells. Furthermore, the inhibition of phospholipid synthesis had no effect on the labelling patterns of the individual PBPs. The significance of these results is discussed below.

II. 2. Dependence of the Lysis Induction Stage on Phospholipid Synthesis

To determine whether phospholipid synthesis was required for the lysis induction stage, the ability of cerulenin to inhibit the lysis of cells which had been primed with ampicillin was tested. Strain VC8 (*relA*) was deprived of isoleucine and treated with ampicillin at 0 min as shown in Fig. 11A. In agreement with previous results (Pisabarro *et al.*, 1990), 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 performed with isoleucine-deprived cells of strain VC7 which were simultaneously treated with ampicillin

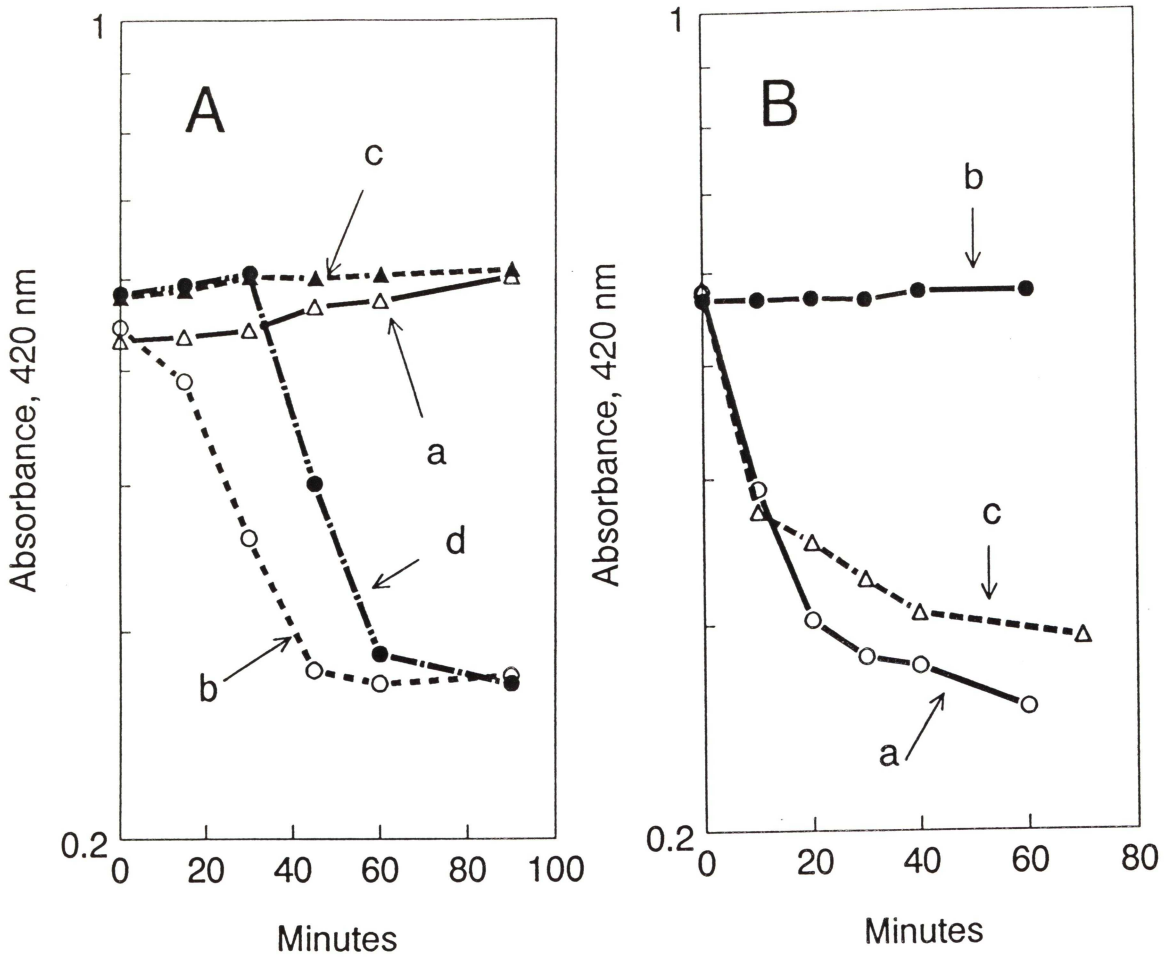

Figure 9. Dependence of the priming stage on phospholipid synthesis. **Panel A.** Isoleucine-deprived cultures of strain VC7 were primed with ampicillin (200 μg/ml) for 20 minutes in the presence (c and d) and in the absence (a and b) of cerulenin (50 μg/ml). At 0 minutes, the cells were washed free of ampicillin (and cerulenin in the case of cultures c and d) and resuspended in fresh isoleucine starvation medium containing a mixture of oleate and palmitate (50 μg/ml each) to reverse the effect of cerulenin for determination of the lysis induction. The subcultures primed in the absence of cerulenin received no further treatment (culture a), or chloramphenicol (100 μg/ml) to relax the stringent response (culture b). The subculture primed in the presence of cerulenin received 100 μg/ml of chloramphenicol (culture c), or a combination of 100 μg/ml of chloramphenicol and 50 μg/ml of ampicillin (culture d). **Panel B.** Isoleucine-deprived cultures of strain VC8 (*relA*) were primed with 200 μg/ml of ampicillin for 20 minutes with (b and c) of without (a) cerulenin. Culture c also received a mixture of oleate and palmitate (100 μg/ml each). At 0 minutes, priming was terminated and the cells from each culture were resuspended in fresh isoleucine deprivation medium for determination of the lysis induction.

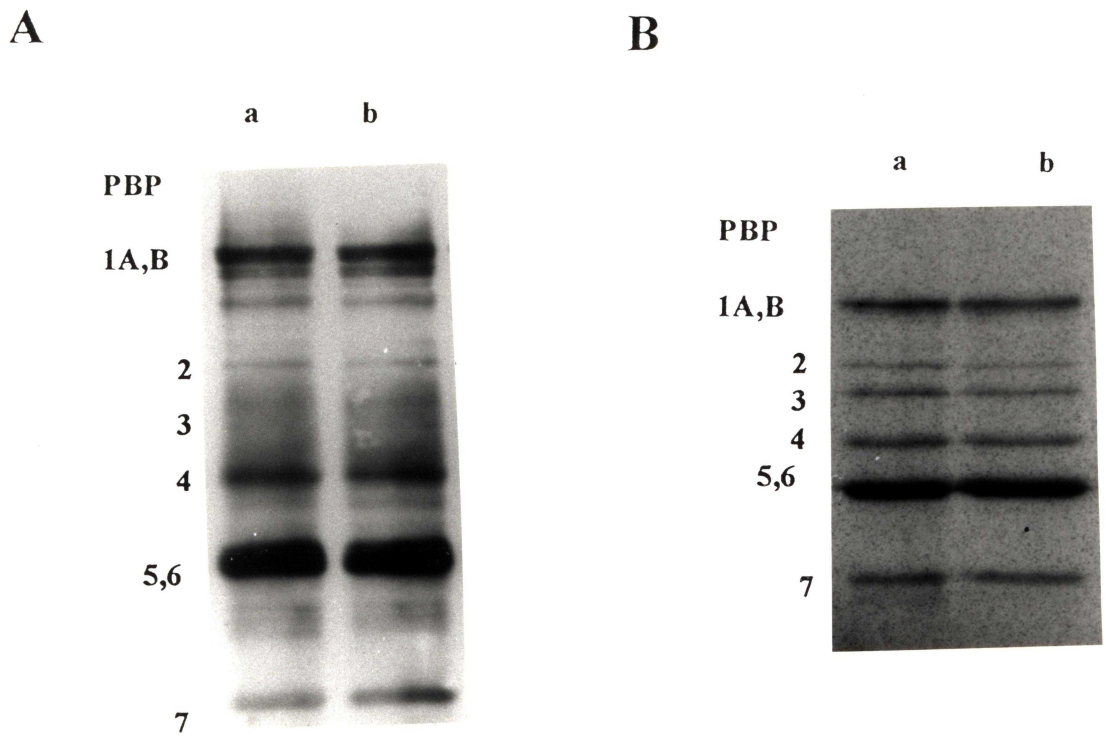

Figure 10. PBP labelling profiles in the presence and in the absence of phospholipid synthesis. **Panel A.** An exponential phase culture of strain VC7 was deprived of isoleucine and the stringent response was relaxed with chloramphenicol (100 μg/ml). The culture was then divided into two portions. One portion received no further treatment, and the other received 50 μg/ml of cerulenin. Both subcultures were incubated for further 20 minutes and their PBPs were labelled with biotinylated ampicillin. The PBPs were separated by SDS polyacrylamide gel electrophoresis and visualized by chemiluminescence. Lane a: PBPs from the cells labelled in the absence of cerulenin; lane b: PBPs from the cells labelled in the presence of cerulenin. **Panel B.** An exponential phase culture of strain VC7 was deprived of isoleucine and the stringent response was relaxed with chloramphenicol. Both subcultures were incubated for further 20 minutes and their PBPs were labelled with [^{3}H]-benzylpenicillin. The PBPs were separated by SDS polyacrylamide gel electrophoresis and visualized by autoradiography. Lane a: PBPs from the cells in which phospholipid synthesis was inhibited; lane b: PBPs from the cells in which phospholipid synthesis continued.

and chloramphenicol to relax the stringent response (not shown). Also, similar results were obtained when viable cell counts were used to monitor lysis instead of optical density readings (not shown). Therefore, phospholipid synthesis was required for lysis induction by ampicillin treatment.

It was also determined whether phospholipid synthesis was required for lysis induced by non-β-lactam agents. As shown in Fig. 11B, strain VC7 was isoleucine-deprived at 0 minutes, relaxed with chloramphenicol, and treated with phosphonomycin. 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). Similar results were obtained with D-cycloserine (not shown). Furthermore, the same results were obtained when this experiment was performed with isoleucine-deprived cells of strain VC8 (*relA*) (not shown). Therefore, lysis induced by non-β-lactams was also dependent on phospholipid synthesis.

II. 3. PG Synthesis in Amino Acid-Deprived *E. coli* is Independent of Stable RNA Synthesis

According to the surface stress model of bacterial cell wall growth (e.g., Koch, 1993), the cell walls expand in response to increasing cytoplasmic mass. Stable RNAis a major component of cell mass and accounts for over 20% of cell dry weight (Neidhardt, 1987). Stable RNA synthesis continues in amino acid-deprived bacteria at even higher rates than in normal growing cells when the stringent response is relaxed (Cashel & Rudd, 1987). In this regard, Cooper (1991) has suggested that the normal levels of PG synthesis in the relaxed nongrowing bacteria may be driven by the increase in the cell mass due to the relaxed synthesis of stable RNA. Therefore, it was essential to gain evidence that the inhibition of PG metabolism during amino acid deprivation was directly related to the inhibition of phospholipid synthesis and not to the inhibition of stable RNA synthesis.

To minimize the possible side effects of cerulenin treatment on other intracellular processes, the minimum concentration of cerulenin that still conferred penicillin tolerance

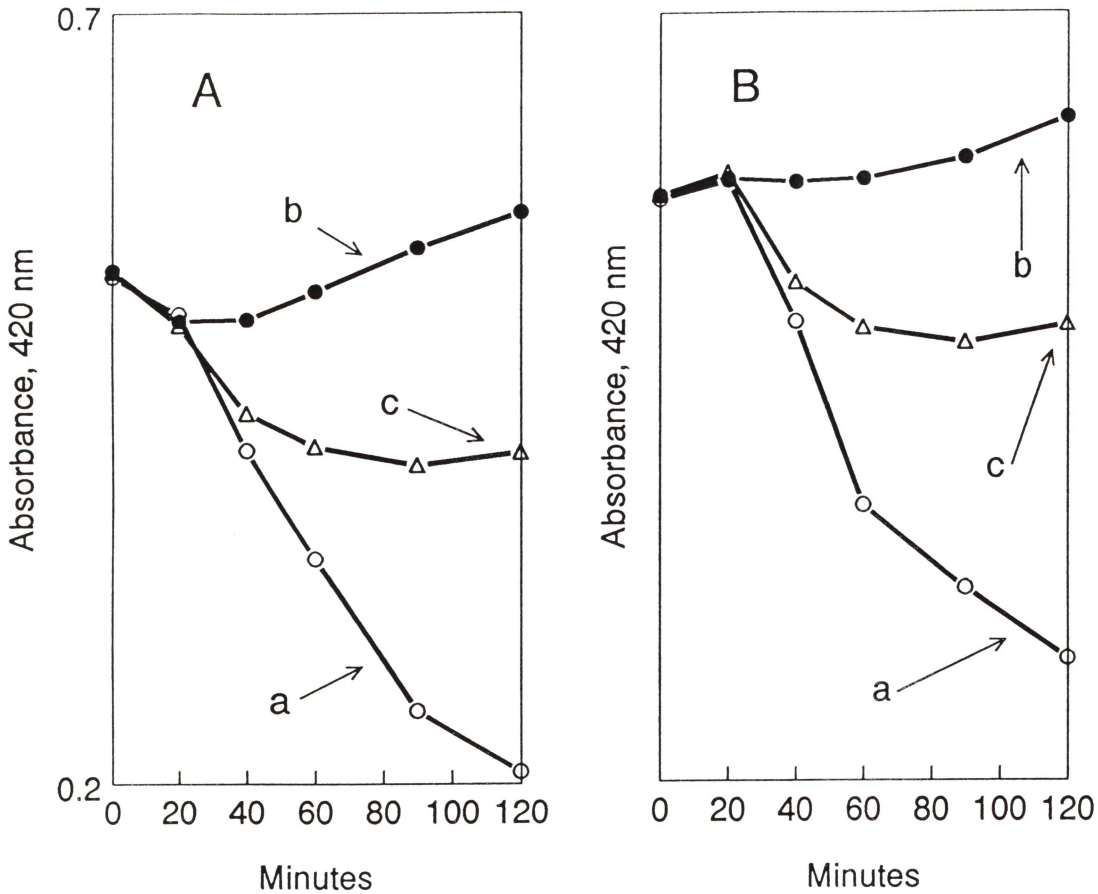

Figure 11. Dependence of the lysis induction stage on phospholipid synthesis. **Panel A.** An exponential phase culture of strain VC8 (*relA*) was isoleucine deprived and divided into three portions. All three subcultures received ampicillin (50 μg/ml) at 0 minutes. Culture a received no further treatment, whereas cultures b and c were treated with cerulenin (50 μg/ml) at 20 and 30 minutes, respectively. **Panel B.** Exponential phase culture of strain VC7 was isoleucine deprived and divided into three portions. All three subcultures were treated with a combination of chloramphenicol (100 μg/ml to relax the stringent response) and phosphonomycin (150 μg/ml) beginning at 0 minutes. Culture a received no further treatment; cultures b and c were treated with cerulenin (50 μg/ml) at 20 and 30 minutes, respectively.

to amino acid-deprived cells of strains VC8 (*relA*$^-$) and VC7 (this strain was phenotypically relaxed by the addition of chloramphenicol) was determined. This concentration was 6 μg/ml of cerulenin, which is close to its MIC of 10 μg/ml. Rifampicin was used at 10 μg/ml, which was sufficient to completely inhibit RNA synthesis under the experimental conditions (see below).

Figure 12 shows the effect of inhibiting of RNA synthesis on synthesis of PG by amino acid-deprived relaxed bacteria. An exponentially growing culture of VC8 was deprived of isoleucine and divided into four portions. PG synthesis continues at normal rates in such cells (Ishiguro & Ramey, 1976; compare curves a and b). Treatment with rifampicin had no effect on the relaxed PG synthesis (curve c). In contrast, cerulenin at a concentration below its MIC inhibited PG synthesis (curve d). Similar results were obtained when amino acid-deprived chloramphenicol-treated cultures of strain VC7 were used (not shown). These results indicate that RNA synthesis can not be the driving force for PG sacculus expansion during relaxed PG synthesis as proposed by Cooper (1991); i.e., relaxed PG synthesis continued even when RNA synthesis was inhibited. On the other hand, these results confirmed the obligatory link between phospholipid synthesis and PG synthesis.

In view of the correlation between phospholipid and PG syntheses, the effect of the inhibition of RNA synthesis on phospholipid synthesis in relaxed cells was determined. Fig. 13 shows that cerulenin at 6 μg/ml inhibited relaxed phospholipid synthesis in isoleucine-deprived VC8 cells as expected (compare curves b and d). On the other hand, there was no inhibition of phospholipid synthesis when a portion of the amino acid-starved culture was treated with rifampicin (curve c). Again, similar results were obtained with an amino acid-deprived culture of strain VC7 relaxed with chloramphenicol (not shown). Therefore, the inhibition of stable RNA synthesis did not affect phospholipid synthesis.

Fig. 14 shows the effect of antibiotic treatment on relaxed RNA synthesis in amino acid-deprived culture of strain VC8. Rifampicin at 10 μg/ml completely inhibited the relaxed RNA synthesis (compare curves a and b). Cerulenin at 6 μg/ml also inhibited RNA synthesis but less effectively than rifampicin (curve c). Relaxed RNA synthesis was also inhibited when phospholipid synthesis was inhibited by cerulenin treatment in

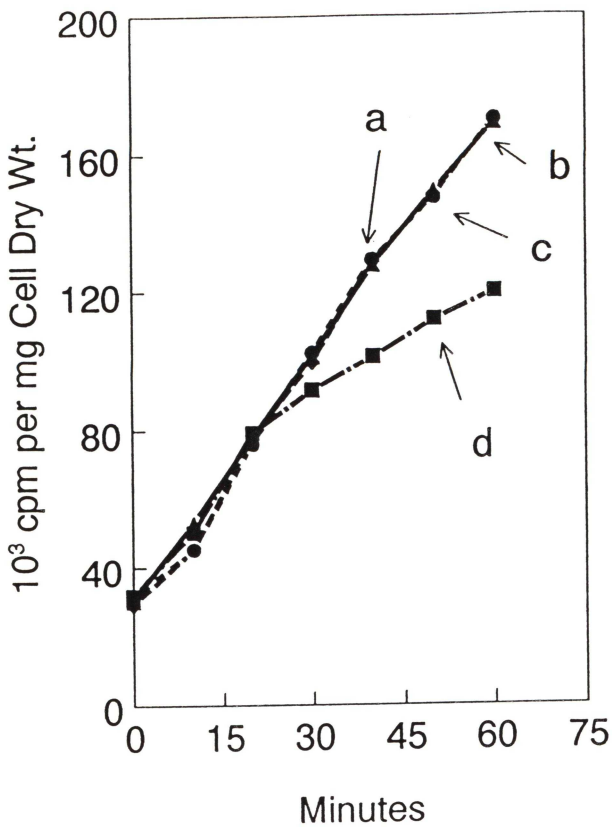

Figure 12. Effect of inhibition of RNA and phospholipid syntheses on PG synthesis in amino acid-deprived *E. coli*. [^{3}H]-DAP was added to an exponential phase culture of strain VC8 (*relA*) to a final concentration of 0.2 μg/ml (10 μCi/ml) at -20 minutes. The culture was divided into four portions at 0 minutes. One portion was left untreated (curve a) whereas three others were isoleucine-deprived. These subcultures received: no further treatment (curve b), 10 μg/ml of rifampicin (curve c), or 6 μg/ml of cerulenin (curve d).

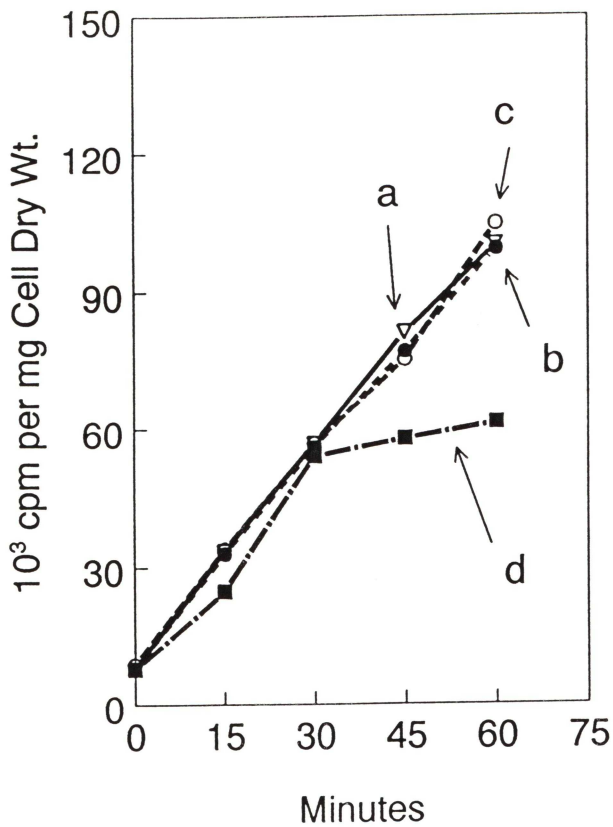

Figure 13. Effect of inhibition of RNA synthesis on phospholipid synthesis in amino acid-deprived *E. coli*. $^{32}P_i$ was added to an exponential phase culture of strain VC8 (*relA*) to a final concentration of 5 μCi/ml at -15 minutes. The culture was divided into four portions at 0 minutes. One portion was left untreated (curve a) whereas three others were isoleucine-deprived. These subcultures received: no further treatment (curve b), 10 μg/ml of rifampicin (curve c), or 6 μg/ml of cerulenin (curve d).

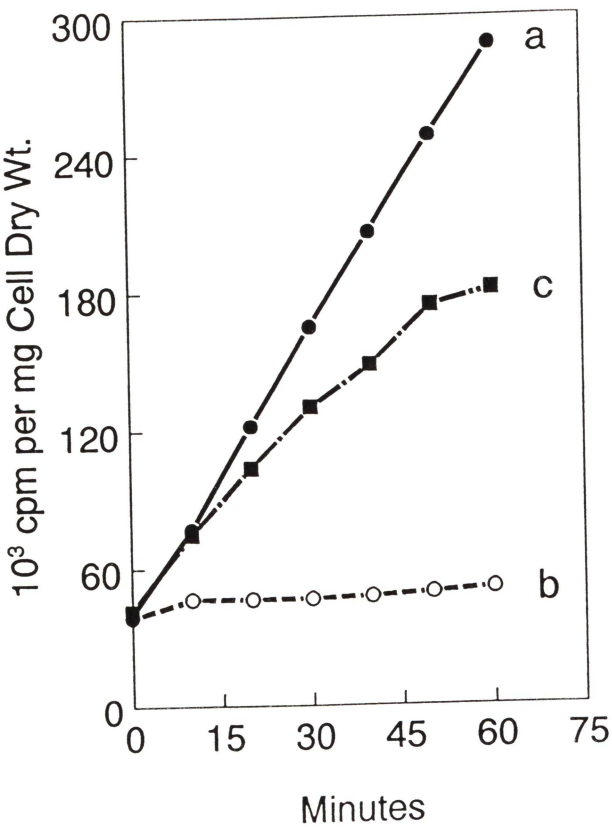

Figure 14. The effect of inhibition of phospholipid synthesis on stable RNA synthesis in amino acid-deprived *E. coli*. [^{14}C]-uracil was added to an exponential phase culture of strain VC8 (*relA*) to a final concentration of 1μg/ml (0.5 μCi/ml) at -20 minutes. The culture was isoleucine deprived at 0 minutes and divided into four portions. These subcultures received: no further treatment (curve a), 10 μg/ml of rifampicin (curve b), and 6 μg/ml of cerulenin (curve c).

amino acid-deprived culture of strain VC7 or by glycerol deprivation of amino acid-deprived strains VC58 and BBfe2 (data not shown). Thus, inhibition of phospholipid synthesis in nongrowing *E. coli* led to the inhibition of stable RNA synthesis, whereas the opposite did not take place.

The effect of the inhibition of RNA synthesis on penicillin tolerance of amino acid-deprived *E. coli* was studied in strain VC8, and the results are shown in Fig. 15. Ampicillin-induced lysis was observed in cells treated with rifampicin (compare curves a and b), whereas the treatment with cerulenin, as already noted, resulted in tolerance to ampicillin (compare curves a and c). Therefore, phospholipid synthesis, but not stable RNA synthesis, is required for PG synthesis in amino acid-deprived *E. coli* and its susceptibility to lysis by β-lactam antibiotics.

III. A Direct Correlation Between Overproduction of ppGpp and Inhibition of PG and Phospholipid Syntheses in *E. coli*

It has been suggested, that ppGpp was the sole effector responsible for the inhibition of phospholipid and PG synthesis and for the development of penicillin tolerance observed in the course of the stringent response (Kusser and Ishiguro, 1985; Ishiguro *et al.*, 1994). However, these suggestions were made on the basis of the association of these phenomena with the stringent response and their suppression upon relaxation of the stringent response. One could thus argue that these results reflected some indirect manifestation of the stringent response rather than ppGpp accumulation. It was therefore important to investigate the relationship between intracellular ppGpp levels and inhibition of phospholipid and PG syntheses.

The plasmid pDR24 in strain VC891 contained the *relA* gene fused to a *tac* promoter. Therefore, the addition of IPTG to a culture of VC891 resulted in the induction of *relA* and in the overproduction of ppGpp as previously described (Svitil *et al.*, 1992), bypassing the induction of the stringent response. Fig. 16 shows the effects of ppGpp overproduction by VC891 on growth (panel A), phospholipid synthesis (panel B), and PG synthesis (panel C). If ppGpp accumulation was induced by isoleucine deprivation, growth, phospholipid synthesis, and PG synthesis were inhibited as expected

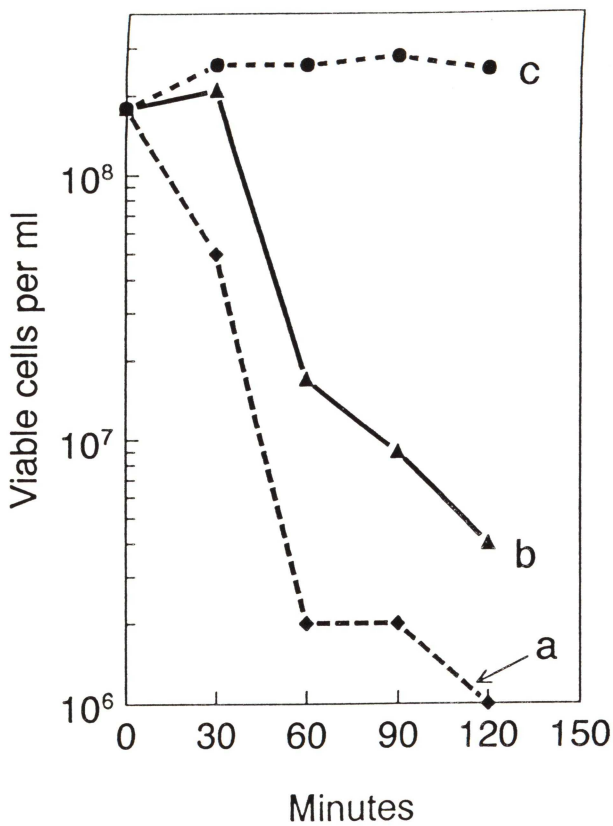

Figure 15. The effect of inhibition of RNA and phospholipid syntheses on ampicillin-induced lysis of amino acid-deprived *E. coli*. An exponential phase culture of strain VC8 was starved for isoleucine at 0 minutes and divided into several parts. These subcultures were treated with 50 μg/ml of ampicillin with no further additions (culture a), 10 μg/ml of rifampicin (culture b), and 6 μg/ml of cerulenin (culture c). Treatment of another culture with rifampicin alone did not decrease the viable cell counts during a 2 hour period of treatment.

(compare curves a and b). The induction of the of the *relA* gene on pDR24 had the same general inhibitory effects, but the degrees of inhibition showed a dependence on IPTG concentration. For example, Fig. 16 shows that 50 μM IPTG was close to the minimum concentration required to cause inhibition of growth, phospholipid synthesis, and PG synthesis (curves d) whereas 25 μM IPTG exhibited almost no inhibitory activity (curves c).

III. 1. Overproduction of ppGpp Causes Ampicillin Tolerance

Fig. 17 summarizes the effects of ampicillin on strains VC891 and VC892. A growing culture of strain VC891 was susceptible to ampicillin-induced lysis (compare curves a and b). The treatment of VC891 with 50 μM IPTG to induce the overproduction of ppGpp resulted in the inhibition of growth (curve c) and in the development of tolerance to ampicillin-induced lysis (curve d). When chloramphenicol was added to an IPTG-treated culture to inhibit the activation of RelA, the susceptibility of VC891 to ampicillin-induced lysis was restored (curve e). In contrast to VC891, strain VC892, which carries pDR25 (encoding a truncated inactive derivative of the *relA* gene), was prone to ampicillin-induced lysis even in the presence of 100 μM IPTG (curve f).

The minimum concentration of IPTG required to cause ampicillin tolerance was determined. Fig. 18 shows the effects of different concentrations of IPTG on the viability of ampicillin-treated cultures of strain VC891. IPTG at 50 μM prevented loss in cell viability. Lower concentrations of IPTG had no such protective effect. Therefore, the minimum amount of IPTG required to cause ampicillin tolerance was approximately 50 μM.

The maximum levels of ppGpp accumulated by strains VC7 and VC891 were quantified, and these results are shown in Fig. 19. Under the experimental conditions, the steady state concentration of ppGpp in a normal exponential phase culture of strain VC7 was determined to be about 35 pmol per mg cell dry weight (sample a, Fig. 19). The addition of IPTG to cultures of VC891 resulted in the maximum accumulation of ppGpp after about 40 min of treatment. Treatments with 50 μM and 25 μM IPTG

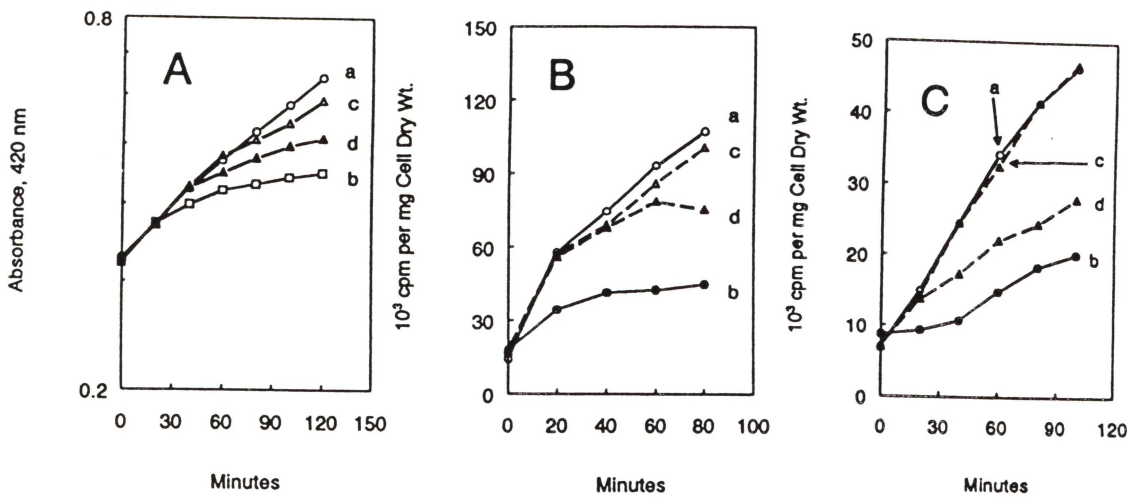

Figure 16. Effect of ppGpp overproduction on growth (**panel A**), phospholipid synthesis (**panel B**), and PG synthesis (**panel C**) in strain VC891. Exponential phase cultures of strain VC891 (carrying plasmid pDR24 (*tacP-relA*$^+$)) were divided into four parts. The cultures used for curves labelled a were untreated controls. The cultures used for curves labelled b were subjected to isoleucine deprivation. The cultures used for curves labelled c and d were treated with 25 and 50 μM IPTG at 0 minutes, respectively, to induce ppGpp overproduction. The cultures in panel B received $^{32}P_i$ (5 μCi/ml) at -15 minutes, whereas the cultures in panel C received [^{3}H]-DAP (0.2 μg/ml, 10 μCi/ml) at -20 minutes.

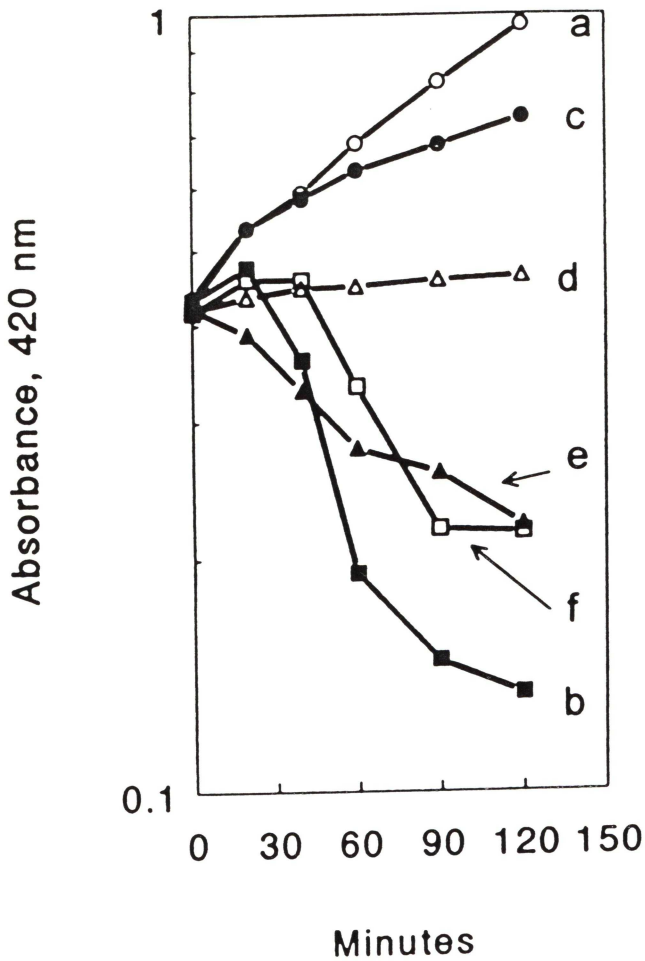

Figure 17. Effect of ppGpp overproduction on ampicillin tolerance. Exponential phase culture of strain VC891 (carrying plasmid pDR24 (*tacP-relA*$^+$)) was divided into five portions. Culture a represents an untreated control. A portion of this culture was treated with ampicillin at 0 minutes (curve b). Three other portions received 50 μM IPTG at -15 minutes to induce the accumulation of ppGpp. One of these received no further treatment (curve c); the other two were treated at 0 minutes with ampicillin alone (curve d), or with a combination of ampicillin and chloramphenicol (curve e). For comparison, an exponential phase culture of strain VC892 (carrying plasmid pDR25 (*tacP-relA*$^-$)) was treated with 100 μM IPTG at -15 minutes and then with ampicillin at 0 minutes (curve f). The concentrations of ampicillin and chloramphenicol used were 50 and 100 μg/ml, respectively.

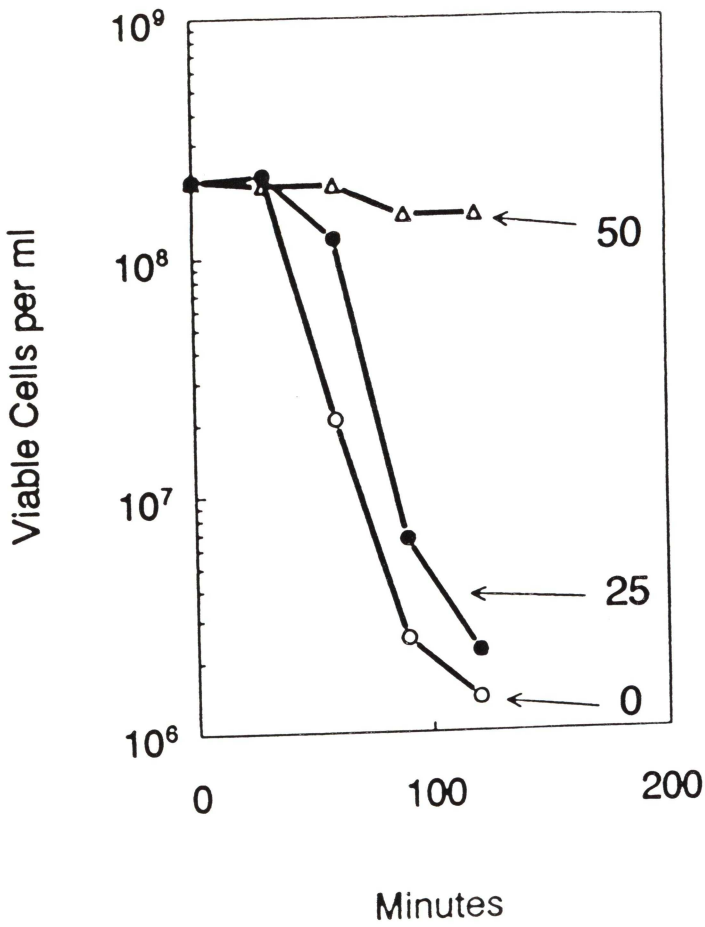

Figure 18. Effect of ppGpp overproduction on viability of ampicillin-treated cultures of strain VC891 (carrying plasmid pDR24 (*tacP-relA*$^+$)). Three cultures were treated with 0, 25, and 50 μM IPTG (as indicated) at -15 minutes. They were then treated with 50 μg of ampicillin per ml beginning at 0 minutes, and viable cell counts were determined as indicated.

resulted in peak accumulations of ppGpp amounting to 870 and 720 pmol per mg cell dry weight, respectively (samples b and c, Fig. 19). These concentrations are significantly lower than the amount accumulated during the stringent response. For example, isoleucine deprivation resulted in an increase in the ppGpp level to 1,200 pmol per mg cell dry weight (sample d, Fig. 19). These results indicate that the minimum concentration of ppGpp required for ampicillin tolerance was about 870 pmol per mg cell dry weight.

III. 2. Reversal of ppGpp-Induced Ampicillin Tolerance by Overexpression of PlsB

Heath *et al.* (1994) have recently reported that the overexpression of *plsB* , which encodes *sn*-glycerol-3-phosphate acyltransferase, reversed the ppGpp-induced inhibition of phospholipid synthesis in experiments similar to those described here. Furthermore, the results from Section II of this Chapter have shown that lysis induced by treatment with β-lactam antibiotics or by other inhibitors of PG synthesis is dependent on phospholipid synthesis. Therefore, the effects of *plsB* overexpression on ppGpp-inhibited PG synthesis and IPTG-induced ampicillin tolerance were tested.

Two derivatives of strain VC891 were constructed for this purpose. Strain VC7004 carried plasmid pRJ10 and consequently overproduced PlsB. The second strain, VC7005, carried plasmid pRJ12 and overproduced an inactive truncated derivative of PlsB. Both strains were grown in the presence of 50 μM IPTG to induce ppGpp synthesis and then subjected to ampicillin treatment. The effect of PlsB overproduction on PG synthesis is shown in Fig. 20A. Overproduction of ppGpp caused dramatic inhibition of PG synthesis in the control strain VC7005 (compare curves c and d), but had virtually no effect on PG synthesis in strain VC7004 (compare curves a and b). In a similar set of experiments, strains VC7004 and VC7005 grown in the presence of 50 μM IPTG were treated with ampicillin (Fig. 20B). Culture of strain VC7005 was ampicillin tolerant under these conditions like the parental strain VC891 (compare curves c and d). Strain VC7004, in contrast, was prone to ampicillin-induced lysis (compare curves a and b). Therefore, overexpression of PlsB reversed the ppGpp-induced inhibition of PG synthesis and abolished ampicillin tolerance. These results provide further support to the hypothesis that ppGpp negatively controls bacterial PG metabolism

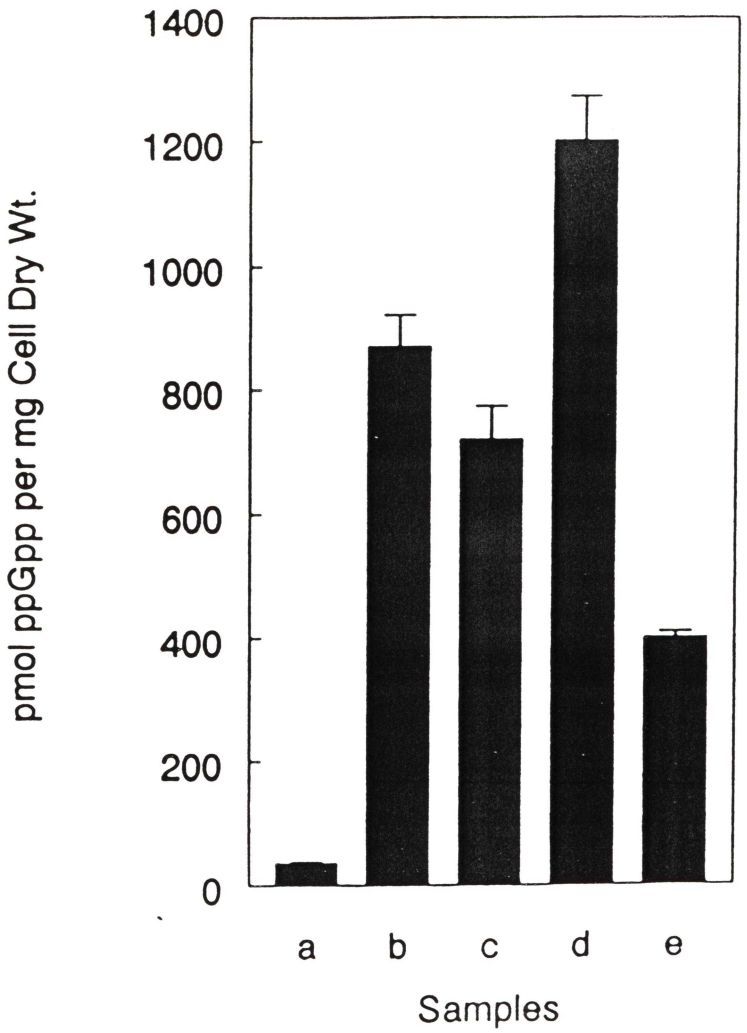

Figure 19. Quantification of ppGpp accumulation in strains VC7 and VC891 (carrying plasmid pDR24 (*tacP-relA*$^+$)). Sample a represents a steady-state level of ppGpp in an untreated culture of strain VC7. Samples b-d represent peak intracellular ppGpp concentration from cultures of strain VC891 treated with 50 μM IPTG (sample b); 25 μM IPTG (sample c); or isoleucine-deprived (sample d). Sample e represents a maximum accumulation of ppGpp in strain VC7 treated with 10 μg/ml of ampicillin (see Fig. 22). The data presented here are the average values from at least three experiments.

and confers penicillin tolerance to *E. coli* by means of inhibition of phospholipid synthesis.

III. 3. Treatment with β-Lactam Antibiotics Causes ppGpp Accumulation

During the course of the ppGpp quantification experiments, it was discovered that the treatment of growing cultures of VC7 with ampicillin resulted in the accumulation of ppGpp. Figure 21 shows a typical accumulation of ppGpp in response to ampicillin treatment. This was a novel observation and required additional investigation. Fig. 22 shows that the time course of ppGpp accumulation was dependent on ampicillin concentration. Furthermore, the accumulation of ppGpp continued up to the point of lysis induction. For the curves shown in Fig. 22, the ppGpp determinations were terminated when the cultures began to lyse except in the case of the culture which was treated with the sublethal dose of 3 μg of ampicillin per ml. The largest accumulation observed was about 400 pmol of ppGpp per mg cell dry weight which was induced by 80 min of treatment with the lethal dose of 10 μg of ampicillin per ml. It is noteworthy that this level represents about half of the minimum amount of ppGpp required to confer ampicillin tolerance (compare samples b and e in Fig. 19). The accumulation of ppGpp was also observed during treatment with sublethal amounts of ampicillin. For example, Fig. 22 shows that growth in the presence of 3 μg of ampicillin per ml resulted in an accumulation of 160 pmol of ppGpp per mg of cell dry weight after 90 min; the maximum level of accumulation in this case is not known since measurements were not made beyond this point. Additional experiments indicated that cephaloridine also caused the accumulation of ppGpp but rifampicin and norfloxacin did not (not shown).

It was important to identify the ppGpp synthetase activity that was responsible for the ppGpp accumulation observed. To this end, strains VC7000 (Δ*relA*) and VC7001 (Δ*relA* Δ*spoT*) were constructed. As expected, the double deletion strain VC7001 completely lacked ppGpp during the steady-state growth, amino-acid deprivation, and carbon source deprivation, whereas strain VC7000 accumulated ppGpp in response to carbon source limitation but not to amino acid deprivation (not shown). Neither strain, however, exhibited ppGpp accumulation under the conditions of ampicillin treatment (Fig.

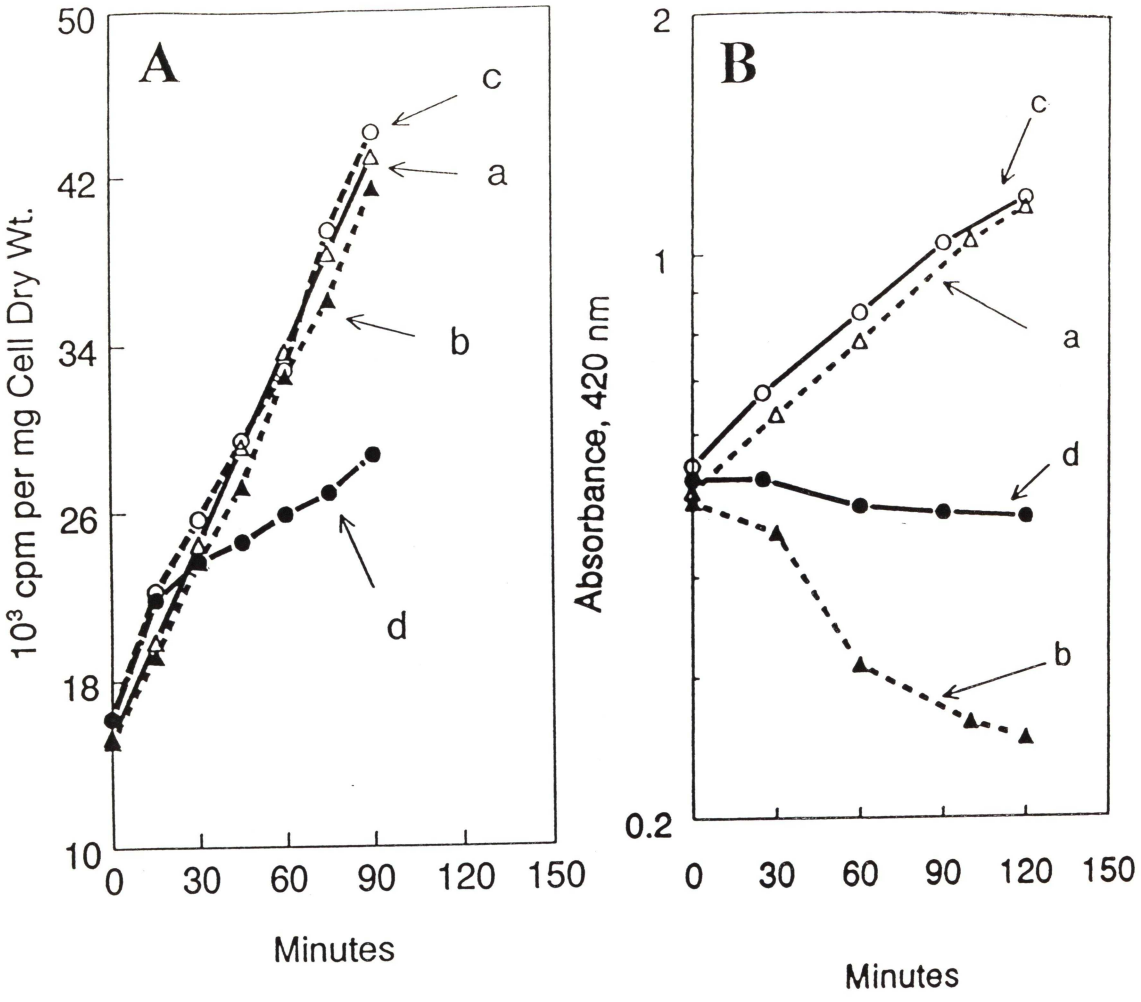

Figure 20. Effect of overexpression of PlsB on ppGpp-induced inhibition of PG synthesis and ampicillin tolerance. **Panel A.** PG synthesis was measured in exponential phase cultures of strain VC7004 (overexpressing RelA and active PlsB) that were either left untreated (curve a) or treated with 50 μM IPTG at 0 minutes (curve b) and in exponential phase cultures of the control strain VC7005 (overexpressing RelA and inactive PlsB) that were likewise untreated (curve c) or treated with 50 μM IPTG at 0 minutes (curve d). **Panel B.** Ampicillin-induced lysis in strains VC7004 (overexpressing RelA and active PlsB) and VC7005 (overexpressing RelA and inactive PlsB). Exponential phase cultures of strain VC7004 were either untreated (culture a) or treated with 50 μM IPTG at -15 minutes and 50 μg/ml of ampicillin at 0 minutes (curve b). For comparison, exponential phase cultures of strain VC7005 were also either untreated (curve c) or treated with 50 μM IPTG at -15 minutes and 50 μg/ml of ampicillin at 0 minutes (curve d).

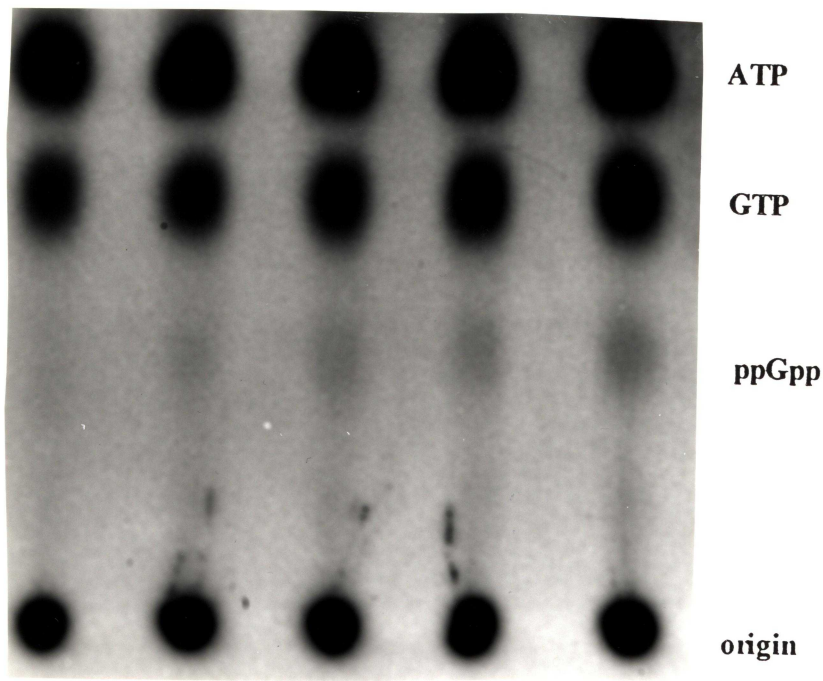

Figure 21. Accumulation of ppGpp in strain VC7 in response to ampicillin treatment. Exponential phase culture of strain VC7 was treated with 6 μg/ml of ampicillin at 0 minutes. Samples were taken at 0, 20, 40, 60, and 90 minutes (left to right). Lysis started between 90 and 100 minutes after administration of ampicillin.

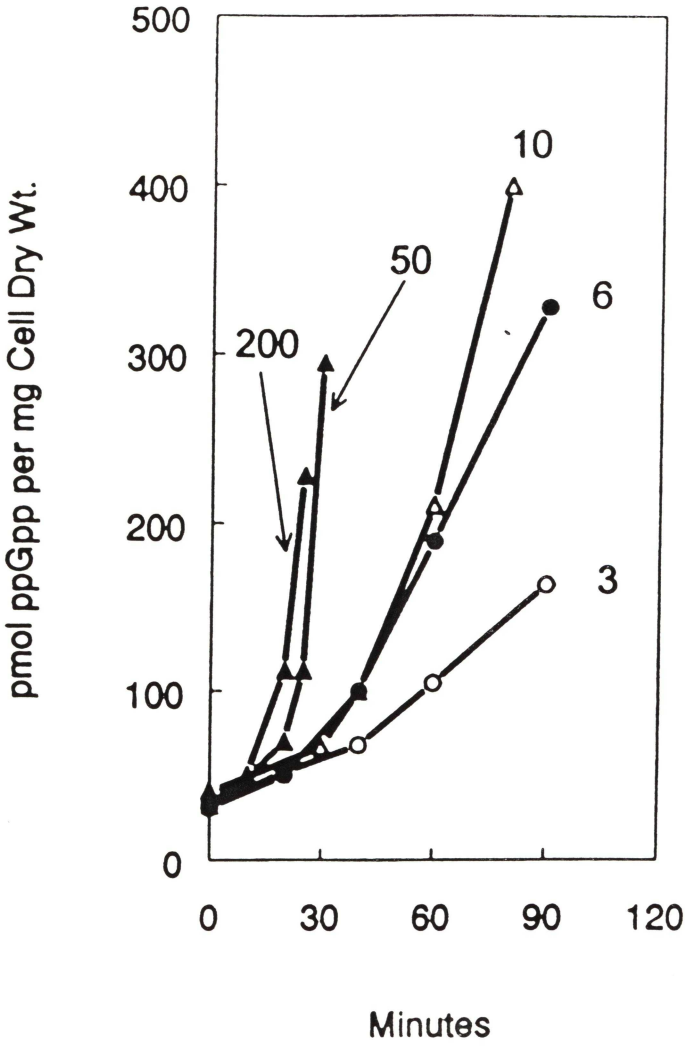

Figure 22. Dose-dependent accumulation of ppGpp in response to ampicillin treatment. At 0 minutes, exponential phase cultures of strain VC7 were treated with ampicillin at the concentrations represented by the numerals on the curves (in mg per ml). Quantification of ppGpp was terminated at the first sign of lysis, except in the case of the culture that was treated with a sublethal dose of ampicillin (3 μg/ml).

23, compare curves a and b, and a and c). This was to be expected in the case of the control strain VC7001. Since strain VC700 also failed to synthesize ppGpp under these conditions, ampicillin (and other β-lactams tested) caused ppGpp accumulation by a *relA* (ppGpp synthetase I)-dependent mechanism. This β-lactam-dependent ppGpp accumulation was not the consequence of a simple inhibition of bacterial growth just before the onset of the lysis induction, since norfloxacin and rifampicin, though at least as potent as β-lactams in inhibiting growth of VC7, failed to cause any noticeable ppGpp accumulation (not shown). Therefore, treatment of growing bacterial cultures with β-lactam antibiotics promoted a *relA*-dependent increase in the intracellular ppGpp levels, that was, however, not sufficient to provide such cells with tolerance to the antibiotic.

IV. Phospholipid Synthesis is Required for β-Lactam-Induced Lysis of Growing Bacteria

Cerulenin selectively blocks fatty acid and phospholipid synthesis in growing cells. Macromolecular synthesis (DNA, RNA, and protein synthesis) continues in cells treated with high concentrations of cerulenin for some time, whereas the lipid synthesis is blocked within minutes (Goldberg *et al.*, 1973; D. Vanderwel & E. Ishiguro, unpublished). PG synthesis is also blocked in such cells shortly after the initiation of cerulenin treatment in a concentration-dependent manner (D. Vanderwel & E. Ishiguro, unpublished). The original interpretation of this result was that PG synthesis in normal growing bacteria was dependent on phospholipid synthesis as has already shown to be the case in amino acid-deprived bacteria. However, Seyfzadeh *et al.* (1993) have recently demonstrated that inhibition of fatty acid synthesis in growing *E. coli* by cerulenin or by a genetic manipulation resulted in rapid accumulation of ppGpp. The synthesis of ppGpp by fatty acid-starved cells was attributed to ppGpp synthetase II (SpoT). Therefore, the inhibition of PG synthesis in growing bacteria during cerulenin treatment could be attributed to ppGpp accumulation. In view of this report by Seyfzadeh *et al.* (1993), the role of phospholipid synthesis in PG metabolism of growing bacteria was re-examined.

Strain VC7001 (Δ*relA* Δ*spoT*) carries null mutations in the genes encoding ppGpp synthetases I and II and consequently is completely incapable of synthesizing

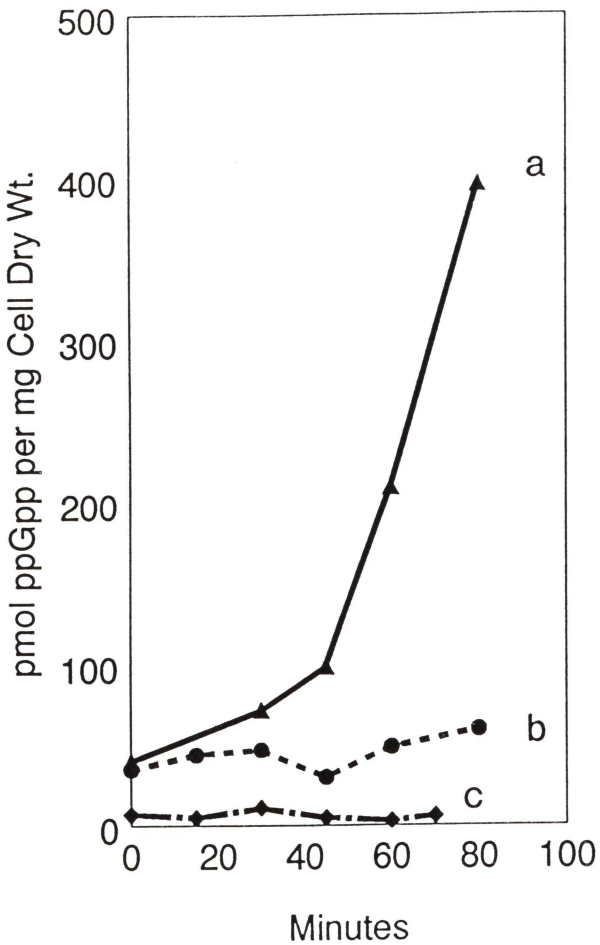

Figure 23. Accumulation of ppGpp in response to treatment with ampicillin by strains VC7 (wt), VC7000 (*ΔrelA*), and VC7001 (*ΔrelA ΔspoT*). Exponential phase cultures were treated with 10 μg/ml of ampicillin at 0 minutes, and the intracellular ppGpp levels were quantified at the designated time intervals. Quantification of ppGpp was terminated at the first sign of lysis, which started at 80 minutes in cultures of strains VC7 (curve a) and VC7000 (curve b), and at 70 minutes in the culture of strain VC7001 (curve c).

ppGpp. This strain was used to determine the role of phospholipid synthesis in penicillin-induced lysis of growing cells in the experiment described in Fig. 24. Growth was inhibited about 40 minutes after the addition of cerulenin to a growing culture of VC7001 (compare curves a and b). Ampicillin caused lysis about 25 minutes after the initiation of treatment (curve c). In contrast, lysis did not occur when the cells were treated simultaneously with cerulenin and ampicillin (curve d). Furthermore, ampicillin-induced lysis was restored when the inhibitory effect of cerulenin on phospholipid synthesis was reversed by the addition of exogenous fatty acids (curve e). This experiment unequivocally shows that the ampicillin tolerance induced by cerulenin is directly related to the inhibitory action on fatty acid and therefore phospholipid synthesis and not to the accumulation of ppGpp.

Seyfzadeh *et al.* (1993) reported that the accumulation of ppGpp they observed was due to fatty acid deprivation but not to the inhibition of phospholipid synthesis. For example, the expression of mutations specifically blocking phospholipid synthesis (i.e., not fatty acid synthesis) did not result in ppGpp accumulation. These results were verified in this study (not shown). Therefore, the effect of blocking phospholipid synthesis on penicillin-induced lysis was determined in strain VC58 (*gpsA*). The incorporation of [^{32}P]-phosphate into the phospholipid fraction was inhibited about 40 minutes after the onset of glycerol deprivation in this strain (not shown). Fig. 25 shows that the rate of cell mass increase was unaffected for about 90 minutes after glycerol deprivation (compare curves a and b). Growth of a portion of the glycerol-deprived culture was inhibited by the addition of ampicillin at 40 minutes, but no lysis occurred (curve c). However, if glycerol was added along with ampicillin, the ampicillin tolerance was abolished (curve d). Similar results were obtained when strain BBfe2, which carries a mutation in *plsB* gene, was used in this experiment (not shown). These experiments clearly demonstrate that the inhibition of phospholipid synthesis in growing bacteria results in penicillin tolerance. Furthermore, this penicillin tolerance was directly attributable to the inhibition of phospholipid synthesis and not to ppGpp accumulation because ppGpp did not accumulate under these conditions.

As shown in Fig. 25, growth rate of the glycerol-deprived culture was normal at the time of ampicillin treatment and the onset of ampicillin-induced lysis. Therefore, it

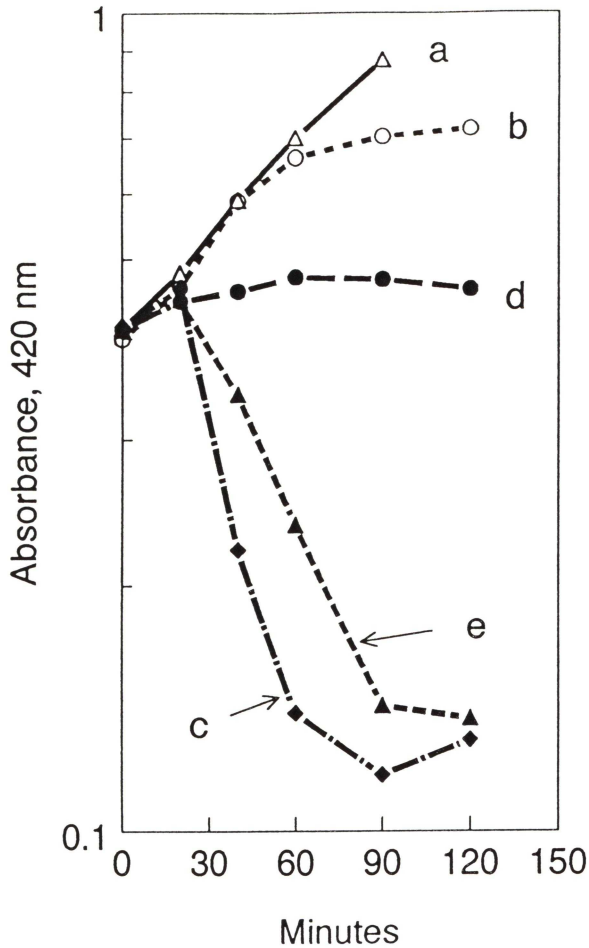

Figure 24. Inhibition of ampicillin-induced lysis by cerulenin in growing *E. coli*. A series of exponential phase cultures of strain VC7001 (*ΔrelA ΔSpoT*) received at 0 minutes: no further treatment (culture a), cerulenin (culture b), ampicillin (culture c), or a mixture of ampicillin and cerulenin (culture d). In addition, another culture received a mixture of oleate and palmitate (100 μg/ml each) and was also treated with a combination of cerulenin and ampicillin beginning at 0 minutes (curve e). The cerulenin and ampicillin concentrations used were 50 μg/ml.

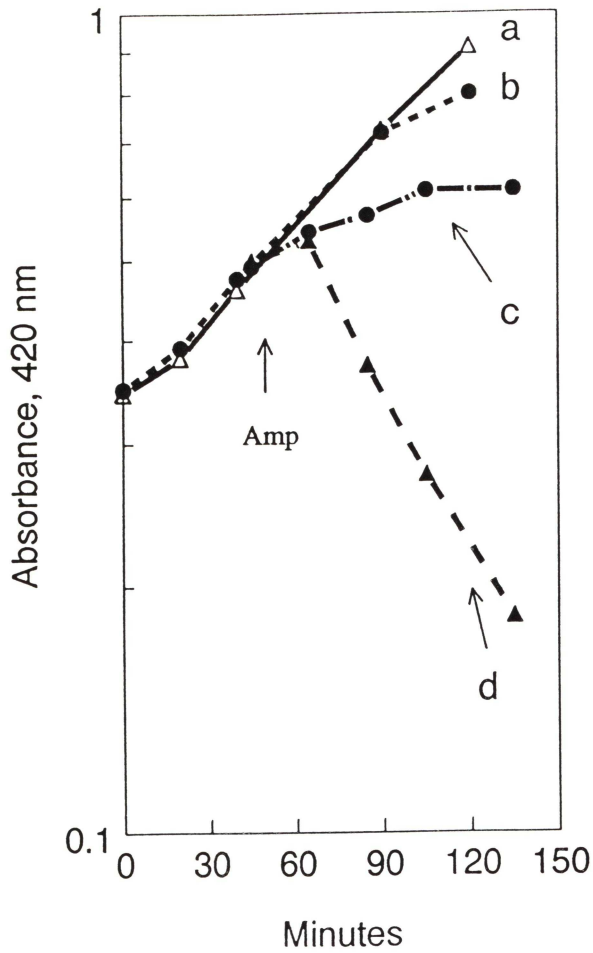

Figure 25. Inhibitory effect of glycerol deprivation on ampicillin-induced lysis in growing culture of strain VC58 (*gpsA*). An exponential phase culture of strain VC58 was deprived of glycerol at 0 minutes (cultures b, c, and d), or left untreated for comparison (culture a). At 40 minutes, the glycerol-deprived culture was divided into three parts. One part received no further treatment (culture b). Other cultures were treated with ampicillin (50 μg/ml) in the absence (culture c) or in the presence (culture d) of the exogenous glycerol.

was unlikely that the observed ampicillin tolerance was linked to a change in growth rate induced by glycerol deprivation. Further evidence for this is presented in Fig. 26. The treatment of strain VC7 with chloramphenicol resulted in an abrupt inhibition of growth (compare curves a and b). However, effective growth inhibiting activity of chloramphenicol was not sufficient to prevent ampicillin-induced lysis (compare curves c and d). In fact, lysis induction was initiated reproducibly faster in the cells treated with chloramphenicol and ampicillin than in the cells treated with ampicillin alone. This phenomenon will be addressed further in Chapter 4. Chloramphenicol treatment did not cause inhibition of phospholipid and PG syntheses for at least one hour after antibiotic administration (not shown). Therefore, the inhibition of phospholipid synthesis, but not the inhibition of growth, was primarily responsible for the acquisition of penicillin tolerance in growing *E. coli* cells.

V. Variation of the Levels of Acidic Phospholipids Has no Effect on Penicillin-Induced Lysis

Experiments described in the previous sections established the central role of net phospholipid synthesis in the regulation of PG metabolism and penicillin tolerance. Next, it was important to determine whether individual phospholipids played a role in the control over these phenomena.

Acidic phospholipids (phosphatidylglycerol and cardiolipin) are indispensable for the cell growth (Raetz & Dowhan, 1990) and take part in a number of cellular activities, including protein export (Kusters *et al.*, 1994) and initiation of DNA replication (Sekimizu and Kornberg, 1988). The levels of acidic phospholipids can be genetically manipulated in *E. coli* strain HDL11 through the controlled IPTG-induced expression of the *pgsA* gene which encodes for phosphatidylglycerol-3-phosphate synthetase (Heacock & Dowhan, 1989; Kusters *et al.*, 1991).

Strain HDL11 was grown without IPTG or in the presence of 1 mM IPTG for over 8 mass doublings, and the levels of individual phospholipids were quantified. The results are shown in Table 1: when HDL11 was grown in the presence of 1 mM IPTG, concentrations of individual phospholipids were that characteristic of exponentially

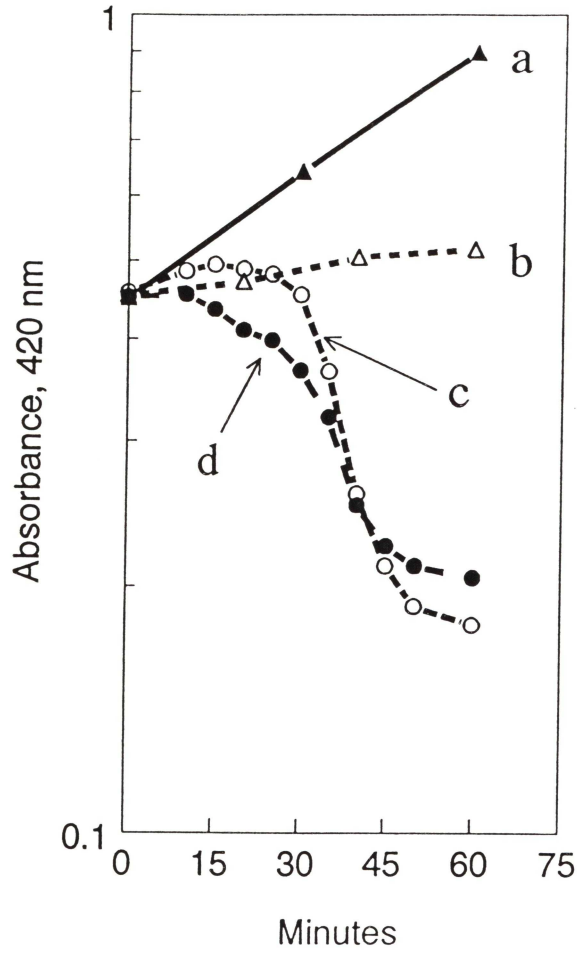

Figure 26. Inhibition of cell growth by treatment with chloramphenicol does not protect *E. coli* from ampicillin-induced lysis. An exponential phase culture of VC7 was divided into four parts. One of the subcultures received no further treatment (culture a), whereas others received chloramphenicol (culture b), ampicillin (culture c), or a mixture of chloramphenicol and ampicillin (culture d). The ampicillin and chloramphenicol concentrations used were 50 and 100 μg/ml, respectively.

growing wild-type *E. coli* (Heacock & Dowhan, 1989). In the absence of IPTG, the levels of acidic phospholipids were about ninefold lower than normal (Table 1).

The effect of ampicillin on strain HDL11 was determined. In Fig. 27A, strain HDL11 was grown in the absence of IPTG (curve a) or with 1 mM IPTG (curve b). Portions of these cultures were treated with ampicillin. As shown in Fig. 27A, the cultures grown with or without IPTG were sensitive to ampicillin-induced lysis (curves c and d). In Fig. 27B, cultures of strain HDL11 were also grown in the presence or absence of IPTG. The stringent response was induced in these cultures by the addition of serine hydroxamate and then relaxed by treatment with gentamicin (curves a and b). The treatment of portions of these cultures with ampicillin resulted in lysis (curves c and d). Cultures of strain HDL11 that were grown in the presence of intermediate concentrations of IPTG also demonstrated normal lysis patterns (not shown). These results suggest that penicillin tolerance does not depend upon the membrane levels of individual phospholipids, but is rather a consequence of inhibition of the net phospholipid synthesis.

VI. General Discussion

VI. 1. Phospholipid Synthesis and β-Lactam-Induced Lysis in Amino Acid-Deprived *E. coli*

This study demonstrated for the first time that the penicillin tolerance of amino acid-deprived *E. coli* is based on the dependence of both the PG synthetase and PG hydrolase activities on phospholipid synthesis. Fig. 28 summarizes the current working model on the regulation of PG metabolism in *E. coli* by the stringent response. A correlation between the inhibition of penicillin-sensitive PG synthesis and the onset of the stringent response in amino acid-deprived *relA*$^+$ cells has been previously demonstrated (Ishiguro *et al.*, 1980). Furthermore, the inhibition of transpeptidation is reversible, and PG 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 (Kusser & Ishiguro, 1985). It has also been demonstrated both *in vivo* (Ishiguro, 1983) and *in vitro* (Ishiguro, 1993) that PG

Table 1. Levels of individual phospholipids in the strain HDL11 grown in the presence or in the absence of IPTG. Bacteria were grown in the presence of 1 mM IPTG or without IPTG for 8 doublings of cell mass. Phospholipids were labelled with [^{32}P]-orthophosphate and extracted with chloroform-methanol. Individual phospholipids were separated on a silica gel G thin-layer chromatography plate. The amounts of radioactivity corresponding to each spot were quantified (the results presented are the average values of three determinations).

IPTG concentration	Phosphatidyl-glycerol	Phosphatidyl-ethanolamine	Cardiolipin
0	2 ± 0.5 %	97 ± 2 %	≥0.5%
1 mM	18 ± 1 %	77 ± 1.5 %	5 ± 1 %

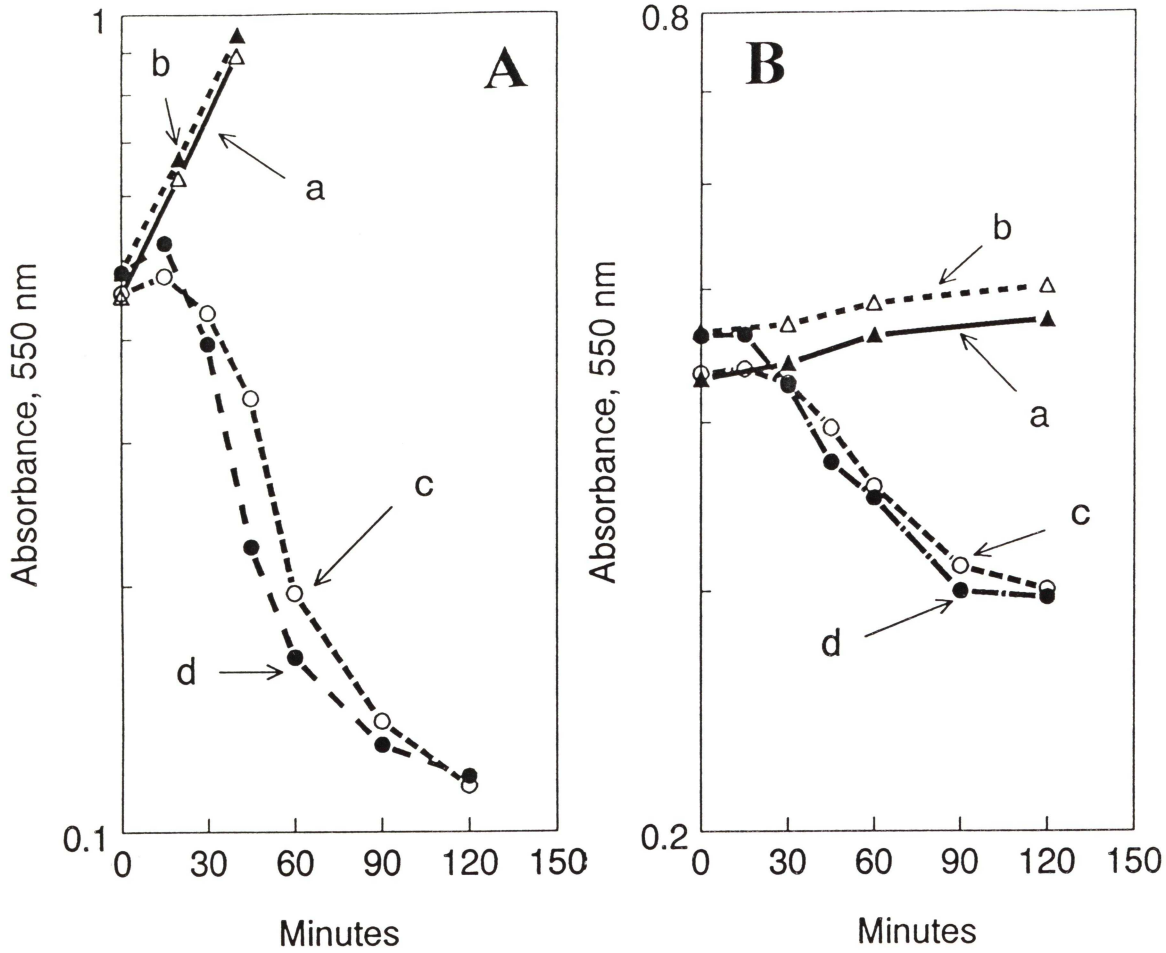

Figure 27. Effect of varying concentrations of acidic phospholipids on ampicillin-induced lysis in growing and nongrowing *E. coli*. Cultures of strain HDL11 (*pgsA*$^-$, *Φ(lacOP-pgsA*$^+$)) were grown for 8 generations in the absence or in the presence of 1 mM IPTG to vary the levels of acidic phospholipids. **Panel A.** An exponential phase culture of strain HDL11 grown in the absence of IPTG was divided into two portions. One portion was left untreated (culture a), whereas the other one received 50 μg/ml of ampicillin (culture c) at 0 minutes. Likewise, the culture grown in the presence of 1 mM IPTG was also divided into two parts: one received no further treatment (culture b), whereas the other received ampicillin (50 μg/ml) at 0 minutes (culture d). **Panel B.** Exponential phase cultures of strain HDL11 (*pgsA*$^-$, *Φ(lacOP-pgsA*$^+$)) grown without IPTG or in the presence of 1 mM IPTG were treated with serine hydroxamate (500 μg/ml) to induce the stringent response and with gentamicin (100 μg/ml) to relax it at 0 minutes. The culture that was grown in the absence of IPTG was then divided into two parts: one part received no further treatment (culture a), and the other was treated with ampicillin (50 μg/ml, culture c). The culture grown in the presence of 1 mM IPTG was also divided into two portions which received no further treatment (culture b) or 50 μg/ml of ampicillin (culture d).

polymerization exhibits a strict requirement for ongoing phospholipid synthesis. Phospholipid synthesis is known to be inhibited during the stringent response (Sokawa *et al.*, 1968, Heath *et al.*, 1994). Therefore, Fig. 28 proposes that ppGpp inhibits PG synthesis indirectly during the stringent response through its inhibitory action on phospholipid synthesis as previously reported (Ishiguro, 1983).

It is generally thought that at least some PG hydrolase activity is essential for normal PG synthesis (e.g., Höltje, 1993), but the exact nature of this requirement is far from understood. The model in Fig. 28 reflects the views of Pelzer and Weidel (1964) who proposed that PG synthesis involved the coordinated activities of the PG synthetases and the PG hydrolases. The bacteriolysis resulting from treatment with an inhibitor of PG synthesis is PG hydrolase-mediated (Tomasz *et al.*, 1970). It has have previously shown (Pisabarro *et al.*, 1990), and confirmed here, that the role of PG 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 theactivities of the PBPs and PG hydrolases are regulated independently during the stringent response. Despite their independent regulation, both activities appear to be tightly coupled. Although the kinetics of inactivation of the PBPs and PG hydrolases during the stringent response cannot be measured accurately with the existing technology, it has been previously estimated that both activities are inhibited at approximately the same time during the course of amino acid deprivation (Pisabarro *et al.*, 1990). 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. A minimum of 20 min of penicillin treatment is essential to fulfil 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 (Pisabarro *et al.*, 1990). In summary, both the PBP and PG hydrolase activities are inhibited by the stringent response within 10 to 20

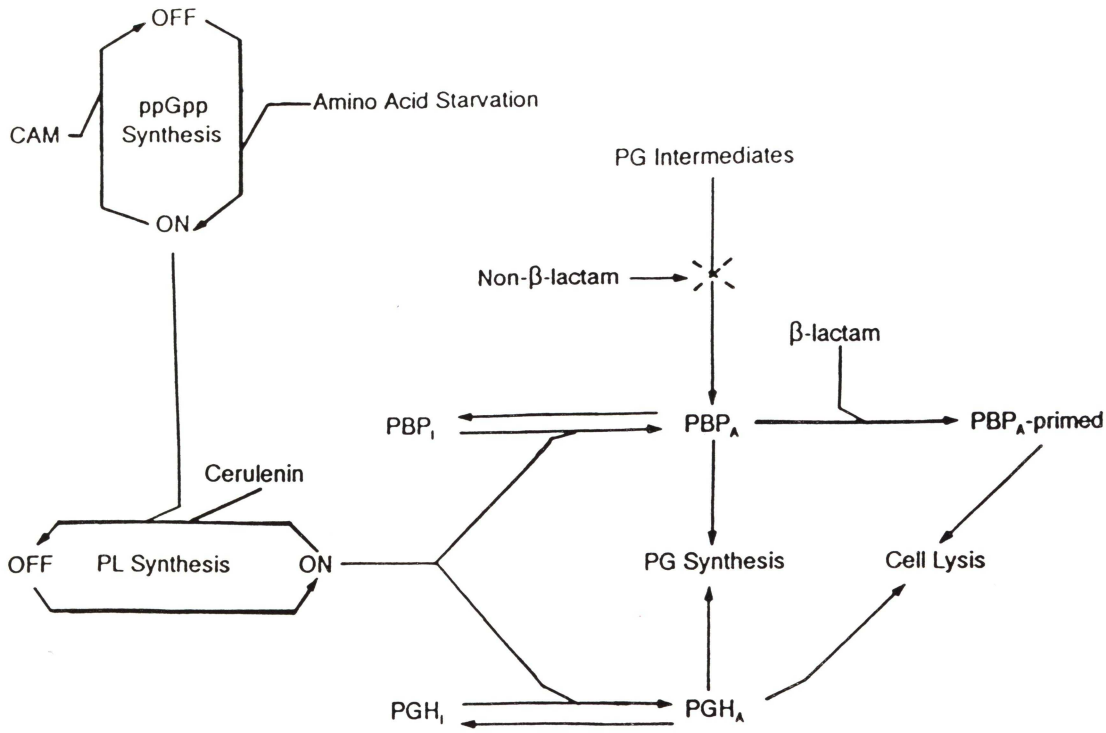

Fig. 28. Model for antibiotic-induced bacteriolysis of amino acid-deprived *E. coli*. The PBPs and PG hydrolases (PGH) may exist in two alternate states, i.e., in inactive (PBP_i and PGH_i) and active (PBP_A and PGH_A) forms. The active forms of these enzymes are dependent on phospholipid (PL) synthesis. Normal 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 PG 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 uncouple PG hydrolase activity to cause lysis whereas non-β-lactams achieve the same effect by interrupting the supply of PG biosynthetic intermediates.

minutes after the start of amino acid deprivation. The current study indicates that the activities of the PBPs and PG 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. 28.

The observations summarized in Fig. 28 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 (Pisabarro *et al.*, 1990). 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), 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 labelled "PBP_A-primed"; and (ii) the inhibition of PG hydrolase activities by the stringent response, i.e., the formation of the component labelled "PGH_i". As already noted, the PBPs and PG 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 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 (Pisabarro *et al.*, 1990). Consequently, penicillin tolerance in this case must be solely due to the RelA-dependent inactivation of PG 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 PG biosynthesis appears to be sufficient to uncouple the activities of PG 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 PG precursors. Furthermore, amino acid-deprived *relA*$^+$ cells develop tolerance to lysis induced by these agents as they do to β-lactams, and inhibitors of the stringent response restore lysis competence. It is shown here that amino acid-deprived cells which were committed to lysis induction by non-β-lactam agents, through relaxation of the stringent response, can be rescued by treatment

with cerulenin. This observation was used to support the conclusion that phospholipid synthesis is necessary for the lysis induction process. Furthermore, these results indicate that tolerance to non-β-lactams could be attributed to the inhibition of PG hydrolases by the stringent response. However, it is possible that non-β-lactam tolerance may also occur upon the inactivation of PBPs (i.e., the formation of PBP_i) by the stringent response. As depicted in Fig. 28, this event would block the biosynthetic pathway and would consequently prevent the detection of a deficiency of PG precursors resulting from non-β-lactam treatment before the PG hydrolase activities can be uncoupled.

The finding that the priming stage, which apparently represents the binding of β-lactam by PBPs, was dependent on phospholipid synthesis is consistent with the earlier observations indicating that phospholipid synthesis is required for PG synthesis (Ishiguro, 1983), and specifically for the transpeptidation activity (Ishiguro, 1993). An attempt was made to demonstrate the dependence of PBP activity on phospholipid synthesis more directly through *in vivo* labelling of PBPs with either biotinylated ampicillin or [^{3}H]-benzylpenicillin. It was found in both cases that the inhibition of phospholipid synthesis by cerulenin during amino acid deprivation did not affect the labelling efficiencies of the individual PBPs. In each case, the normal full complement of PBPs was labelled, and a comparison of cells deficient in phospholipid synthesis and control cells revealed no quantitative differences in their PBP labelling patterns. These results may not be unexpected, since it has been reported previously that the labelling efficiencies of individual PBPs were not affected by the stringent response, i.e., under conditions where PG synthesis and penicillin-induced lysis are inhibited (Tuomanen, 1986; Kusser *et al.*, 1990). In order to explain her results, Tuomanen (1986) has proposed that only a small fraction of the total existing PBPs was required for normal PG synthesis in *E. coli* and *Streptococcus pneumoniae*. For instance, bacteria exhibited normal growth rates even when the activities of over 90% of their PBPs were inhibited by β-lactam antibiotics. An interesting and relevant observation was that this critical PBP subset was newly synthesized; the recovery of cell growth upon removal of penicillin could not be explained by the deacylation of inactivated PBPs. According to Tuomanen, this PBP subset undergoes continuous turnover; i.e., PBPs involved in PG synthesis apparently have short functional half-lives, and their replacement by newly synthesized PBPs is a

process which is essential for normal PG synthesis. Tuomanen also proposed that penicillin-induced lysis was dependent on the acylation of this small subset of PBPs. Therefore, according to Tuomanen's hypothesis, the bulk of PBPs are catalytically active, with respect to penicillin-binding activity, but do not participate in PG synthesis. Consequently, one unresolved problem is the role of these PBPs which she has named the "silent majority" and which appear to be dispensable as far as PG synthesis is concerned. In light of Tuomanen's hypothesis, the priming stage may require the penicillin-induced inactivation of only the small subset of PBPs specifically involved in PG synthesis. The results presented here suggest that it is this small subset which is inactivated when phospholipid synthesis is inhibited.

Höltje (1993) has recently proposed a simple, highly feasible model for the growth of *E. coli* PG sacculus which couples the processes of PG turnover and biosynthesis. A multienzyme complex composed of PBPs and PG hydrolases is hypothesized to facilitate this process, and evidence for such a multienzyme complex has been presented (Said & Höltje, 1983; Romeis & Höltje (1994). It is possible that the minor fraction of PBPs, referred to above, is associated with Höltje's hypothetical multienzyme complex. According to this view, only the activities of the multienzyme complexes will exhibit a dependence on phospholipid synthesis. The hypotheses of Tuomanen (1986) and Höltje (1993) are also relevant to the discussion of the results presented in Chapter 4.

The stringent response inhibits all major macromolecular synthesis in bacteria. The inhibition of protein synthesis and RNA synthesis occurs within minutes of the stringent response induction (reviewed in Cashel & Rudd, 1987; see Svitil *et al.*, 1992). DNA replication continues until all rounds of replication in progress are completed, but the initiation of new rounds of replication are blocked by the stringent response, maybe due to inhibition of RNA synthesis (von Meyenburg & Hansen, 1987). This study demonstrated that the inhibition of stable RNA synthesis had no effect on relaxed PG synthesis and penicillin sensitivity of amino acid-deprived *E. coli*. This contradicts the proposal made by Cooper (1991), who suggested that the driving force of the relaxed PG synthesis was the relaxed stable RNA synthesis. According to the surface stress model (e.g., Koch, 1993), PG sacculus expands in order to accommodate the growing cytoplasm. It appears, that the relaxed PG synthesis is independent of synthesis of the main

components of cytoplasm, such as stable RNA and total protein.

Inhibition of phospholipid synthesis in amino acid-deprived cells resulted not only in the inhibition of PG synthesis, but also in the inhibition of RNA synthesis. These results confirm the earlier observations of Goldberg *et al.* (1973) and Bell (1974), who reported that inhibition of phospholipid synthesis in growing *E. coli* resulted in eventual inhibition of all major macromolecular synthesis. Phospholipid synthesis, therefore, may control not only PG synthesis (Ishiguro, 1983; Ishiguro, 1993), but also RNA synthesis.

VI. 2. Intracellular ppGpp Levels and β-Lactam-Induced Lysis

In this study, a direct relationship between the accumulation of ppGpp and the regulation of PG metabolism was demonstrated for the first time. To accomplish this, the accumulation of ppGpp was induced directly through the controlled overexpression of the cloned *relA* gene, and the necessity to employ amino acid deprivation was thereby bypassed. One important conclusion from this study was that the inhibition of PG synthesis and the development of penicillin tolerance were both directly associated with the accumulation of ppGpp. The effects of ppGpp on these processes were concentration-dependent. Furthermore, the minimum effective concentrations of ppGpp in both cases were identical; the required concentration was about 30% lower than the maximum concentration accumulated during the stringent response.

While this work was in progress, Heath *et al.* (1994) reported that phospholipid synthesis in *E. coli* was inhibited when ppGpp accumulation was induced by the overexpression of RelA. This study has confirmed their result and, in addition, have shown that the concentration of ppGpp which was necessary to inhibit phospholipid synthesis was approximately the same as that required to inhibit PG synthesis and to induce penicillin tolerance. This study has also demonstrated that the overproduction of PlsB abolished both ppGpp-associated penicillin tolerance and ppGpp-induced inhibition of PG synthesis. This provides still further support for the proposal that PG metabolism and penicillin-induced bacteriolysis are dependent on ongoing phospholipid synthesis.

Interestingly, treatment of *E. coli* with either ampicillin or cephaloridine (the only β-lactams tested) resulted in the accumulation of ppGpp. The kinetics of ppGpp

accumulation were directly dependent on the concentrations of the β-lactam employed. It is known that intracellular levels of ppGpp are inversely related to growth rate (Sarubbi *et al.*, 1988; Hernandez & Bremer, 1991). Therefore, the simplest explanation for these observations would be one based on the fact that treatment with β-lactam antibiotics resulted in the inhibition of growth with the degree of inhibition being directly dependent on β-lactam concentration. This inhibitory effect on growth could, in turn, have been responsible for the induction of ppGpp accumulation. However, further studies on this phenomenon suggest that the mechanism is apparently more complex than this. For example, although norfloxacin and rifampicin were at least as effective as inhibitors of growth, they failed to induce ppGpp accumulation. On the other hand, it is unlikely that this effect is specifically associated with β-lactam agents. Gramicidin and polymyxin have been shown previously to cause ppGpp accumulation, and this was attributed to the abilities of these antibiotics to interfere with ppGpp degradation by an, as yet, uncharacterized mechanism (Cortay & Cozzone, 1983). Another unexpected result was the RelA dependence of the β-lactam-induced ppGpp accumulation. As already noted, the growth rate-controlled synthesis of ppGpp has been attributed to SpoT or ppGpp synthetase II (Hernandez & Bremer, 1991; Xiao *et al.*, 1991) whereas the RelA activity has long been considered as being dedicated to sensing amino acid deprivation (Cashel & Rudd, 1987). The link between amino acid limitation and treatment with β-lactam agents is not obvious, and this matter clearly requires further investigation. Finally, the maximum levels of ppGpp accumulated during the course of treatment with β-lactam antibiotics, while significant, were insufficient to induce tolerance to β-lactam antibiotics. For example, the highest concentration of ppGpp achieved during ampicillin treatment was at least two-fold lower than the minimum concentration required for the induction of ampicillin tolerance (Fig. 23).

A correlation between ppGpp accumulation and mecillinam resistance has been previously demonstrated in *E. coli* (Joseleau-Petit *et al.*, 1994; Vinella & d'Ari, 1994; Vinella *et al.*, 1992), and it has been of interest to determine whether this phenomenon is related to the ppGpp-dependent penicillin tolerance described here. Mecillinam is a β-lactam agent which specifically inhibits penicillin-binding protein 2 (PBP2). The inhibition of PBP2 results in the production of spherical cells and is lethal when bacteria

are grown in rich medium; these observations indicate that PBP2 is essential for the lateral elongation of cell wall PG (Bouloc *et al.*, 1992). However, PBP2 is dispensable when the ppGpp levels in *E. coli* are elevated; such bacteria are in fact resistant to mecillinam (Bouloc *et al.*, 1992, Vinella *et al.*, 1992). For example, bacteria continue to grow as spherical cells in the presence of mecillinam when the stringent response is partially induced (Vinella *et al.*, 1992). Joseleau-Petit *et al.* (1994) have recently quantified the minimum concentration of ppGpp required to confer mecillinam resistance and have found this value to be about 140 pmol of ppGpp per A_{600}. This value is over 7-fold lower than the minimum amount of ppGpp required to induce penicillin tolerance as determined in this study; i.e.,the minimum level of ppGpp for penicillin tolerance was determined to be 870 pmol per mg cell dry weight or about 1,000 pmol per A_{420}. It is also noteworthy that the proposed role of ppGpp in mecillinam resistance is very different from the hypothesis concerning phospholipid synthesis and PG metabolism. The inactivation of PBP2 results in the inhibition of cell division which can be reversed by the introduction of a multicopy plasmid carrying *ftsZ* (Vinella *et al.*, 1993). Furthermore, mecillinam-resistant spherical cells have diameters which are almost four times larger than those of their normal rod-shaped counterparts, and it is thought that such cells may require more FtsZ protein to carry out septation (Joseleau-Petit *et al.*, 1994). It has therefore been suggested that the role of ppGpp in mecillinam resistance may be to stimulate the production of FtsZ, possibly at the level of transcription of *ftsZ*. On the bases of the amounts of ppGpp required and the proposed modes of action, it can be concluded that the ppGpp-dependent mecillinam resistance studied by D'Ari's group is not directly related to the phenomenon of penicillin tolerance described here.

VI. 3. Phospholipid Synthesis and β-Lactam-Induced Lysis in Growing Bacteria

Inhibition of phospholipid synthesis in growing *E. coli* also resulted in tolerance to the killing by penicillin. It must be noted that this effect was observed during the initial stages of cerulenin treatment, i.e., before the antibiotic had visible effects on growth.

The inhibition of autolysis by cerulenin treatment has been observed previously.

In their experiments with growing cultures of *E.coli*, Leduc *et al.* (1982) demonstrated that extensive (30 minute) pre-treatment with cerulenin protected cells from moenomycin and cephaloridine-induced lysis. However, these results were interpreted as the necessity of fatty acid synthesis for the induction of lysis. The experiments presented in this study demonstrate that inhibition of phospholipid synthesis rather then fatty acid synthesis is required for achievement of phenotypic tolerance by either growing or amino acid-starved bacteria.

In view of the results of Seyfzadeh *et al.* (1993), phenotypic tolerance observed in the course of cerulenin treatment could be explained as a consequence of ppGpp accumulation due to inhibition of fatty acid synthesis. This study quantified the maximum levels of ppGpp induced in the course of cerulenin treatment: though significant (450 pmol per mg dry cell weight), they were lower than the minimum concentration necessary to achieve inhibition of phospholipid and PG syntheses and penicillin tolerance (870 pmol per mg dry cell weight). Moreover, it was demonstrated, that phenotypic tolerance is induced in growing *E. coli* cells when phospholipid synthesis is inhibited in the absence of any ppGpp synthesis (i.e., when phospholipid synthesis is inhibited either by glycerol deprivation of a *plsB* or a *gpsA* mutant, or by cerulenin treatment of Δ*relA* Δ*spoT* double mutant). It is also important to note, that SpoT-dependent ppGpp synthetic pathway is blocked in amino acid-deprived cells due to reasons that are not yet understood (Cashel & Rudd, 1987). During the course of this work it was confirmed, that ppGpp accumulated neither in amino acid-deprived strain of VC7 in the presence of chloramphenicol nor in amino acid-starved VC8 cells in response of cerulenin treatment (unpublished results). Therefore, the results obtained in amino acid-deprived cells could not be influenced by the elevated intracellular ppGpp levels.

In their studies with *Bacillus subtilis*, Rogers and Thurman (1985) used a subinhibitory concentration of cerulenin which only partially inhibited phospholipid synthesis and permitted exponential growth to continue. The protein and PG contents of these cerulenin-treated cells were the same as those of the untreated control, whereas the cerulenin-treated cells reduced their phospholipid content by 50%. It was thus suggested, that the inhibitory effect of cerulenin on autolytic activity of *B. subtilis* could reflect the decrease in phospholipid-to-protein ratio in the membrane (Rogers and Thurman, 1985).

This hypothesis could also explain the phenotypic tolerance to penicillin observed by growing *E. coli*. However, the experiments performed in amino acid-deprived cells (i.e., in the absence of protein synthesis) rule out such possibility for the control of penicillin-induced lysis in *E. coli*.

The exact mechanism of coupling of phospholipid synthesis to the regulation of PBP and PG hydrolase activities is unknown. Presumably, inhibition of net phospholipid synthesis reversibly affects some important property of the inner membrane. Interestingly, depletion of the inner membrane of acidic phospholipids that are critical for a number of vital cell functions, including the initiation of DNA replication (Sekimizu & Kornberg, 1988) and protein transport across the inner membrane (Kusters *et al.*, 1994) did not change lytic properties of the bacteria.

Chapter 4: Other Factors that Control Autolytic Activity of *E. coli*

I. Overview of Chapter Contents

The results presented in this Chapter are derived from attempts to determine the basis for the coupling of PG metabolism to phospholipid synthesis. Although these attempts were negative, the following useful conclusions were made. (i) De-energization of the *E. coli* cytoplasmic membrane resulted in penicillin tolerance due to the inhibition of both the priming and the lysis induction stages. (ii) The temperature sensitivity of β-lactam-induced lysis was not due to the induction of the heat-shock response, as previously reported, but from a reversible inhibition of an unidentified thermosensitive enzyme(s) involved in the lysis induction stage. (iii) Inhibition of protein synthesis in the absence of the stringent response promoted both the priming and the lysis induction stages resulting in a faster onset of β-lactam-induced lysis.

II. Energy Uncouplers Inhibit β-Lactam-Induced Lysis of Amino Acid-Deprived *E. coli*

In preliminary experiments, a variety of energy uncouplers were found to inhibit the penicillin-induced lysis of amino acid-deprived bacteria. The minimum concentrations that completely prevented lysis were determined to be 10 mM for sodium azide, 5 mM for potassium cyanide, and 50 μM for CCCP. It is notable that these concentrations were higher than the MICs for these uncouplers and were sufficient to dissipate the proton-motive force in the strains used. Therefore, these agents were used at these concentrations in all the experiments reported here.

Figure 29 shows the effect of sodium azide on ampicillin-induced lysis of strain VC8 (*relA*). An isoleucine-deprived culture was divided into four parts. Curve a represents a control which received no additional treatment. The addition of sodium azide had no further effect (curve c). Treatment with ampicillin caused lysis (curve b). This lysis was inhibited by sodium azide (curve d). Similar results were obtained with amino acid-deprived cells of strain VC7 (*relA*$^+$) that were relaxed by chloramphenicol treatment (not shown).

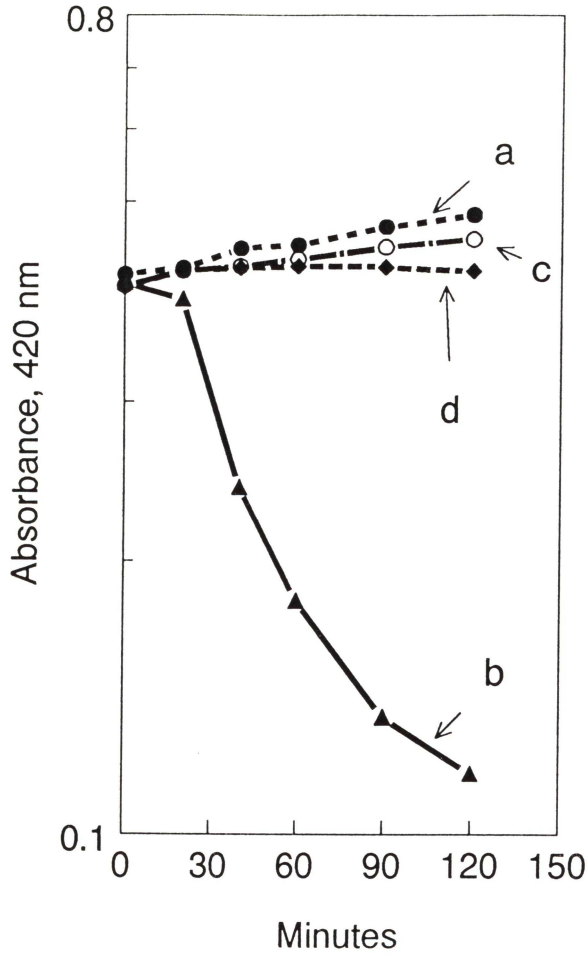

Figure 29. Effect of sodium azide on ampicillin-induced lysis of amino acid-deprived *E. coli*. An exponential phase culture of strain VC8 (*relA*) was deprived of isoleucine at 0 minutes and divided into four portions. These subcultures received: no further treatment (culture a); ampicillin (culture b); sodium azide (culture c), and a combination of sodium azide and ampicillin (curve d). Concentrations of ampicillin and sodium azide used were 50 μg/ml and 10 mM, respectively.

The same results were obtained when other β-lactams (i.e., benzylpenicillin, cephaloridine, and imipenem) were used instead of ampicillin (not shown). Furthermore, potassium cyanide and CCCP also induced penicillin tolerance (not shown).

It is noteworthy that the effect of energy uncouplers on these cells was transient and depended upon the constant presence of an uncoupler: cells washed free of uncoupler regained susceptibility to ampicillin-induced lysis. Also, viable cell counts did not decrease over a two hour incubation of amino acid-starved cells of strains VC7 and VC8 in the presence of a combination of penicillin and either sodium azide or potassium cyanide. When amino acid-deprived cultures of strains VC7 and VC8 were incubated in the presence of CCCP and penicillin, no loss in cell viability was observed within first 40 minutes. A two hour incubation, however, resulted in 70% loss in cell viability, although optical density remained unchanged. A similar loss in viability was observed when a parallel culture of amino acid starved cells was incubated in the presence of CCCP alone. Therefore, the observed loss in cell viability is probably due to the fact that CCCP may act as a sulfhydryl agent as well as a protonophore (Kaback *et al.*, 1974) and may affect some vital cell function this way. Thus, energy uncouplers prevented penicillin-induced lysis of *E. coli*.

II. 1. The Priming Stage is Inhibited by De-Energized Membrane

The effect of energy uncouplers on the priming stage of ampicillin-induced lysis was investigated. Figure 30 shows the effect of sodium azide on the priming stage. Portions of an isoleucine-deprived culture of strain VC7 were primed with ampicillin in the presence and in the absence of sodium azide. Priming was terminated, and both cultures were resuspended in the isoleucine starvation medium. Cells which were primed in the absence of sodium azide did not lyse (curve a) unless the stringent response was relaxed by treatment with chloramphenicol (curve b) as already described. Cells that were primed in the presence of sodium azide also did not lyse when left untreated (not shown). Moreover, the presence of sodium azide during the priming stage clearly inhibited priming as evidenced by the fact that these cells did not lyse upon relaxation of the stringent response with chloramphenicol (curve c, compare with curve b). On the

other hand, the inhibitory effect of azide was transient and depended upon the continuous presence of the uncoupler. Therefore, cells primed in the presence of sodium azide lysed when simultaneously treated with a combination of chloramphenicol and ampicillin as indicated by curve d.

In another experiment, sodium azide was added 5 minutes after the initiation of priming. The results were the same as those shown in Fig. 30, indicating that the uncoupler action was rapid and not specific to the initial stages of priming (not shown). Furthermore, this effect was not specific for azide, as potassium cyanide and CCCP also inhibited ampicillin priming (not shown).

PG synthesis was monitored in amino acid-deprived cultures of strain VC8 (*relA*) in the presence and in the absence of sodium azide (Fig. 31). Bacteria were pre-labelled with [^{3}H]-DAP for 20 minutes, and the exogenous [^{3}H]-DAP was removed. The cells were then isoleucine-deprived. As shown previously (Wientjes *et al.*, 1985), the intracellular levels of labelled PG precursors, under these conditions, were sufficient to sustain normal rates of the radiolabel incorporation into PG for at least 1 hour. Incorporation of the label into PG fraction was normal in the control culture (curve a). Sodium azide inhibited the incorporation of the radiolabelled precursors into PG within 5 minutes (curve b), indicating that this process was dependent on energized membrane. It is important to note that the inhibitory effect of azide on PG synthesis can not be attributed to the inhibition of [^{3}H]-DAP transport.

An attempt was made to determine whether the penicillin binding to PBPs was inhibited by azide treatment. Amino acid-starved relaxed cells of strains VC7 and VC8 were pre-treated with 10 mM of sodium azide for 20 minutes and the PBPs were labelled *in vivo* with [^{3}H]-benzylpenicillin. Importantly, sodium azide was also present throughout the entire labelling stage. The labelling patterns of individual PBPs were indistinguishable in azide-treated and control cells (Fig. 32). Similar results were obtained when the PBPs were labelled with biotinylated ampicillin (not shown). Therefore, azide apparently does not inhibit penicillin-binding. As discussed below, these results may indicate that the PBPs involved in penicillin-induced lysis represent a minor fraction of the total PBPs; i.e., a fraction which can not be distinguished by the existing methods for the PBP assay.

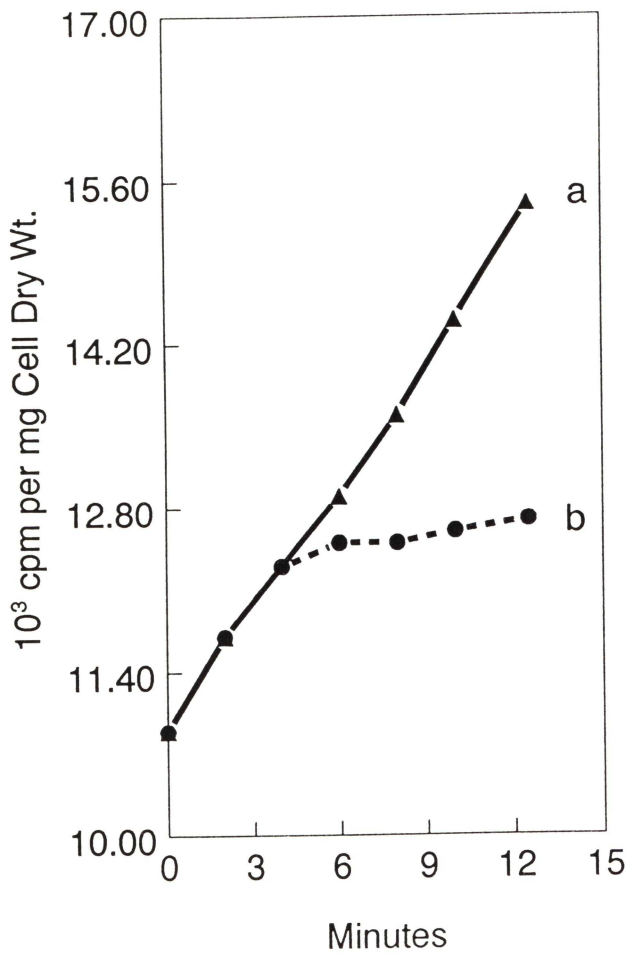

Figure 31. Inhibition of PG synthesis in amino acid-deprived *E. coli* by sodium azide treatment. An exponential phase culture of strain VC8 (*relA*) was pre-labelled with [^{3}H]-DAP (final concentration, 0.2 μg/ml, 10 μCi/ml) for 20 minutes, washed free of exogenous [^{3}H]-DAP and resuspended in isoleucine deprivation medium for measurements of PG synthesis. One culture received no further treatment (curve a), and the other received 10 mM sodium azide (curve b).

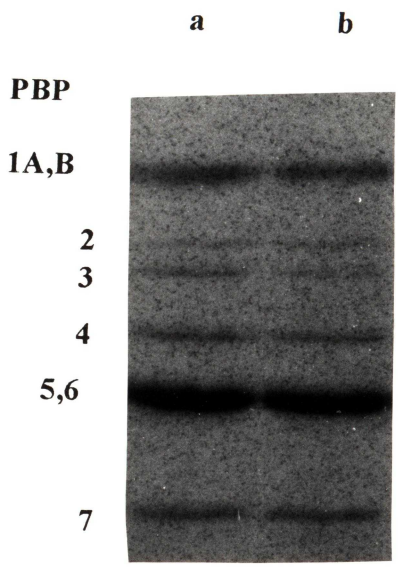

Figure 32. PBP labelling profiles of amino acid-deprived *E. coli* in the presence and in the absence of sodium azide. An exponential phase culture of strain VC7 was deprived of isoleucine and the stringent response was relaxed with chloramphenicol. Cells were further incubated for 20 minutes in the presence or absence of sodium azide (10 mM). The PBPs were labelled with [^{3}H]-benzylpenicillin, separated on SDS PAGE, and visualised by autoradiography. Lane a: the PBPs from the cells treated with sodium azide; lane b: the PBPs from the control cells.

II. 2. The Lysis Induction Stage is Inhibited by De-Energized Membrane

The following experiments were performed to determine the effects of energy uncouplers on the lysis induction stage. In the first experiment, an isoleucine-deprived culture of strain VC7 was primed with ampicillin, resuspended in isoleucine starvation medium, and divided into three parts. As shown in Fig. 33A, a portion which received no further treatment did not exhibit lysis (curve a) whereas a portion which was treated with chloramphenicol to relax the stringent response did lyse (curve b). These two controls indicated that the priming procedure was successful. The third portion was treated with a mixture of chloramphenicol and 10 mM sodium azide. This culture did not lyse (curve c), indicating that sodium azide inhibited the lysis induction stage. These results were verified by determining viable cell counts instead of optical density measurements (not shown). Furthermore, additional experiments indicated that potassium cyanide and CCCP also inhibited the lysis induction stage.

In the second experiment, ampicillin (200 μg/ml) was continuously present during the lysis induction stage. Exponentially growing culture of strain VC8 was deprived of isoleucine and treated with ampicillin. Sodium azide was added to the portions of this culture at different times, and the results are shown in Fig 33B. The culture that received ampicillin only started to lyse about 20 minutes after its addition (curve a), whereas no lysis was observed in the portion of the culture which was treated with sodium azide and ampicillin concomitantly (curve b). Moreover, the addition of sodium azide 15 minutes after the addition of ampicillin (i.e., just before the onset of lysis) to another portion of the culture still completely protected bacteria from lysis (curve c), whereas the addition of sodium azide 20 minutes after the initiation of ampicillin treatment, i.e. when lysis already started, rescued the majority of the cells from lysis (curve d). These results were verified with viable cell counts (not shown). Therefore, energized membrane is important for a successful achievement of the lysis induction stage.

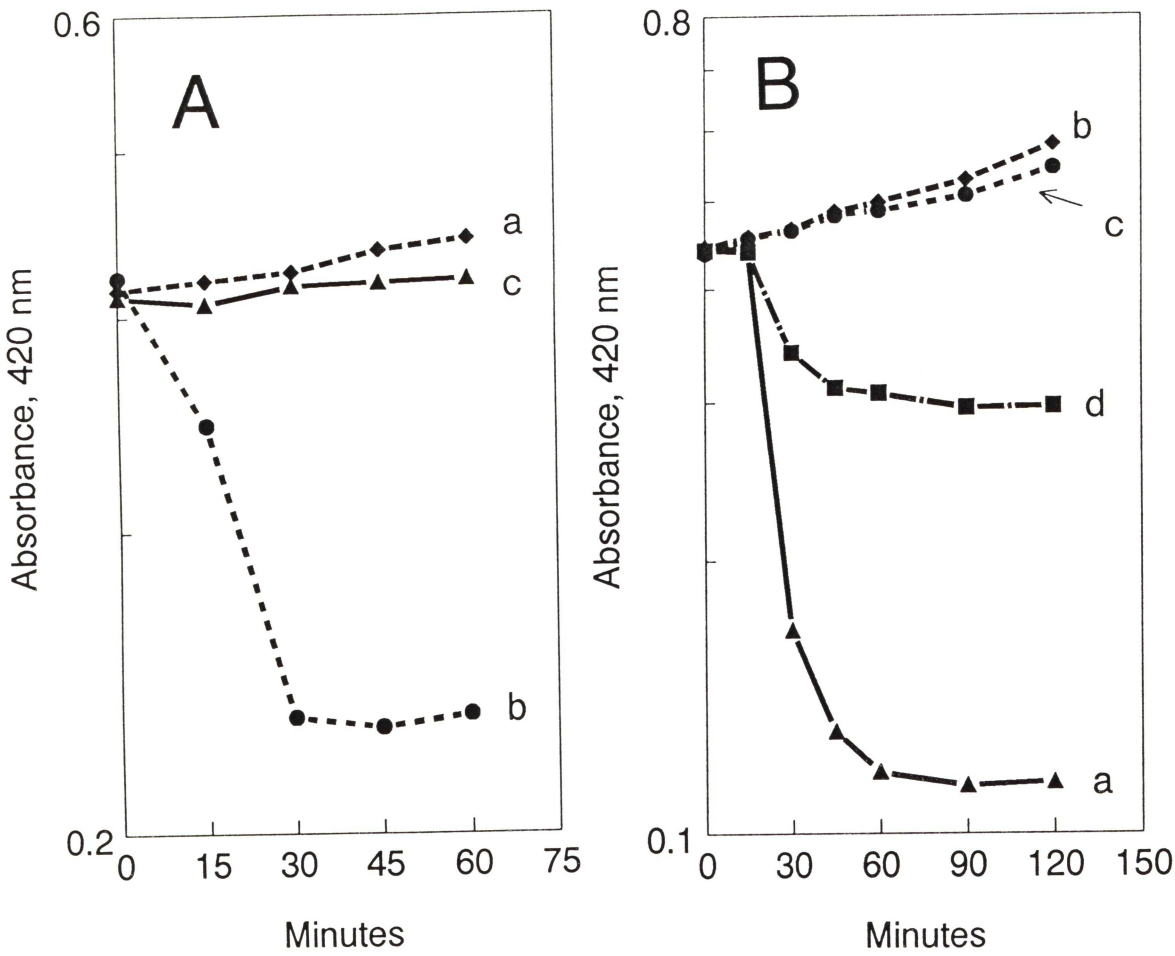

Figure 33. Inhibition of the lysis induction stage by sodium azide. **Panel A.** An exponential phase culture of strain VC7 was deprived of isoleucine and primed with 200 μg/ml of ampicillin for 20 minutes. Unbound ampicillin was then washed out. The primed cells were resuspended in isoleucine starvation medium and divided into three portions. These subcultures received: no further treatment (culture a), chloramphenicol (100 μg/ml) (culture b), and chloramphenicol (100 μg/ml) plus sodium azide (10 mM) (culture c). **Panel B.** An exponential phase culture of strain VC8 (*relA*) was isoleucine deprived and treated with ampicillin (200 μg/ml) at 0 minutes. A portion of this culture received no further treatment (culture a), whereas other portions received sodium azide (10 mM) at 0 minutes (culture b), 15 minutes (culture c) and 20 minutes (culture d).

II. 3. Effects of Cerulenin and Energy Uncouplers on Proton-Motive Force and Intracellular ATP Levels in Amino Acid-Deprived *E. coli*

The inhibition of phospholipid synthesis and the action of energy uncouplers both result in the inhibition of PG metabolism. One objective of experiments discussed here was to determine whether there were common underlying mechanisms involved in these phenomena.

Figure 34 shows that the energy uncouplers in the concentrations used caused a rapid (within 2 minutes) drop in the intracellular ATP levels. Cerulenin treatment, on the other hand, did not affect ATP concentration inside the cell (Fig. 34). Table 2 shows that cerulenin-treated cells exhibited normal membrane potential and ΔpH. On the other hand, the uncouplers used in this study completely dissipated membrane potential and ΔpH. They also caused a two-fold decrease in ATP levels. Therefore, inhibition of phospholipid synthesis does not cause dissipation of the proton-motive force across the inner membrane of *E. coli* or rapid depletion of intracellular ATP levels. Thus, the obligate coupling of PG metabolism and phospholipid synthesis can not involve membrane de-energization.

III. Temperature Sensitivity of β-Lactam-Induced Lysis in Amino Acid-Deprived *E. coli*

Another factor that interferes with β-lactam-induced lysis of amino acid-deprived *E. coli* is temperature up-shift. The temperature sensitivity of penicillin-induced lysis was originally discovered in this laboratory (Kusser & Ishiguro, 1987). Penicillin-induced lysis of amino acid-deprived relaxed bacteria was temperature-dependent and was markedly inhibited at temperatures approaching 42^{0}C. This temperature sensitivity was not as significant in growing bacteria. Powell and Young (1991) subsequently reported a positive correlation between overexpression of heat-shock genes and tolerance to some β-lactams. They showed that strains expressing certain mutant heat shock genes (including mutant *dnaJ* and *dnaK* genes) were lysis-susceptible, whereas strains overexpressing normal heat-shock genes were penicillin-tolerant. The objective here was

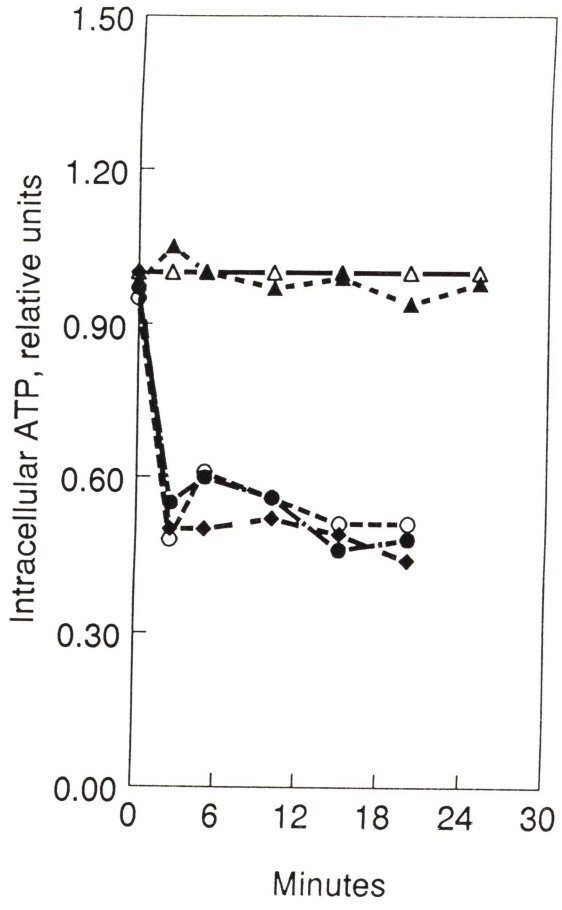

Figure 34. Changes in the intracellular ATP levels in amino acid-deprived *E. coli* cells in response to treatment with cerulenin and energy uncouplers. Exponential phase culture of strain VC8 was deprived of isoleucine and divided into portions, which either received no further treatment (△) or were treated with cerulenin or various energy uncouplers at 0 minutes. At designated time intervals, samples were removed from the subcultures, and ATP was extracted from the cells and quantified. Symbols: (△), culture that received no additions; (▲), cerulenin (50 μg/ml); (○), sodium azide (10 mM); (●), potassium cyanide (5 mM); and (◆), CCCP (50 μM).

Table 3. Determinaion of the proton-motive force and intracellular ATP concentrations in amino acid-deprived strain VC8 (*relA*) treated with cerulenin and energy uncouplers. Membrane potential, ΔpH, and intracellular ATP concentrations were measured as stated in Materials and Methods (Chapter 2). The results presented are the average values of three determinations. The proton-motive force (Δp) was calculated on the basis of the following equation: $\Delta p = \Delta\psi - 59\Delta pH$.

Treatment	Membrane potential, mV	ΔpH	Δp, mV	Intracellular ATP, relative units
None	-145 ± 3	0.95 ± 0.05	-201 ± 12	1
Cerulenin (50 μg/ml)	-142 ± 2	0.8 ± 0.1	-189 ± 22	0.98
Sodium azide (10 mM)	0	0	0	0.51
KCN (5 mM)	0	0	0	0.48
CCCP 50 μM	0	0	0	0.46

to re-investigate the effect of temperature up-shift on penicillin tolerance. The question was whether the temperature sensitivity of penicillin-induced lysis of amino acid-deprived bacteria was due to the induction of the heat-shock response.

III. 1. Induction of the Heat-Shock Response is not Responsible for the Temperature Sensitivity of Penicillin-Induced Lysis in Amino Acid-Starved *E. coli*

Strains VC895 and VC896 were derivatives of strain VC7 carrying the *dnaJ259* and *dnaK756* mutations, respectively. The *dnaJ259* and *dnaK756* alleles were temperature-sensitive mutations. Therefore, both VC895 and VC896 grew normally at 30°C but neither strain exhibited a heat shock response when subjected a temperature upshift to the nonpermissive temperature of 42°C due to the thermoinactivation of DnaJ and DnaK, respectively. These strains were constructed by phage P1-mediated transduction using the closely linked *thr*::Tn*10* insertion as a selectable marker. Therefore, a control strain, VC899, carrying only the *thr*::Tn*10* insertion was also constructed. These strains were used to study the effects of the heat shock response on penicillin-induced lysis of amino acid-deprived bacteria in the experiment described in Fig. 35. Exponential phase cultures grown at 30°C were isoleucine-deprived and 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 (corresponding to 0 min in Fig. 35), chloramphenicol was added to relax the stringent response, and the cultures were treated with ampicillin. 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 (1987) indicating that penicillin-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. It is significant that 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 penicillin-induced killing. (ii) Treatment with the β-lactams, benzylpenicillin, cephaloridine, and imipenem, or the non-β-lactam, phosphonomycin, gave the same results as treatment with ampicillin. Collectively, these experiments indicate that the temperature sensitivity of the penicillin-

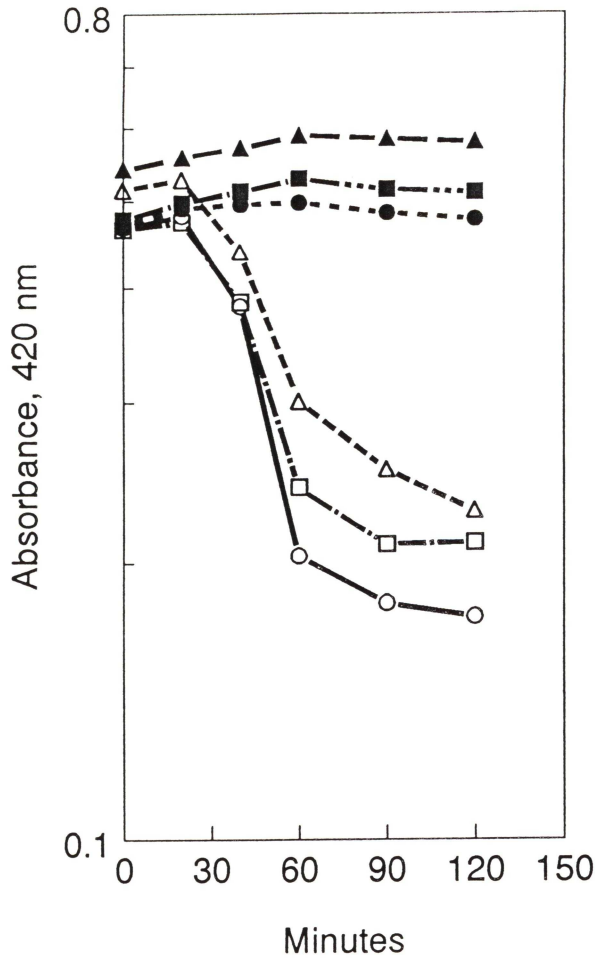

Figure 35. Effect of temperature up-shift on ampicillin-induced lysis of amino acid-deprived *E. coli*. Exponential phase cultures of strains VC899 (wt), VC895 (*dnaJ*), and VC896 (*dnaK*) grown at 30°C were deprived of isoleucine and divided into two portions each. One portion was returned to the 30°C waterbath shaker, whereas the other one was incubated at 42°C waterbath shaker. Both portions of each culture received a combination of 50 μg/ml of ampicillin and 100 μg/ml of chloramphenicol. Open symbols indicate portions of the cultures that were incubated at 30°C, and filled symbols indicate portions of the cultures that were up-shifted to 42°C: VC899 (○ and ●); VC895 (△ and ▲); and VC896 (□ and ■).

induced lysis process in amino acid-deprived bacteria cannot be attributed to the induction of the heat shock response. This means that the observation of Kusser and Ishiguro (1987) cannot be related to that of Powell and Young (1991).

III. 2. Temperature Sensitivity of Penicillin-Induced Lysis in Amino Acid-Deprived *E. coli* Results from the Compromised Lysis Induction

The effect of temperature on the two stages of ampicillin-induced lysis was investigated. Figure 36 demonstrates the effect of temperature upshift on the priming stage. For this experiment, a culture of strain VC7 was grown at 30°C, isoleucine-starved, and divided into two parts. One part was primed with ampicillin at 30°C. Cells from this culture did not lyse in the absence of further treatment (curve a), but did lyse when the stringent response was relaxed by treatment with chloramphenicol (curve b). This indicates that the priming process was successfully achieved at 30°C. The second part of the culture was primed at 42°C. The cells from this culture also did not lyse in the absence of further treatment when incubated at 42°C or when down-shifted to 30°C (curves c and d, respectively). However, this culture lysed when it was down-shifted to 30°C and treated with chloramphenicol (curve e). These results clearly indicate that the priming stage was not inhibited at 42°C.

Two additional results in the experiment shown in Fig. 36 are important with regard to the effect of temperature on the lysis induction stage. (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 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). Therefore, the temperature-sensitive step in penicillin-induced lysis was the lysis induction stage.

In another experiment, incorporation of [^{3}H]-DAP into PG upon temperature up-shift was studied in amino acid-starved relaxed strain VC7. Incorporation of the label was normal in such cells upon the temperature upshift (not shown), indicating that the PBP activities involved in PG synthesis were not affected by the temperature up-shift. This further supports the results shown in Fig. 36.

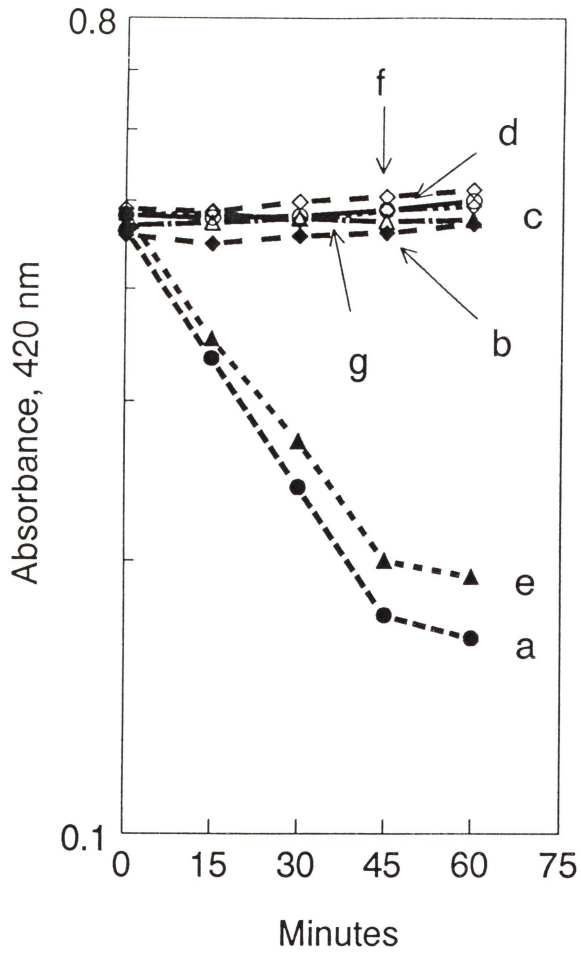

Figure 36. Effect of temperature up-shift on the priming and the lysis induction stages of ampicillin-induced lysis. An exponential phase culture of strain VC7 grown at 30°C was isoleucine-deprived and divided into two portions. One portion was returned to the 30°C waterbath shaker, whereas the other one was up-shifted to the 42°C waterbath shaker. Both portions were primed with ampicillin (200 μg/ml) for a standard period of 20 minutes and further divided into several parts each. Cultures a and b represent cells that were primed at 30°C and were returned to the 30°C waterbath shaker with no further treatment (culture a) or with chloramphenicol (100 μg/ml) to relax the stringent response (culture b). Cultures c, d, and e represent cells that were primed at 42°C. Culture c was returned to the 42° waterbath shaker with no further treatment, whereas cultures d and e were down-shifted to 30°C with no further treatment (culture d) or with chloramphenicol (100 μg/ml) to relax the stringent response (culture e). Culture f was primed at 30°C and up-shifted to 42°C upon the chloramphenicol treatment. Culture g was primed at 42°C and was returned to 42°C upon the chloramphenicol treatment.

To further investigate the temperature sensitivity of the lysis induction stage, cultures of strain VC7 were grown at 30°C and 42°C. These cultures were amino acid-deprived and transferred as described in Fig. 37. Portions of the culture grown at 42°C were treated with a combination of ampicillin and chloramphenicol at 42°C and at 30°C. Ampicillin-induced lysis occurred at 30°C (curve b), but was inhibited at 42°C (curve a). However, lysis was somewhat delayed in the former case and not as pronounced as that in a similarly treated culture grown originally at 30°C (curve c). On the other hand, lysis of the 30° grown culture was inhibited by temperature upshift to 42°C (curve d). These results were further verified with viable cell counts (not shown). Similar results were obtained when other β-lactams, cephaloridine and benzylpenicillin) and non-β-lactam, phosphonomycin, were used instead of ampicillin. These results indicate that the lysis tolerance of nongrowing bacteria caused by the temperature upshift is reversible and does not require *de novo* protein synthesis. Therefore, it may represent temperature sensitivity of an enzymatic autolytic activity.

IV. The Effect of Inhibition of Protein Synthesis on β-Lactam-Induced Lysis of *E. coli*

Previous sections of this Chapter dealt with the factors that interfere with the β-lactam-induced lysis in amino acid-deprived relaxed bacteria. Protein synthesis is inhibited in such bacteria and apparently does not interfere with the lysis; however, several reports have indicated, that inhibition of protein synthesis provides *E. coli* cells with phenotypic tolerance to penicillins (Tomasz, 1979; Goodell & Tomasz, 1980; Leduc *et al.*, 1982). This apparent discrepancy is addressed below. The results presented suggest that a simple inhibition of protein synthesis (i.e., without induction of the stringent response) does not confer rapid tolerance to β-lactam antibiotics. Moreover, inhibitors of protein synthesis promote both the priming and the lysis induction stages of β-lactam-induced lysis.

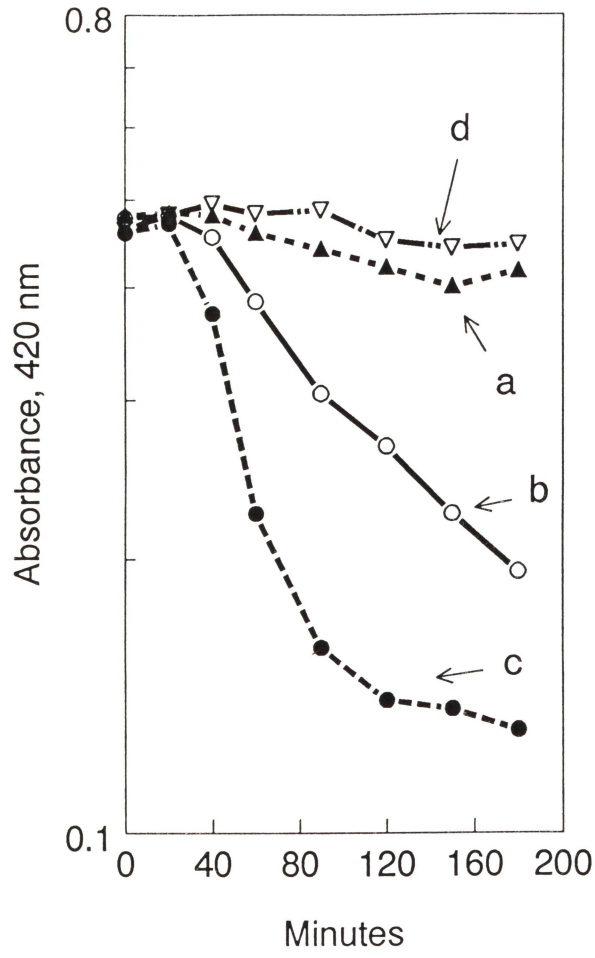

Figure 37. Effect of temperature down-shift on ampicillin-induced lysis of amino acid-deprived *E. coli*. An exponential phase culture of strain VC7 grown at 42°C was deprived of isoleucine at 0 minutes and divided into two parts. One part was left at 42°C (culture a), whereas the other was down-shifted to 30°C (culture b). Both parts were treated with a combination of chloramphenicol (to relax the stringent response) and ampicillin. For comparison, a culture of strain VC7 was grown at 30°C, isoleucine-deprived and treated combination of chloramphenicol and ampicillin at 30°C (culture c) and 42°C (culture d). The concentrations of ampicillin and chloramphenicol used were 50 and 100 μg/ml, respectively.

IV. 1. Inhibition of Protein Synthesis does not Inhibit β-Lactam-Induced Bacteriolysis

Fig. 38 illustrates the effect that chloramphenicol treatment had on ampicillin-induced bacteriolysis of strain VC7. Chloramphenicol 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. 38A). A series of chloramphenicol-treated subcultures were treated with ampicillin at the indicated times. As reported previously (Kusser & Ishiguro, 1986), treatment with chloramphenicol for periods of up to 20 min did not inhibit ampicillin-induced lysis (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 (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 minutes after the addition of chloramphenicol did not result in lysis (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 hours of ampicillin treatment. The results are summarized in Fig. 38B. 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 (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 minutes were ampicillin-tolerant; they did not lyse as noted above, and less than 1% of the cells lost viability (not shown).

The inhibition of protein synthesis and the concomitant stringent response induced by amino acid deprivation results in the rapid onset of ampicillin tolerance as previously reported (Goodell & Tomasz, 1980; Kusser & Ishiguro, 1985). Therefore, the relative

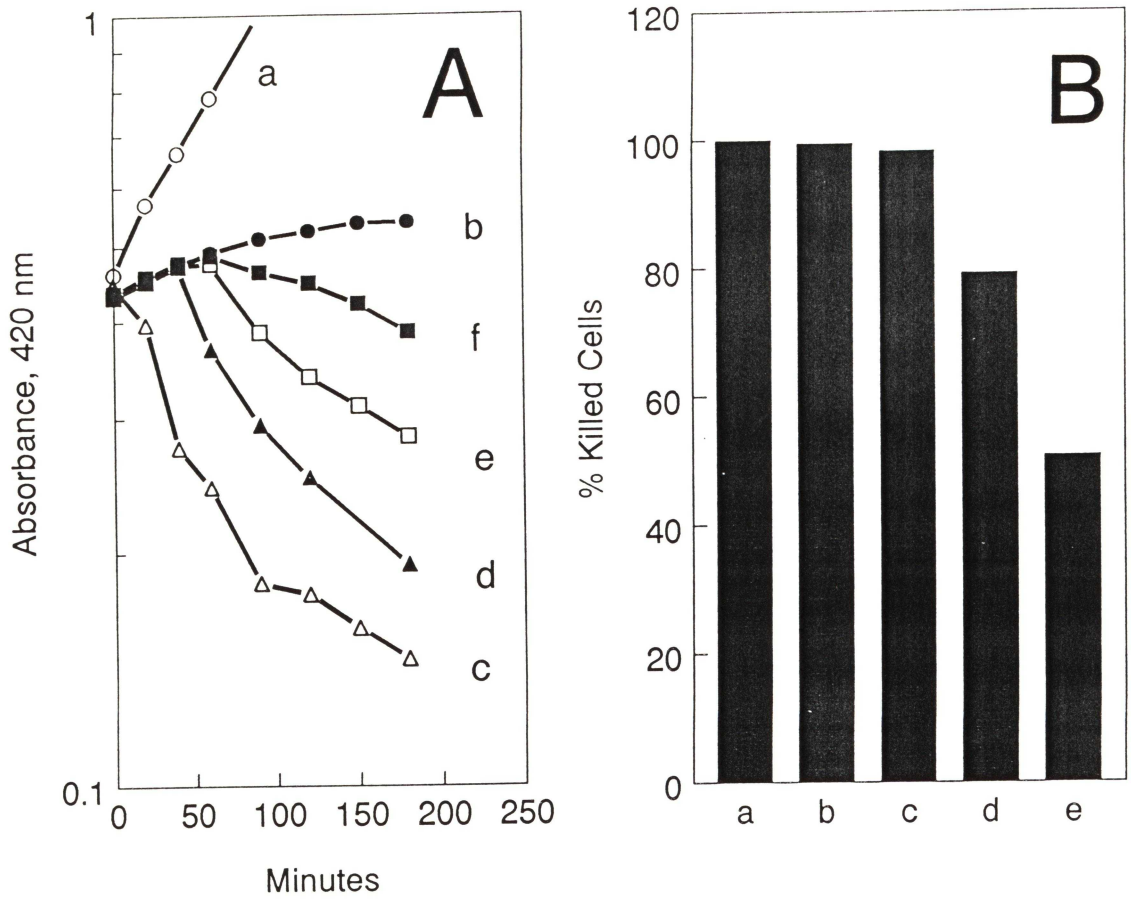

Figure 38. Effect of inhibition of protein synthesis on ampicillin-induced lysis and bactericidal activity of ampicillin. **Panel A.** The effect of chloramphenicol treatment on ampicillin-induced lysis of strain VC7. Culture a represents normal growth of exponential phase culture of strain VC7. A number of portions of this culture was treated with chloramphenicol (100 μg/ml) at 0 minutes. These portions were left without further treatment (culture b), or received ampicillin (50 μg/ml) at 0 (culture c), 20 (culture d), 40 (culture e), and 60 (culture f) minutes. **Panel B.** The effect of chloramphenicol treatment on the bactericidal activity of ampicillin. In the experiments described in panel A, viable cell counts were determined in each of the subcultures before the addition of ampicillin and after 2 hours of ampicillin treatment. The percentage of cells killed are shown for the chloramphenicol-treated subcultures, which were treated with ampicillin at 0 (sample b), 20 (sample c), 40 (sample d), and 60 (sample e) minutes after initiation of chloramphenicol treatment. For comparison, sample a represents percentage of the cells killed in a culture that was treated with ampicillin alone (i.e., in the absence of chloramphenicol) for 2 hours.

ineffectiveness of ribosome inhibitors on countering ampicillin-induced lysis as shown in Fig. 38 was considered unusual. The following experiments address this issue. They show that chloramphenicol (and other inhibitors of protein synthesis) and amino acid deprivation affected β-lactam-induced bacteriolysis of *relA*$^+$ bacteria in different ways.

IV. 2. Inhibition of Protein Synthesis Promotes Both the Priming and the Lysis Induction Stages of β-Lactam-Induced Lysis

The system for dissociating the priming and lysis induction stages was originally developed using amino acid-deprived bacteria (Pisabarro *et al.*, 1990). This procedure was modified by achieving the priming stage in normal growing bacteria. The remainder of the strategy was essentially the same as the original procedure; i.e., after priming with ampicillin, the development of the lysis induction stage was inhibited by inducing the stringent response; the lysis induction stage was subsequently activated by relaxing the stringent response with chloramphenicol. In the initial experiments, samples from an exponential phase culture of strain VC7 (*relA*$^+$) were treated with ampicillin (200 μg/ml) for various time intervals to determine the minimum time of treatment required for the priming of growing bacteria. After the treatment, the unbound ampicillin was removed and the cells were resuspended in fresh complete M9 minimal medium. The results of 10-min and 15-min treatments with ampicillin are shown in Figs. 39A and 39B, respectively. Bacteria which were ampicillin-treated for 10 min recovered from the treatment and resumed growth about 10 min after resuspension in fresh M9 medium (curve a, Fig. 39A). 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 (curve a, Fig. 39B). Isoleucine deprivation, achieved by the addition of L-valine at 500 μg per ml, prevented the lysis of bacteria which were treated with ampicillin for 15 min (curve b, Fig. 39B). 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 (curve c, Fig. 39B). 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 (curve a, Fig. 39B) indicates that the 15-min treatment with ampicillin was more than sufficient to fulfil 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. 39B). With respect to the bacteria subjected to a 10-min treatment with ampicillin, a comparison of curves a and b in Fig. 39A 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 (curve c; Fig. 39A). 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 (curve b, Fig. 39A) 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 in curve c 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 (curve a, Fig. 39A). 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 proteinsynthesis 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. 40A and 40B, respectively. Both strains recovered from the 13-min ampicillin treatment and grew when resuspended in ampicillin-free medium (curves labelled a). In strain VC7, isoleucine deprivation inhibited the growth recovery process (curve c; Fig. 40A), but the combination of isoleucine deprivation and chloramphenicol treatment resulted in lysis (not shown). This situation is analogous to that described in Fig. 39A; the lysis observed here is unusual

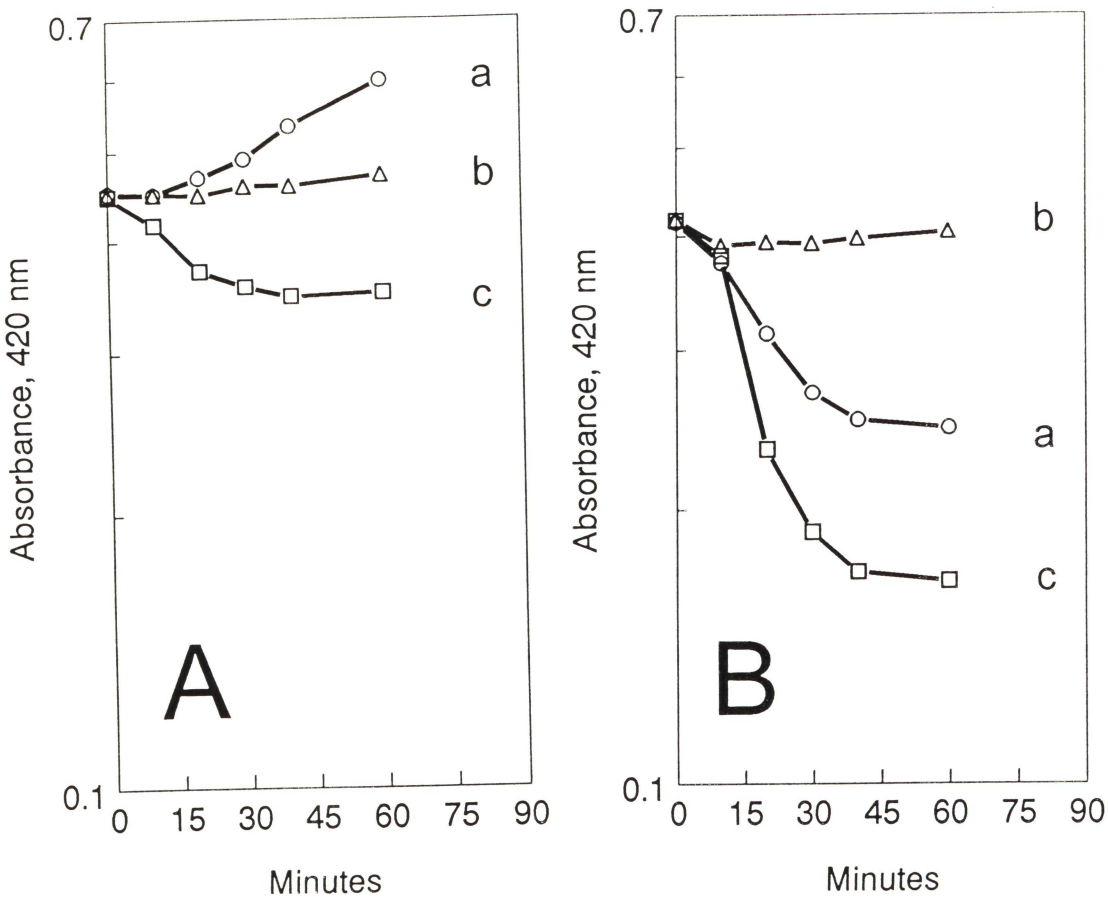

Figure 39. Establishment of conditions for a modified two-stage ampicillin-induced lysis in growing *E. coli*. **Panel A.** An exponential phase culture of strain VC7 were treated with ampicillin (200 μg/ml) for 10 minutes. **Panel B.** An exponential phase culture of strain VC7 were treated with ampicillin (200 μg/ml) for 15 minutes. In both sets of cultures (i.e., shown in panels A and B) the unbound ampicillin was removed and the bacteria resuspended in fresh M9 medium supplied with all required growth factors. The resuspended cultures was divided into three portions each. One part received no further treatment (cultures labelled a in both panels). The other two parts received L-valine (500 μg/ml) to induce the stringent response (cultures labelled b), or a combination of L-valine (500 μg/ml) and chloramphenicol (100 μg/ml) (cultures labelled c).

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 (curve b, Fig. 40A). Interestingly, isoleucine deprivation of strain VC8 (*relA*) resulted in lysis (curve c; Fig. 40B) rather than in inhibition of growth as was the case with strain VC7 (curve c, Fig. 40A). Chloramphenicol treatment had the same effect (curve b, Fig. 40B). Other protein synthesis inhibitors, e.g., gentamicin and tetracycline, also induced lysis of both strains after they had been subjected to the 13-min ampicillin treatment (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 per ml, rather than chloramphenicol, to the ampicillin-treated cultures resulted in the arrest of growth recovery but not lysis (not shown). Under these conditions, norfloxacin did not cause the accumulation of ppGpp and had no effect on the rate of protein synthesis for at least 10 min (not shown). Therefore, the observed lysis was specifically related to the inhibition of protein synthesis and not simply to the inhibition of growth. In summary, these results may be interpreted 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 where the process also provoked the stringent response, i.e., during amino acid deprivation of *relA*$^+$ bacteria.

The experiment described in Fig. 41 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 ampicillin (200 μg/ml) alone to the second subculture. The antibiotics were removed by centrifugation after 13 min of treatment, and the cells were resuspended in fresh M9 medium. In agreement with data shown in Fig 40A (curves a and b), the subculture treated with ampicillin alone eventually recovered and started to grow after the removal of the ampicillin (curve a; Fig. 41), but the addition of chloramphenicol resulted in the induction of lysis (curve b; Fig. 41). In contrast to the subculture treated solely with ampicillin, the subculture which was

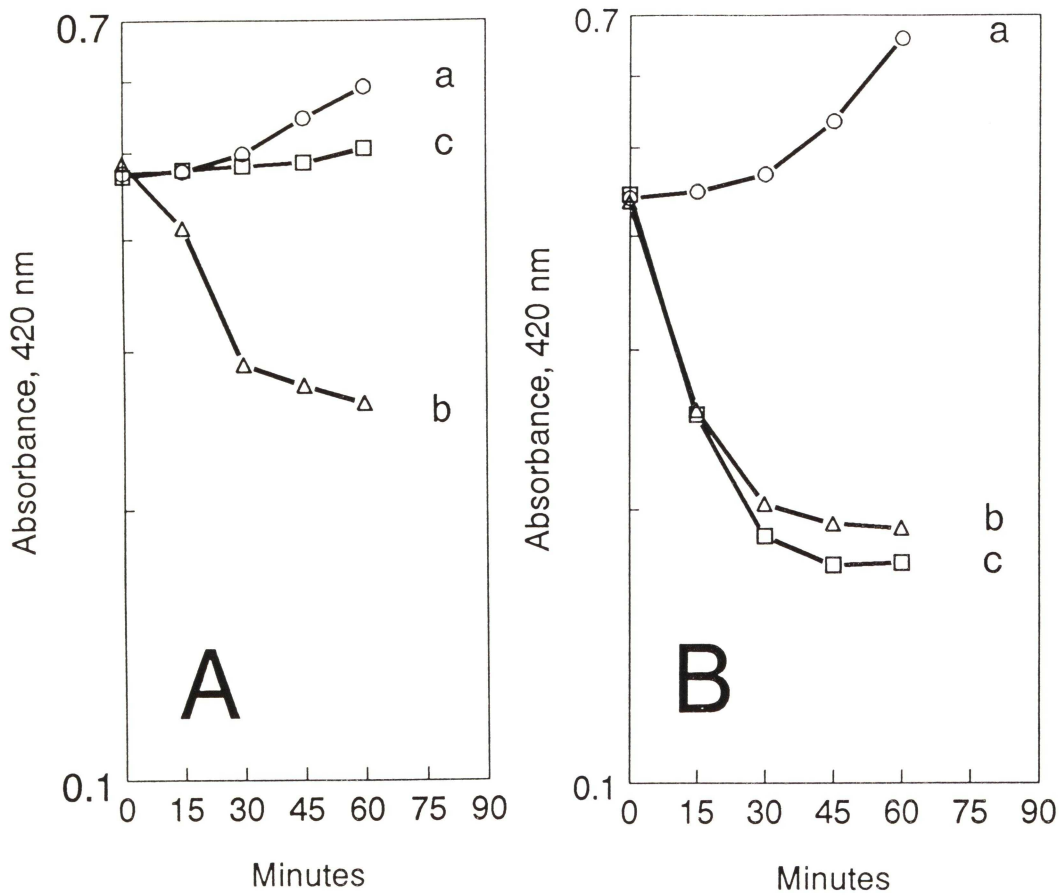

Figure 40. The effect of chloramphenicol on the lysis induction stage in growing *E. coli*. Exponential phase cultures of strains VC7 (**panel A**) and VC8 (*relA*, **panel B**) were primed with ampicillin (200 μg/ml) for 13 minutes. The unbound ampicillin was removed and the cells were resuspended in fresh M9 medium supplied with all required growth factors. Culture of each strain was divided into three parts. One part received no further treatment (cultures labelled a in both panels). The other two parts received either 100 μg/ml of chloramphenicol (cultures labelled b), or 500 μg/ml of L-valine (cultures labelled c).

ampicillin-treated in the presence of chloramphenicol underwent lysis after resuspension in fresh medium (curve c; Fig. 41). 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 (not shown). Furthermore, if the *relA* mutant, strain VC8, was used in this experiment, the enhanced effect on ampicillin priming reaction was observed when the ampicillin treatment was combined with isoleucine deprivation instead of treatment with a ribosome inhibitor (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.

V. General Discussion

V. 1. Energized Membrane and β-Lactam-Induced Lysis

Leduc *et al.* (1982) previously reported that pretreatment of growing *E. coli* cells for a period of 20 min with 20 mM sodium azide inhibited lysis upon subsequent treatment with cephaloridine. It was possible that the inhibition of cephaloridine-induced lysis in this case was directly attributable to the inhibition of bacterial growth by sodium azide rather than to a specific requirement for energized membranes. In the experiments described here, the effects of energy uncouplers on β-lactam-induced lysis were re-examined in amino acid-deprived cells; i.e., in this case, bacterial growth was already inhibited by amino acid deprivation, and the effect of the energy uncouplers on lysis could be tested directly. The results unequivocally confirm that energy uncouplers inhibited β-lactam-induced lysis. Under the experimental conditions, the uncouplers completely dissipated membrane potential and ΔpH and caused a 2-fold decrease in intracellular ATP concentration.

Energy uncouplers inhibited PG synthesis as well as both the priming and the lysis induction stages of penicillin-induced lysis. These results suggest that the activities of both PBPs and PG hydrolases were dependent on energized membrane. However, the PBP labelling experiments indicated that the majority of PBPs were apparently not

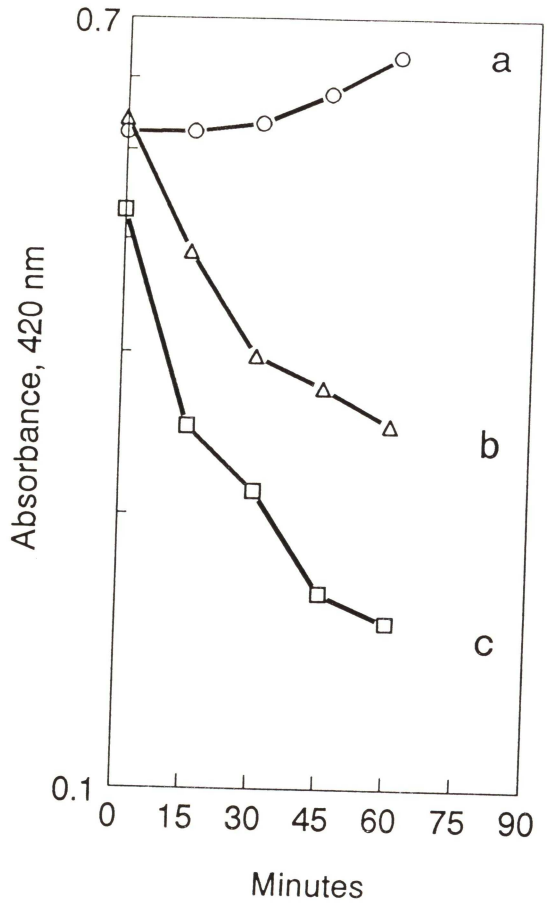

Figure 41. Effect of chloramphenicol treatment on ampicillin priming efficacy in growing *E. coli*. An exponential culture of strain VC7 was divided into two portions. Both subcultures were primed with ampicillin (200 μg/ml) for 13 minutes. On of the subcultures was also treated with chloramphenicol (100 μg/ml) simultaneously with ampicillin. The unbound ampicillin (and exogenous chloramphenicol in one case) were removed from each subculture, and the bacteria were resuspended in fresh M9 medium supplied with all required growth factors. A portion of the subculture that was primed with ampicillin alone received no further treatment (curve a), whereas another portion was treated with 100 μg/ml of chloramphenicol (curve b). The subculture that was primed with ampicillin in the presence of chloramphenicol received no further treatment (curve c).

inhibited by energy uncouplers, and no differences were observed in the PBP.labelling patterns of control bacteria and bacteria treated with energy uncouplers. Therefore, in accordance with Tuomanen's observations (1986), it is proposed that the activities of only the minor fraction of PBPs actually responsible for PG synthesis were dependent on energized membranes. Furthermore, as already noted, PG synthesis is currently thought to be catalyzed by a multienzyme complex composed of PBPs and PG hydrolases (Höltje, 1993). It is possible that only this multienzyme complex is dependent on energized membranes.

It is noteworthy that the inhibition of phospholipid synthesis by treatment with cerulenin had no effect on energized membrane. Therefore, the basis for the coupling of PG metabolism to phospholipid synthesis was not based on membrane de-energization. On the other hand, the possibility that phospholipid synthesis was inhibited when membranes were de-energized has not been excluded.

Lysis of *E. coli* cells by bacteriophage ϕX174 gene E product also requires proton-motive force across the inner membrane of bacteria (Witte *et al.*, 1987). The mechanism of E protein-induced lysis is poorly understood. However, it apparently involves the activation of endogenous PG hydrolases that degrade the cell wall PG (Keller & Simon, 1988; Lubitz *et al.*, 1984) and therefore, may be similar in to native β-lactam-induced lysis.

Interestingly, the energized membrane has been shown to control the autolytic activity in *Bacillus subtilis* in a very different way from that observed in *E. coli* (Jolliffe *et al.*, 1981). When the membrane of *B. subtilis* was de-energized, cellular autolysis was induced rather than inhibited. Both exponential or stationary cultures of *B. subtilis* SR22 strain were rapidly lysed when treated with millimolar concentrations of sodium azide (Jolliffe *et al.*, 1981). The results of Jolliffe *et al.* (1981) were confirmed in the course of this work using *B. subtilis* strain 168Trp (unpublished data). Thus, autolytic systems of *E. coli* and *B. subtilis* are controlled differently by energized membrane, but the basis for the observed differences is not understood and requires further investigation.

V. 2. Temperature Sensitivity of β-Lactam-Induced Lysis

The temperature sensitivity of the penicillin-induced lysis of amino acid-deprived bacteria, first observed by Kusser and Ishiguro (1987), was confirmed. Furthermore, this phenomenon was shown to independent of the heat shock response and therefore cannot be the same phenomenon that was described by Powell and Young (1991). It was also discovered that the temperature-sensitivity was restricted to the lysis induction stage. It is important to point out that the temperature-sensitive event seems to be directly and specifically associated with the lysis induction stage. For example, the fact that the priming stage and PG synthesis were not inhibited by temperature up-shift eliminates phospholipid synthesis as the possible temperature-sensitive target.

The basis for the temperature sensitivity of lysis induction is currently unknown. This has not been easy to determine because the mechanism of lysis induction is not well understood. The experiments reported here indicate that the temperature sensitivity was reversible and occurred in the absence of *de novo* protein synthesis. Alterations in membrane fluidity were considered to be a possibility, but this seemed unlikely in view of the fact that bacteria, pre-grown at 42°C, still exhibited temperature-sensitive lysis induction. Therefore, the phenomenon could be due to the direct thermoinactivation of a key PG hydrolase(s) which is naturally thermosensitive, and future efforts will be concentrated in this area. In this connection it should be noted, that the *in vitro* activity of at least one *E. coli* PG hydrolase, the membrane-bound lytic transglycosylase known as Mlt38, is temperature sensitive at 42°C (Ursinus & Höltje, 1994).

Thermosensitivity of penicillin-induced lysis was also observed in growing cells, but the degree of temperature sensitivity was significantly less than that in amino acid-deprived cells (Kusser & Ishiguro, 1987). The reason for this is not obvious. It is possible that there is simply more active PG hydrolases (component labelled PGH_a in Fig. 28) in growing cells, than in amino acid-starved bacteria. This may be relevant to the proposal that PG biosynthetic enzymes have short half-lives and undergo constant turnover (Tuomanen, 1986). In view of the proposed multienzyme complex composed of both the PBPs and PG hydrolases (Höltje, 1993), the concept of short half-lives and constant turnover could be extended to PG hydrolases. In this regard, the pool of active

PG hydrolases would be progressively depleted in amino acid-starved bacteria because the synthesis of new PG hydrolases is inhibited. This would not be the case in normal growing cells.

V. 3. Protein Synthesis and β-Lactam-Induced Lysis

This study confirmed and extended a previous report (Kusser & Ishiguro, 1986) 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. This is 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 study 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, the efficiency of the stringent response in the establishment of ampicillin tolerance was further documented: the lysis induction stage of primed *relA*$^+$ bacteria was inhibited within 10 min of amino acid deprivation (Fig. 39A).

The observations reported here seemingly contradict certain previous reports (e.g., Leduc *et al.*, 1982) that demonstrate protection from penicillin-induced lysis by chloramphenicol treatment. The reason for these apparent discrepancies are uncertain; they may be due to differences in experimental conditions, specifically in growth media. The experiments reported here were performed in minimal medium whereas the other study (Leduc *et al.*, 1982) reported the results of experiments performed in complex media. It has already been noted that the degree of lysis induced by ampicillin in the presence of chloramphenicol was markedly reduced in complex media as compared to minimal medium (Kusser & Ishiguro, 1986). Currently there is no explanation for this, and further studies are warranted.

Two new findings are important in the development of a working hypothesis. They both relate to observations made on bacteria which were subjected to only partial priming with ampicillin, i.e., a 13-min treatment with 200 μg per 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 (e.g., Fig. 41). The second observation was that the inhibition of protein synthesis during the growth recovery process of partially primed bacteria resulted in lysis induction (Fig. 40). Therefore, the inhibition of protein synthesis promoted the development of both the priming and lysis induction stages. According to Tuomanen (1986), the PBPs involved in PG synthesis apparently have short functional half-lives, and their replacement by newly synthesized PBPs is a process which is essential for normal PG synthesis. It is therefore proposed 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 and PG hydrolases involved in PG metabolism are also inhibited.

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