# Cloning and Identification of the *lytB* Locus in *Escherichia coli*

ACCEPTED
ILTY OF GRADUATE STUDIES
[redacted]
DEAN
91/09/24

by

**Corinne Elaine Gustafson**
**B.Sc., University of Victoria, 1987**

A Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
MASTERS OF SCIENCE
in the Department of Biochemistry and Microbiology

We accept this thesis as conforming
to the required standard

[redacted]

Dr. E.E. Ishiguro, Supervisor (Department of Biochemistry & Microbiology)

[redacted]

Dr. F.E. Nano, Departmental Member (Department of Biochemistry & Microbiology)

[redacted]

Dr. E.D. Styles, Outside Member (Department of Biology)

[redacted]

Dr. M.J. Ashwood-Smith, External Examiner (Department of Biology)

**Supervisor : Dr. Edward E. Ishiguro**

# ABSTRACT

The phenotype conferred by a mutation in the *lytB* gene of *Escherichia coli* included temperature sensitive growth, and tolerance to penicillin-induced lysis at the restrictive temperature. These properties are correlated with the chronic accumulation of guanosine 5', 3'-bispyrophosphate (ppGpp), the alarmone associated with induction of the stringent response, at the restrictive temperature. Suppression of ppGpp accumulation and the penicillin tolerance of this mutant at 42°C was accomplished by treatment with the ribosome inhibitor chloramphenicol, or by a *relA*$^-$ genetic background, suggesting that the stringent response is induced at the restrictive temperature. Based on these phenotypic properties, it is proposed that LytB may normally be responsible for maintaining RelA (ppGpp synthetase I) in its inactive state in growing bacteria, and the thermoinactivation of the temperature-sensitive mutant LytB protein results in the activation of the RelA enzyme at the restrictive temperature.

The objective of this study was to identify the *lytB* gene. The *lytB* gene was cloned and identified by both deletion mapping and insertion mutagenesis. It was found to be the last open reading frame, designated *orf316*, in the *ileS-lsp* operon. Instability, in the form of spontaneous deletions and possibly point mutations, of plasmids carrying the *lytB* gene in high copy number indicated toxicity of the LytB protein when overexpressed. In support of this, it was shown that multicopy plasmids carrying an insertion-inactivated *lytB* gene were stable in multicopy. The possible basis for LytB toxicity is discussed.

Examiners:

[redacted]

Dr. E.E. Ishiguro, Supervisor (Department of Biochemistry & Microbiology)

[redacted]

Dr. F.E. Nano, Departmental Member (Department of Biochemistry & Microbiology)

[redacted]

Dr. E.D. Styles, Outside Member (Department of Biology)

[redacted]

Dr. M.J. Ashwood-Smith, External Examiner (Department of Biology)

# CONTENTS

**Chapter 1: INTRODUCTION**

**Chapter 2: MATERIALS AND METHODS**

**Chapter 3: RESULTS**

**Chapter 4: DISCUSSION**

# LIST OF FIGURES

# LIST OF TABLES

# ABBREVIATIONS

| | |
|---|---|
| Ap | ampicillin |
| ATP | adenosine 5'-triphosphate |
| Cm | chloramphenicol |
| cpm | counts per minute |
| D-ala | D-alanine |
| DAP | *meso*-diaminopimelic acid |
| D-glu | D-glutamic acid |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediaminetetraacetic acid |
| GlcNAc | *N*-acetylglucosamine |
| HPLC | high performance liquid chromatography |
| IPTG | isopropyl β-D-thiogalactoside |
| Km | kanamycin |
| kb | kilobase pairs |
| kD | kilodalton |
| LPS | lipopolysaccharide |
| mRNA | messenger ribonucleic acid |
| MurNAc | *N*-acetylmuramic acid |
| MurNAc-pentapeptide | *N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-DAP-D-alanyl-D-alanine |
| MW | molecular weight |
| OD | optical density |
| orf | open reading frame |
| PBP | penicillin binding protein |

| | |
|---|---|
| PG | peptidoglycan |
| ppGpp | guanosine 5'-diphosphate 3'-diphosphate |
| pppGpp | guanosine 5'-triphosphate 3'-diphosphate |
| SDS | sodium dodecyl sulfate |
| SDS-PAGE | SDS polyacrylamide gel electrophoresis |
| Sm | streptomycin |
| Sp | spectomycin |
| Tn | transposon |
| tRNA | transfer ribonucleic acid |
| TSA | tryptic soy agar |
| TSB | tryptic soy broth |
| Tris | tris-(hydroxymethyl)aminomethane |
| UDP | uridine 5'-diphosphate |
| UV | ultraviolet |
| X-gal | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |

# ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. E. E. Ishiguro for his support and guidance throughout this study.

Thanks also to members of the department for discussions, with special thanks to A. Labossiere and S. Scholz for their help and computer assistance.

Sincerest thanks to Dr. Richard Alm for many stimulating discussions, assistance, and encouragement.

Finally, thanks to my parents for their patience, love and support, which has made everything possible.

# Chapter 1

# INTRODUCTION

## 1.1 Chapter Overview

The introduction of this thesis covers three topics; i) the stringent response, ii) peptidoglycan biosynthesis, and iii) regulation of peptidoglycan biosynthesis and autolysis by the stringent response. While the first two sections are broad topics and are largely unrelated, they cover the basic information required to understand the third section and the purpose of this thesis.

## 1.2 The Stringent Response

### 1.2.1 General description

Normal bacterial growth involves a balanced synthesis of cellular macromolecules such as RNA, proteins, phospholipids, and peptidoglycan (PG). When bacteria are subjected to nutritional stress, for example amino acid deprivation, the rates of anabolic processes must be readjusted. During amino acid starvation, the ability of the cell to aminoacylate tRNA is unable to keep up with the process of protein synthesis. This calls for a global shutdown of macromolecular syntheses since there is a diminished requirement in amino acid starved bacteria for cell wall growth as well as stable RNA and phospholipid synthesis. The down-regulation of these processes is known as the stringent response. This term originally came about when it was observed that the accumulation of stable RNA depended on the availability of charged tRNA. This dependence was termed as a stringent requirement for RNA control (1).

Analysis of the radiolabeled nucleotide fraction derived from bacteria which were pulse-labeled with [$^{32}$P]-phosphate revealed the accumulation of the unusual nucleotides guanosine 3'-diphosphate 5'-triphosphate (pppGpp), and guanosine 3'-diphosphate 5'-diphosphate (ppGpp) (1). The appearance of elevated levels of pppGpp and ppGpp in the cell has since been correlated with the inhibition of various macromolecular synthetic processes (2, 3, 4). It has been proposed that these unusual nucleotides represent nutritional stress signals which are involved in shutting down superfluous cellular activities. Figures 1 and 2 (5) summarize the phenomenon described above. Figure 1 shows that the levels of ppGpp (○) and pppGpp (●) begin to rise within a few minutes of amino acid starvation. The rise continues for 5 to 15 minutes, reaches a peak, then drops to a new steady-state level which is usually 10 to 20-fold above the original basal level found in growing cells. Cell growth is shut down concomitantly with elevated ppGpp levels. The effect of elevated ppGpp on RNA synthesis is illustrated in Figure 2. The lower panel shows the rise in pppGpp and ppGpp levels, while the upper panel shows a concomitant decrease in RNA synthesis.

Figure 3 provides an overview of the cellular routes of pppGpp and ppGpp synthesis and degradation (6). As discussed below, strains carrying mutations in the *relA* gene do not accumulate pppGpp and ppGpp during

**Figure 1. Effect of ppGpp accumulation on cell growth.**
**Data from Cashel, M. (5).**

The lower panel shows an amino acid auxotroph grown in Tris-glucose minimal medium containing amino acid requirements, filtered, washed, and resuspended in 2 μg per ml of methionine. Acid extracts of aliquots of [$^{32}$P]-phosphate labeled cells were prepared at the indicated times. Chromatography in 1.5 M $KH_2PO_4$ was done to determine the presence of pppGpp (●) and ppGpp (○). Methionine exhaustion occurs at 20 minutes. The top panel monitors cell growth in a cold aliquot of culture.

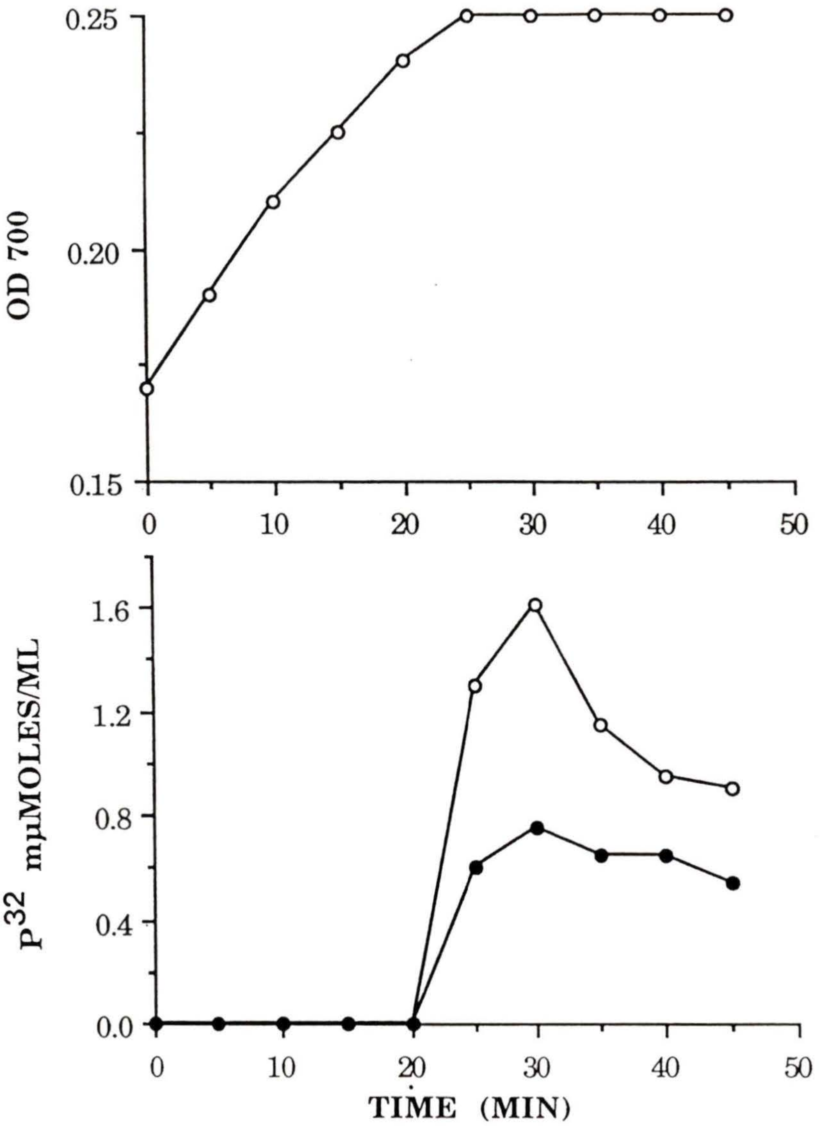

0.25
0.20
0.15
OD 700
0
10
20
30
40
50
1.6
1.2
0.8
0.4
0.0
P$^{32}$ mμMOLES/ML
0
10
20
30
40
50
TIME (MIN)

**Figure 2. Effect of ppGpp accumulation on RNA synthesis**
**Data from Cashel, M. (5).**

The lower panel measures levels of [$^{32}$P]-phosphate labeled pppGpp (●) and ppGpp (○) after addition of thiazole alanine to the amino acid auxotroph described in Figure 1. The upper panel measures RNA synthesis after addition of thiazole alanine by the amount of [$^{3}$H]-uracil incorporated into trichloroacetic acid-insoluble material from aliquots of cells after 20 sec pulse-labeling.

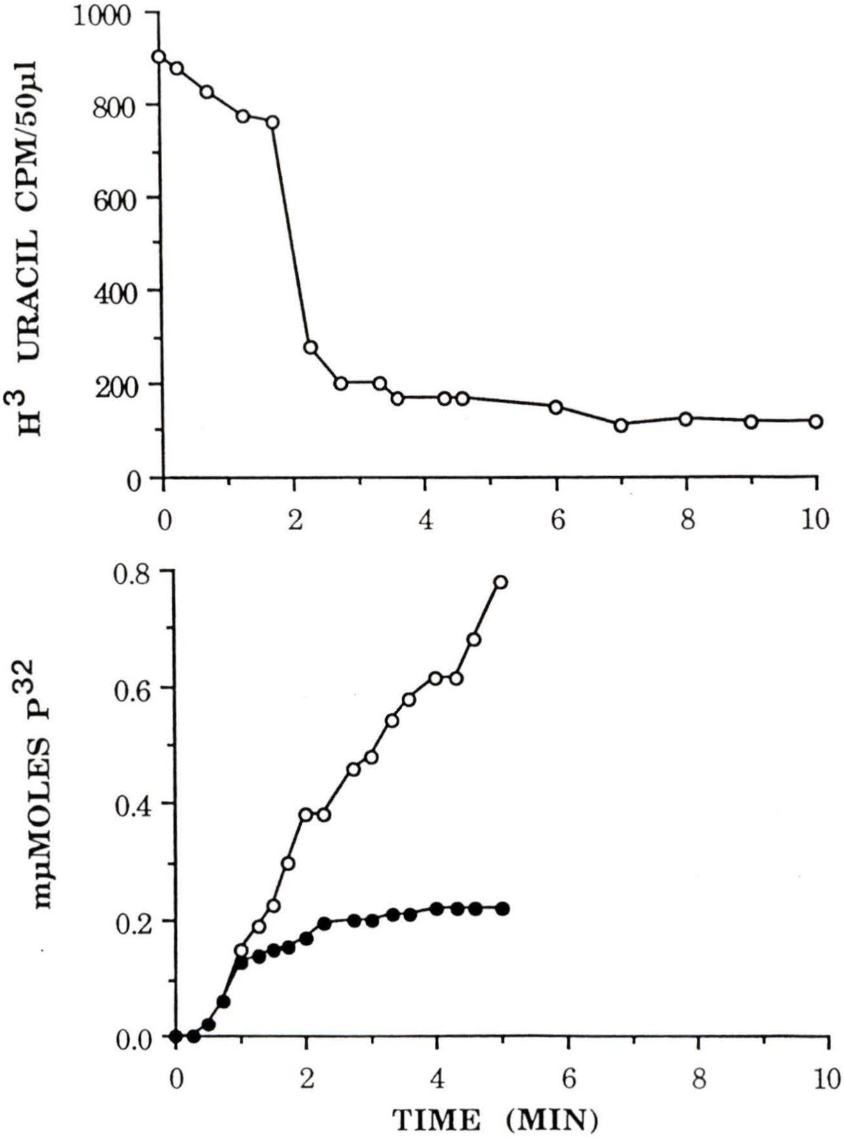
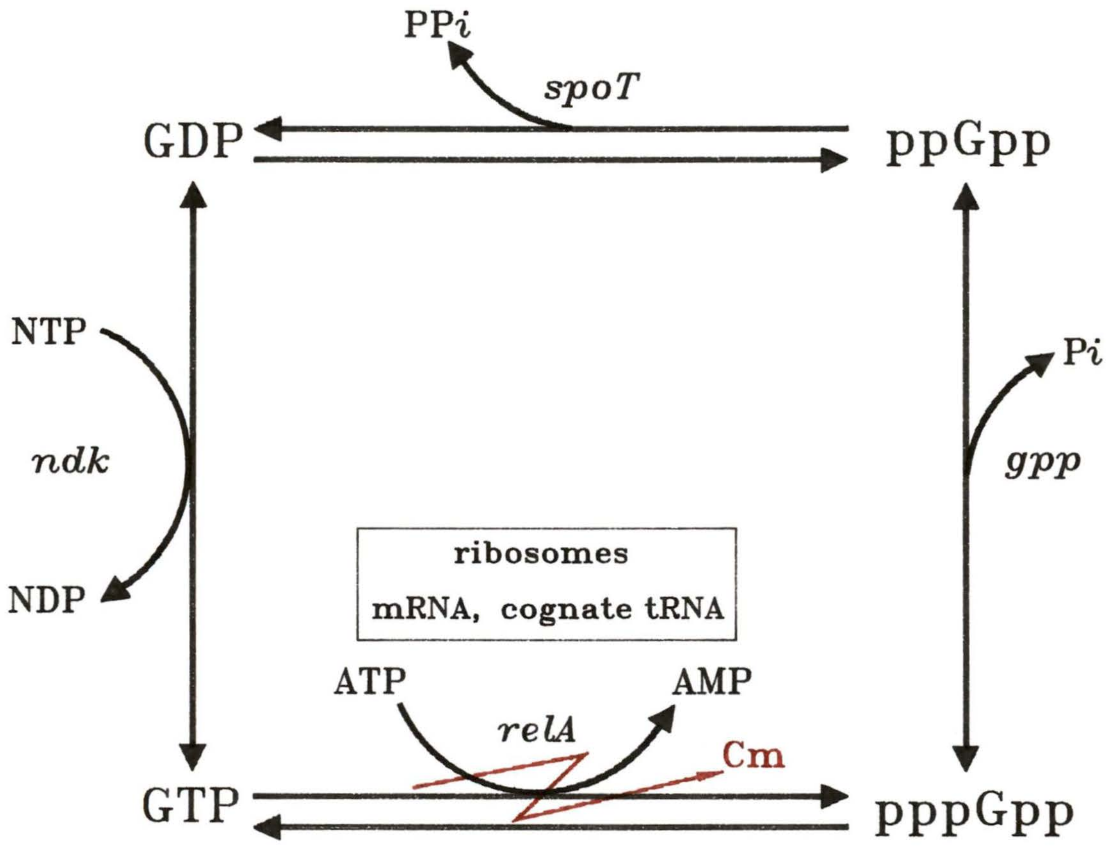

1000
800
600
400
200
0
H 3 URACIL CPM/50μl
0
2
4
6
8
10
0.8
0.6
0.4
0.2
0.0
mμMOLES P 32
0
2
4
6
8
10
TIME (MIN)

**Figure 3. The cellular routes of pppGpp and ppGpp synthesis and degradation.**

RelA is a pyrophosphoryl group transfer enzyme which converts cellular GTP into pppGpp using ATP as the phosphate donor. The synthesis of pppGpp requires ribosomes, bound mRNA, and cognate tRNA. A 5'-pyrophosphohydrolase encoded by the *gpp* gene catalyzes the release of a phosphate to form ppGpp. The *spoT* gene encodes a 3'-pyrophosphohydrolase which catalyzes the release of a pyrophosphate to form GDP. The cycle is completed by a nucleoside 5'-diphosphate kinase encoded by the gene *ndk*. The addition of chloramphenicol (Cm) to cells inhibits RelA function to block pppGpp synthesis, indicated by the red arrow.

Modified from reference 12.


PPi
spoT
GDP
ppGpp
NTP
ndk
NDP
Pi
gpp
ribosomes
mRNA, cognate tRNA
ATP
AMP
relA
Cm
GTP
pppGpp

amino acid deprivation and consequently continue to synthesize macromolecules such as stable RNAs under these conditions. The *relA* gene product is an enzyme known as ppGpp synthetase I which converts cellular GTP into pppGpp by a pyrophosphoryl group transfer using ATP as the pyrophosphate donor. ppGpp can also be formed from GDP *in vitro* (6, 7). However, considering the more abundant GTP pools in the cell (50-fold higher than GDP pools), it is more likely that RelA synthesizes pppGpp as the primary *in vivo* product (6). Certain inhibitors of protein synthesis such as chloramphenicol have been found to inhibit RelA function (8), as indicated in Figure 3 by the red arrow. A 5'-phosphohydrolase encoded by the *gpp* gene catalyzes the formation of ppGpp from pppGpp, with the release of an inorganic phosphate. Levels of ppGpp in the cell are regulated not only by RelA-mediated synthesis, but also by ppGpp degradation. A 3'-pyrophosphohydrolase encoded by the *spoT* gene catalyzes this degradation by the release of two inorganic phosphates from ppGpp to form GDP (Figure 3). The SpoT enzyme plays an important role in ppGpp metabolism and will be discussed in more detail in section 1.2.3. The final enzyme in the cycle of ppGpp metabolism is nucleoside 5'-diphosphate kinase, encoded by the *ndk* gene which uses NTP as a phosphate donor to catalyze the formation of GTP (6, 8, 9, 10, 11).

The stringent response has been observed in many different bacterial species with minor variations, such as the nucleotide that acts as the signal for the induction of the stringent response. Among related bacteria such as *Escherichia coli* and *Salmonella typhimurium*, however, the response appears to be identical (12).

### 1.2.2 *relA* encoded ppGpp synthetase I

A large number of mutants in the *relA* gene have been isolated. Phenotypes of the mutants ranged from a slight to strong relaxation of the stringent response (13). The original mutant, *relA1* was mapped at 60 minutes on the *E. coli* chromosome. The *relA*$^+$ gene was cloned and overexpressed in order to study the ppGpp synthetase I enzyme. A 10-fold overexpression resulted in elevated ppGpp levels and a corresponding decrease in growth rate (14, 15). The ppGpp synthetase I is an 84 kilodalton (kD) hydrophobic protein (14, 16) which is normally ribosome-bound but can be released by salt washes. The purified ribosome-free enzyme is inactive, but agents which induce conformational changes such as methanol or detergents partially restore activity to the enzyme (17, 18). In addition, RelA activity can be reactivated after the salt wash by incubation with 70S wild type ribosomes (19, 20). These results suggest that a conformational change in the enzyme is necessary for the activation of its catalytic activity, possibly induced by the ribosome.

The *relA* gene has been sequenced (14) and the nature of the *relA1* mutation identified. There has been an IS2 insertion in the 5'end of the gene. This insertion results in the formation of two fragments (α and β) which, together, possess some residual activity (16). It was suggested that the fragments form two functional domains of the enzyme - one which binds the ribosome, and the other which exhibits the catalytic activity. Evidence for this suggestion comes from the observation that high levels of overexpressed RelA1 β-fragment are able to abolish the ppGpp synthetase I activity of the *relA*$^+$ gene in single copy. This implies that the β fragment in high copy number outcompetes the single RelA+ copy for ribosome binding (16).

Shreiber *et al.* (2) also gave evidence for two functional domains in RelA. The normal level of RelA protein in the cell is a few copies per 100 ribosomes. The wild type *relA* gene was overexpressed to levels which would progressively saturate ribosomes. In addition, a deletion mutant protein was overexpressed with the carboxyl terminal portion of the protein deleted since the ribosome-binding characteristics were suggested to be located in this domain. The host strains used for determining the ppGpp synthetic activities of the overexpressed wild type or truncated RelA enzymes were either *relC*+ or *relC*. The *relC* gene encodes the L11 protein which is a component of the 50S ribosomal subunit. It was found that the ability of the overexpressed wild type RelA enzyme to synthesize ppGpp under conditions of amino acid starvation was dependent on the L11 protein. Moreover, the ppGpp synthetic activity of the truncated protein, which was no longer able to bind to the ribosome, was independent of L11 (2).

*In vivo* studies by Rojiani et al. (21) involved construction of a plasmid bearing the $tRNA^{Trp}$ gene under the control of the inducible tac promoter. This plasmid was introduced into a tryptophan auxotroph for the purpose of manipulating the total concentration of $tRNA^{Trp}$ in the cell. The levels of $Trp\text{-}tRNA^{Trp}$ were controlled by varying the amount of exogenously added tryptophan. In this system, it was found that the synthesis of ppGpp was induced when a decrease in the ratio of charged/uncharged $tRNA^{Trp}$ was manipulated by increasing the amount of uncharged $tRNA^{Trp}$ in the cell to mimic conditions in the cell under the stringent response. The important point of this experiment was that the ratio of charged to uncharged $tRNA^{Trp}$ was the determining factor for induction of ppGpp synthesis, and not the absolute concentration of charged or uncharged $tRNA^{Trp}$. However, once ppGpp synthesis had been triggered due to the low ratio of charge/uncharged $tRNA^{Trp}$, the magnitude of the response was dependent on the total concentration of $tRNA^{Trp}$, which would be mostly uncharged at the point of induction. In this way, the cells were able to sense the state of amino acid deprivation and responded quantitatively (21).

A model of RelA action during the stringent response, based on *in vivo* and *in vitro* observations, has been proposed. In amino acid deprived cells, the demands of protein translation exceed the ability of the cell to aminoacylate tRNAs. Therefore, uncharged tRNA corresponding to the deficient amino acid inevitably binds to its specific codon on mRNA in the acceptor site of the ribosome. Translation is idled because there is no amino acid on the tRNA to continue synthesis of the peptide chain. Translational idling has been proposed to cause a conformational change in the ribosome, which in turn activates the catalytic domain of the bound RelA enzyme. The synthesis of ppGpp *in vitro* is stoichiometrically regulated by the degree of translational idling; for every uncharged tRNA which binds and causes the ribosome to stall, one ppGpp molecule is synthesized (22). It has been suggested that this stoichiometric relationship permits the continual sensing of the ratio of charged/uncharged tRNA's in the cell, and ensures that the ppGpp synthetic response is strictly quantitative.

### 1.2.3 The SpoT enzyme

A degradative pathway for ppGpp was suggested from early studies on the turnover of ppGpp. The ppGpp pool in amino acid-deprived bacteria was found to be rapidly depleted upon treatment of the cells with chloramphenicol (which inhibits RelA-mediated ppGpp synthesis). The product of the *spoT* gene has been identified as the enzyme which degrades ppGpp. Mutants in *spoT* have been utilized for studies on the effects of increased levels of ppGpp in the cell (8, 15, 23,).

In the presence of manganese ions, SpoT catalyzes the removal of the 3'-pyrophosphate from ppGpp and pppGpp in an ATP-dependent reaction (9, 10). The *spoT* locus at 82 min on the *E. coli* chromosome encodes a ribosome-associated 80 kD protein (10, 23). *In vitro* studies have shown that SpoT is capable of catalyzing the degradation of pppGpp to GTP + pyrophosphate, but the *in vivo* role is more likely a degradation of ppGpp (6), as indicated in Figure 3.

SpoT works in opposition to RelA in maintaining precise levels of ppGpp in a coordinated system which is sensitive to cellular and environmental conditions. The intracellular half-life of ppGpp is about 20 seconds, and this is maintained by the continual degradation of ppGpp by SpoT (5, 11). Although ppGpp synthetic rates may increase dramatically upon nutritional stress, the ppGpp half-life of 20 seconds does not change (8). Similarly, the turnover rate is the same during steady state growth. Thus, SpoT is responsible for maintaining the turnover rate of ppGpp, and RelA is responsible for determining the level of accumulation (8). The SpoT protein is highly related to the RelA protein at the amino acid level, but only distantly related at the nucleotide level (16). This may reflect a functional similarity between the two proteins, and the possible relevance of this is discussed below with respect to RelA-independent ppGpp synthesis.

### 1.2.4 Pleiotropic effects of the stringent response

The stringent response affects a multitude of cellular functions (see Cashel for review, ref. 12). The transcription of stable RNAs is inhibited. The synthesis of about one-half of the cellular proteins detectable in two-dimensional gels is affected either positively or negatively. For example, the synthesis of the ribosomal proteins, various translation factors, and the RNA polymerase $\alpha$, $\beta$, and $\beta'$ subunits are inhibited. On the other hand, the synthesis of the RNA polymerase $\sigma^{70}$ and $\sigma^{32}$ subunits and several heat-shock proteins appear to be positively regulated by the stringent response. Nucleotide, amino acid, and carbohydrate metabolism are inhibited. The synthesis of cell envelope components (phospholipids, peptidoglycan, and lipopolysaccharide) are restricted. How these varied effects are mediated has not been conclusively determined. The most widely studied effect has been that on stable RNA transcription, and these efforts are summarized here.

The mechanism by which ribosomal RNA transcription is downregulated during the stringent response is not fully understood. It has been proposed that a signal molecule, possibly ppGpp, interacts directly or indirectly with the promoter region of rRNA genes, but this has not yet been demonstrated directly. Two differentially regulated tandem promoters, P1 and P2, exist on all rRNA operons and on at least some tRNA genes. Transcriptional studies (15, 24) have shown that P2 is an unregulated constitutive promoter which is unaffected by various growth conditions. This promoter ensures the synthesis of basal levels of rRNA even during stress. A basal level is required for survival since at least one ribosome is necessary for the synthesis of more ribosomes. P1 is regulated by growth rate. Rapid growth results in a high level of expression from P1. In contrast, expression from P1 is severely inhibited during very slow growth and during the stringent response, both of which result in elevated levels of ppGpp. An inverse exponential relationship exists between P1 promoter activity and ppGpp concentration. This implies that ppGpp is a negative feedback effector which mediates the decrease in rRNA promoter activity. One possible mechanism by which ppGpp could accomplish this may be by binding to RNA polymerase and changing its conformation sufficiently to alter its promoter specificity. This idea is not new; promoter specificity has been supposed to be altered in various systems by modifications to RNA polymerase. This may be by such mechanisms as physical associations with some factor such as a protein or nucleotide, or modification by phosphorylation or ADP-ribosylation (25).

Based on a P1 promoter-*lacZ* construction (24), an activator sequence has been identified upstream of P1. This sequence enhances activity from P1 up to 15-fold. The component which interacts here has not been identified, but the possibility exists that ppGpp inhibition of P1 activity may involve either the activator region or the component which interacts here. For example, if ppGpp can bind to either the enhancer sequence or to the unidentified activator molecule it could in this way decrease P1 activity.

A new 10 kD protein, omega (ω) factor, has been identified by Igarashi *et al.*(25) which is tightly associated with RNA polymerase holoenzyme. Interestingly, ω factor is encoded by the *rpoZ* gene which has been located next to the *spoT* gene, suggesting a role for the ω protein in the stringent response. It was found through an *in vitro* mixed transcription study that the addition of ω factor rendered RNA polymerase sensitive to the inhibitory effects of ppGpp on transcription, and RNA polymerase became resistant to ppGpp upon the removal of the ω factor. Thus, it was proposed that either ppGpp interacts with the ω factor bound to the β subunit of RNA polymerase, or the binding of ω to RNA polymerase activates a ppGpp binding site (25). However, subsequent experiments by Gentry *et al.* (26) found that the deletion of the *rpoZ* gene had no effect on stringent control on stable RNA accumulation *in vivo*. Thus, more questions are raised than are answered by the finding of this new ω protein.

### 1.2.5 Effects and mechanisms of action of stringent response antagonists

Several inhibitors of ribosome function are known to block the stringent response, but the exact mechanism is not known. It has been proposed that the stringent response antagonistic action of these agents is directly related to their ability to block translation (27). When cells are amino acid starved, there is a turnover of existing proteins in the cell. This results in a pool of free amino acids. However, the amino acid which the bacteria are being deprived of becomes limiting and cannot keep up with the demands of protein synthesis in the cell. As already noted, this normally causes a decrease in the ratio of charged/uncharged tRNA and the ensuing translational idling results in the synthesis of ppGpp and the induction of the stringent response. However, the addition of a translational inhibitor such as chloramphenicol decreases the demand for charged tRNA because of the blockage of translation. As a result, the charged to uncharged tRNA ratio increases, thereby inhibiting ppGpp synthesis (28).

### 1.2.6 ppGpp synthetase II

As mentioned briefly above, it has been proposed that there exists an alternate route for the synthesis of ppGpp which is *relA*-independent which operates in growing bacteria. In this pathway, ppGpp levels respond in a reciprocal manner to growth-rate, and this may account for the observed inverse relationship between ppGpp levels and growth rate (2, 15, 24). The existence of this pathway was suspected because high levels of ppGpp still accumulate during energy source deprivation or growth rate downshift in *relA* deletion mutants (16). This enzyme which is activated by energy source downshift has been named ppGpp synthetase II (12) in order to distinguish it from the enzyme involving the amino acid starvation pathway.

Although the existence of this enzyme has been proposed based on the above observations, the ppGpp synthetase II enzyme in *E. coli* has not been conclusively identified. It would be expected due to function similarities that the ppGpp synthetase II gene may share nucleotide sequence homology with the gene encoding ppGpp synthetase I. The first search at the nucleotide level was unreavealing ; southern blot analysis under first high, then lower stringency conditions using a labeled probe for the *relA* gene detected no cross-hybridizing DNA (16). New findings reveal the possibility of SpoT as the ppGpp synthetase II enzyme. Indeed, although this protein is not highly related at the nucleotide level, it shares a high degree of homology at the amino acid level with the RelA enzyme (16), indicating a functional similarity between the two enzymes. In addition, a *spoT* deleted strain apparently abolishes ppGpp synthetase II activity (29). Hernandez and Bremer (30), and Xiao *et al.* (29) propose that SpoT is a bifunctional enzyme responsible for both ppGpp degradation in the *relA*-dependent pathway, and ppGpp synthesis in the *relA*-independent pathway.

Both routes of ppGpp synthesis contribute to cellular basal levels of the nucleotide during steady-state growth of *relA*$^+$ strains. The contribution is additive, with neither one able to supply basal levels alone (31).

## 1.3 Peptidoglycan Metabolism in *Escherichia coli*

### 1.3.1 The cell wall of *E. coli*

A general description of the cell wall of *E. coli* is given here for two reasons; i) to give an overview of the environment in which peptidoglycan biosynthesis occurs, and ii) because the synthesis of the major components of the cell wall (outer membrane proteins, phosopholipids, lipopolysaccharides, and peptidoglycan) are regulated by the stringent response. The following discussion will be restricted to enteric bacteria.

*E. coli* is characterized by having an outer membrane and an inner membrane sandwiching a thin layer of peptidoglycan. The outer membrane contains lipopolysaccharides extending out from the cell surface, lipoproteins embedded in the outer membrane and covalently linked to the peptidoglycan sacculus, outer membrane proteins which interact non-covalently with peptidoglycan, and porins forming channels extending from the periplasm to the outside of the cell (32).

The rod shape of *E. coli* cells is genetically determined, and is maintained by the rigid exoskeleton-like structure called a sacculus. The sacculus is composed of peptidoglycan which is strong enough to allow differences in external and internal osmotic pressure without cell lysis occurring. Cell growth and division is a dynamic process requiring both strength and flexibility. The strength of peptidoglycan is obtained through covalent bonds between the subunits within the sacculus matrix (33). Flexibility arises from the 3-dimensional arrangement of peptidoglycan components.

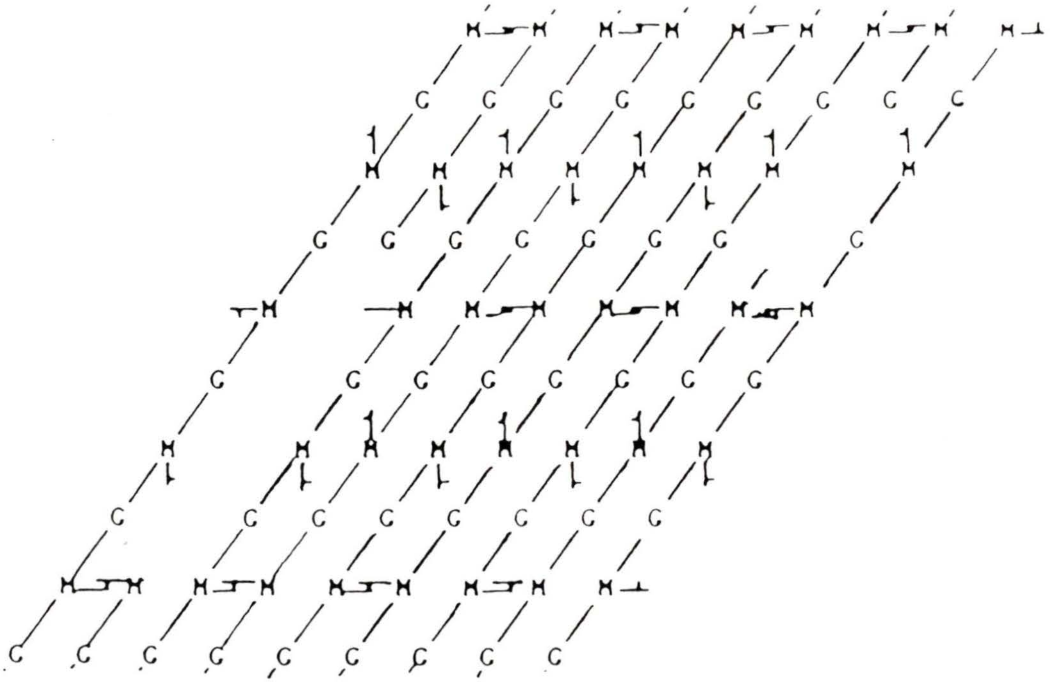
Enzymes involved in peptidoglycan metabolism exist in the cytoplasm, on the cytoplasmic membrane face, or on the periplasmic membrane face.

### 1.3.2 The structure of peptidoglycan

Peptidoglycan is constructed from glycan chains which run parallel to each other, with peptide side chains which intermittently form cross-bridges thereby lending mechanical strength to the entire complex (Figure 4). The glycan chains are made up of a repeating unit of the alternating sugar residues N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). The glycan chains are terminated by nonreducing 1, 6-anhydro MurNAc residues. The length of the chains has been statistically averaged at 70 amino sugars per chain (34). Furthermore, each MurNAc carries a peptide substitution. The peptide side chains are composed of the amino acids L-alanine, D-glutamic acid, *meso*-diaminopimelic acid (DAP), and D-alanine. Between 40-60% of the peptide substitutions on MurNAc residues of adjacent glycan chains may be crosslinked, depending on the growth state of the cell (35). New peptidoglycan shows 66% less cross-linkages and has fewer lipoprotein associations (35,36). The peptide dimers cross-linking

**Figure 4. The structure of peptidoglycan.**

Glycan chains run parallel to each other, with intermittent peptide side chains which may be crosslinked to adjacent side chains. G, N-acetylglucosamine; M, N-acetylmuramic acid. Glycosidic bonds connect the repeating sugar residues, G and M ( ). Peptide side chains are represented by , and crosslinked side chains by . Modified from reference "*Escherichia coli* and *Salmonella typhimurium*. Cellular and molecular biology", (Neidhardt, F.C., ed.), pp 24, American Society for Microbiology, Washington D.C.



glycan chains can be tetrapeptide-tetrapeptide, tripeptide-tetrapeptide, and tetrapeptide-pentapeptide compounds, as determined by HPLC analysis (37, 38). Lipoprotein can be linked to free peptide chains, cross-linked muropeptides, and to anhydro compounds. Typical cross-linking occurs between a D-alanine and *meso*-diaminopimelic acid bond. However, linking between two diaminopimelic acid residues is also possible (35, 37).

The basic 3-dimensional architecture of the peptidoglycan sacculus is as yet unresolved since no direct methods have previously existed for analyzing it in its hydrated form (39). However, indirect evidence comes from the release of trimeric and tetrameric crosslinked units upon muramidase digestion of the peptidoglycan. This would only be possible if three or four separate glycan chains were crosslinked together (40). Direct evidence comes from a recent study by Labischinski et al. (39) who uses a technique called Neutron Small-Angle Scattering. These workers provide direct proof that peptidoglycan is constructed with about 75-80% of the surface single layered, and the remaining 20-25% triple layered. There are two models which propose the region of triple-layered peptidoglycan to be either at the hemispherical caps of the rod, or along the length of the cylinder in growth zones (38). Both models are only speculative at this stage and must be clarified by further experimental work.

### 1.3.3 Peptidoglycan biosynthesis

#### i) Biosynthetic pathway of peptidoglycan

The *E. coli* cell shape is determined by the peptidoglycan exoskeleton. Typical *E. coli* cells are cylindrical rods. The hemispherical caps on each end are formed from septal peptidoglycan during cell division. Many genetic and physiological studies have been carried out to dissect the steps involved in peptidoglycan biosynthesis. An overview of the steps involved in peptidoglycan biosynthesis in *E. coli* is illustrated in Figure 5. Biosynthesis has been subdivided into three different stages which occur in different cellular compartments - the cytoplasm, the cytoplasmic membrane, and the periplasm.

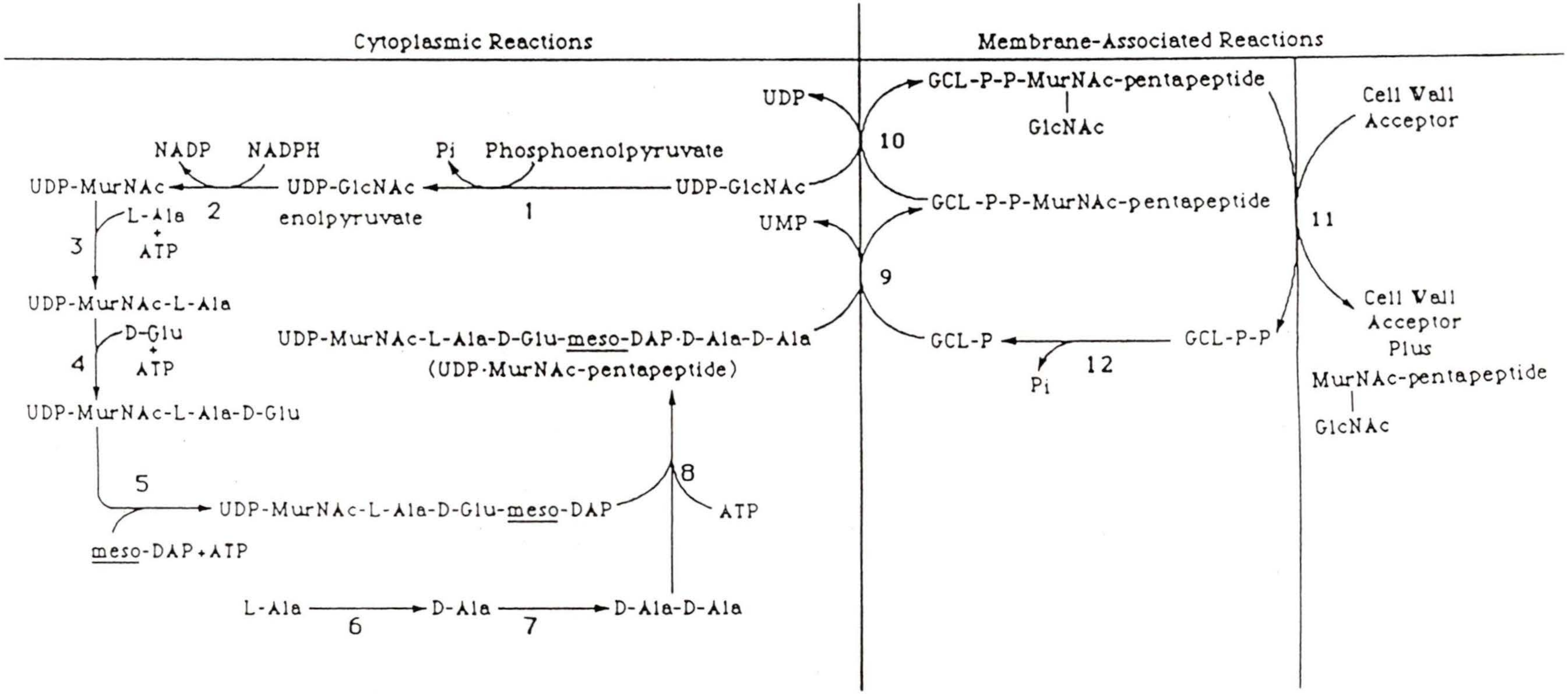
In the first stage, uridine diphosphate (UDP)-activated precursors are synthesized in the cytoplasm by soluble enzymes. These precursors are UDP-N-acetylglucosamine, and UDP-N-acetylmuramic acid with an attached pentapeptide. Specific amino acid ligases catalyze the sequential addition of L-alanine, D-glutamic acid, *meso*-diaminopimelic acid, and D-alanyl-D-alanine to UDP-MurNAc. The second stage involves a series of membrane-bound translocation reactions which result in the formation of lipid-linked intermediates. Undecaprenyl phosphate is a minor membrane lipid which serves to transport the hydrophilic peptidoglycan intermediates across the cytoplasmic membrane to the periplasmic face. This lipid carrier also transports the heteropolysaccharides of LPS and capsular polysaccharide. The limiting supply of this carrier requires that it be recycled (41).

The third stage occurs on the periplasmic face of the cytoplasmic membrane, where polymerization and maturation of macromolecular peptidoglycan occurs. Membrane-bound penicillin binding proteins (PBPs) catalyze the translocation of the disaccharide-pentapeptide units from the lipid carriers into acceptor sites in the pre-existing peptidoglycan matrix.

**Figure 5. Peptidoglycan biosynthesis in *E. coli* .**

The three stages of biosynthesis are subdivided into reactions which occur in the cytoplasm, on the cytoplasmic membrane face, and on the periplasmic membrane face. The enzymes involved in each biosynthetic step have been numbered 1-12. 1, phosphoenolpyruvate:UDP-GlcNAc-pyrophosphorylase; 2, UDP-GlcNAc-enolpyruvate reductase; 3, L-alanine adding enzyme; 4, D-glutamate adding enzyme; 5, *meso*-diaminopimelic acid adding enzyme; 6, alanine racemase; 7, D-alanyl-D-alanine synthetase; 8, D-alanyl-D-alanine adding enzyme; 9, UDP-MurNAc-pentapeptide translocase; 10, UDP-GlcNAc translocase; 11, peptidoglycan transglycosylase and/or transpeptidase (PBPs); 12, undecaprenol pyrophosphate pyrophosphatase.

From the dissertation of D. Dai, University of Victoria (1990).


Cytoplasmic Reactions
Membrane-Associated Reactions
UDP
GCL-P-P-MurNAc-pentapeptide
GlcNAc
Cell Wall
Acceptor
10
NADP
NADPH
Pi
Phosphoenolpyruvate
UDP-MurNAc
2
UDP-GlcNAc
enolpyruvate
1
UDP-GlcNAc
GCL-P-P-MurNAc-pentapeptide
11
L-Ala
+
ATP
3
UMP
9
UDP-MurNAc-L-Ala
D-Glu
+
ATP
4
UDP-MurNAc-L-Ala-D-Glu-meso-DAP-D-Ala-D-Ala
(UDP-MurNAc-pentapeptide)
GCL-P
12
Pi
GCL-P-P
Cell Wall
Acceptor
Plus
MurNAc-pentapeptide
GlcNAc
UDP-MurNAc-L-Ala-D-Glu
5
UDP-MurNAc-L-Ala-D-Glu-meso-DAP
8
ATP
meso-DAP+ATP
L-Ala
6
D-Ala
7
D-Ala-D-Ala

### ii) Penicillin binding proteins

PBPs are the enzymes involved in the insertion of new peptidoglycan subunits into the existing matrix during cell wall elongation and cell division. They acquired their name because they form covalent bonds with β-lactam antibiotics such as penicillin.

There are seven major PBPs which fall into two divisions based on molecular weight and function. Peptidoglycan polymerization in the final steps of biosynthesis is under the control of the high molecular weight PBPs, designated 1A, 1B, 2, and 3 (42, 43). PBPs 1A and 1B are both involved in peptidoglycan synthesis (44, 45). PBP2 is involved in elongation of the cylindrical cell wall as determined by observing mutants which grow as spheres as opposed to normal rods (45). Mutants in PBP3 are unable to form septa, and therefore PBP3 is proposed to be involved in cell division (44).

PBPs 4, 5, and 6 are the low molecular weight PBPs. The exact role of these PBPs is yet to be determined, but they may act as peptidoglycan hydrolases, as discussed in the next section.

### iii) Peptidoglycan hydrolases

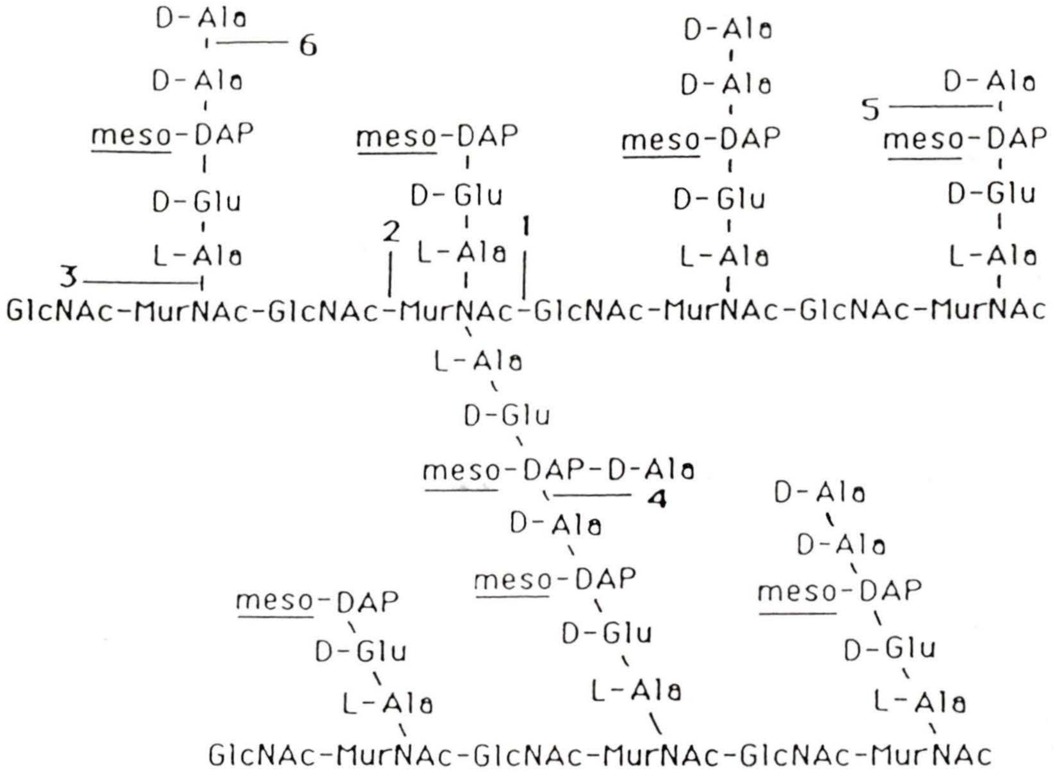
It is generally thought that growth and cell division of bacteria require enzymes capable of cleaving the covalent bonds within the sacculus to allow new peptidoglycan units to be inserted. A continuous state of turnover occurs, with new material being inserted and old material being released at a rate resulting in net growth (46). The set of enzymes responsible for this function are called peptidoglycan hydrolases. The hydrolases must function coordinately with each other and with synthetases during different growth conditions, aging, and cell division (47). If the hydrolases act alone and not in concert with the synthetases they become autolysins (46, 48).

There are nine hydrolytic enzymes in *E. coli* capable of cleaving the covalent bonds in high molecular weight peptidoglycan. These can be subdivided into the glucosidases which cleave interglycan linkages, and the peptidases which cleave interpeptide bonds or a glycan-peptide bond. Figure 6 summarizes the hydrolase activities on their substrates.

The first glucosidase is the lytic transglycosylase. It is an enzyme which cleaves the β-1,4-glycosidic bond between N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc). Cleavage is followed by an intramolecular transglycosylation reaction resulting in the formation of 1,6-anhydromuramic acid from the transfer of the O-muramyl residue onto its own C-6 hydroxyl group (49). There are two distinct enzymes encoded by different genes, one of which is soluble in the cytoplasm. Immunogold labeling demonstrated that this soluble lytic transglycosylase appears to be tightly bound to the peptidoglycan itself (38), even after osmotic shock or boiling in 4% SDS (49). Overproduction of the enzyme by up to 30-fold results in accumulation in the cytoplasm with no autolytic effects observed (50). This suggests that there is a controlled transport through the membrane. There is also a membrane-bound version of the lytic transglycosylase which resides in the envelope. It is proposed that this enzyme is associated with both the inner and outer membrane (51). The second glucosidase is β-*N*-acetylglucosaminidase. This enzyme hydrolyses soluble peptidoglycan fragments but not whole isolated sacculi (52).

**Figure 6. Peptidoglycan hydrolase activities on their substrates.**

The hydrolases have been numbered 1-6. Glucosidases: 1, lytic transglycosylase (soluble and membrane bound); 2, *β-N*-acetylglucosaminidase. Peptidases: 3, *N*-acetylmuramyl-L-alanine amidase. 4, PBP4 is an endopeptidase as well as a carboxypeptidase; 5, L,D-carboxypeptidase; 6, D,D-carboxypeptidases (PBP5 and PBP6). From the dissertation of D. Dai, University of Victoria (1990).


D-Ala
6
D-Ala
meso-DAP
D-Glu
L-Ala
3
meso-DAP
D-Glu
2
1
L-Ala
D-Ala
D-Ala
meso-DAP
D-Glu
L-Ala
5
D-Ala
meso-DAP
D-Glu
L-Ala
GlcNAc-MurNAc-GlcNAc-MurNAc-GlcNAc-MurNAc-GlcNAc-MurNAc
L-Ala
D-Glu
meso-DAP-D-Ala
4
D-Ala
meso-DAP
D-Glu
L-Ala
D-Ala
D-Ala
meso-DAP
D-Glu
L-Ala
meso-DAP
D-Glu
L-Ala
GlcNAc-MurNAc-GlcNAc-MurNAc-GlcNAc-MurNAc

The peptidases are as follows. N-acetylmuramyl-L-alanine amidase is similar to β-N-acetylglucosaminidase in that it accepts only muropeptides as a substrate. It resides in the periplasmic space and may be bound to a cell wall substrate since it can not be released by osmotic shock (38).

Two unrelated D,D-endopeptidases in *E. coli* have been found which catalyze the splitting of the D,D-alanine-diaminopimelic acid crossbridges within the peptidoglycan net. This D,D-peptide bond is crucial to the mechanical stability of the sacculus because it provides cross-linking between peptide bonds on adjacent glycan strands.

PBP4 is an endopeptidase. It is membrane-bound and is penicillin-sensitive. *In vivo*, PBP4 appears to function as a carboxypeptidase as well as an endopeptidase (53). A soluble, penicillin-insensitive enzyme encoded by the *mepA* gene is also an endopeptidase.

Recent studies have found the existence of an L,D crosslinking peptide bond. It exists in *meso*-DAP-*meso*-DAP crossbridges and is present in minor amounts in the peptidoglycan sacculus (37). Therefore, it has been suggested that a specific L,D-endopeptidase must exist, although this hydrolase has not yet been discovered (38).

An L,D-carboxypeptidase cleaves the L,D-peptide bond between meso-diaminopimelic acid and D-alanine. This enzyme is not inhibited by penicillin G. It is located in the periplasm as indicated by its easy release by osmotic shock or Tris/EDTA treatment (54).

PBPs 5 and 6 are D,D-carboxypeptidases (55) which catalayze the cleavage of the D-alanyl-D-alanine peptide bonds of the muramyl-peptide side chains. PBP 5 is highly sensitive to low concentrations of penicillin G.

PBP's 5 and 6 are anchored to the cytoplasmic membrane by short sequences at their carboxyl terminal end (56).

It has been suggested that regulation of the autolysis enzymes occurs mostly at the post-translational level, because the activities of the hydrolases (except the membrane-bound hydrolases) increases exponentially in synchronously growing cultures even when DNA synthesis is inhibited(57). Some post-translational regulatory mechanisms which may function include the following; i) a restricted export of the hydrolase into the periplasm where it acts on its substrate (50), ii) a topological restriction of enzyme distribution in the cell wall, specifically localized in the inner or outer membrane, or on the peptidoglycan matrix itself (38) and, iii) specific activation of the enzyme by substrate modification (58).

In the case of the membrane-bound hydrolases (e.g., PBP 4), the newly synthesized enzymes are capable of initiating hydrolysis, while the old enzymes are functionally inert (59).

### iv) Autolysis by endogenous hydrolases

*E. coli* cells are subject to autolysis induced by either a deprivation in growing cells of the necessary components for peptidoglycan biosynthesis such as diaminopimelic acid, or, if peptidoglycan biosynthesis is blocked by antibiotics (38, 60). The proposed mechanism of antibiotic induced lysis involves a deregulation of the peptidoglycan hydrolases from the synthetases. By analyzing the products of autolysis, the lytic transglycosylases and the endopeptidases have been implicated as autolysins (61). Further evidence for the major role played by the endogenous hydrolases in autolysis is seen by a lysis of cells in which the soluble lytic transglycosylase *(slt)* gene is overexpressed >30-fold (50). Although different antibiotics inhibit biosynthesis of peptidoglycan at different steps in the pathway, the basis for autolysis appears to remain a result of endogenous hydrolases (62).

## 1.4 Regulation of Peptidoglycan Biosynthesis and Autolysis by the Stringent Response

As described in section 1.2, the stringent response to amino acid starvation results in a global shutdown of macromolecular synthesis. Of interest here is the restriction on phospholipid and peptidoglycan synthesis. This response is seen in *relA*$^+$ strains of *E. coli* in correlation with the accumulation of pppGpp and ppGpp, but not in *relA* strains.

It has been observed that non-growing *E. coli* (for example, amino acid starved cells) are tolerant to penicillin-induced autolysis. This is because the stringent response inhibits both peptidoglycan synthesis and β-lactam-induced autolysis in *relA*$^+$ strains of *E. coli.*

Based on recent observations, a preliminary model has been proposed by Ishiguro et al. (63) (Figure 7) to explain the regulation of peptidoglycan synthesis and β-lactam-induced lysis in amino acid-deprived *E. coli* cells. First, amino acid starvation results in an accumulation of ppGpp. The accumulation of ppGpp inhibits phospholipid synthesis. Peptidoglycan synthesis is also blocked, both at an early and a late step in the pathway. The early step involves a block in the synthesis of UDP-MurNAc-pentapeptide and possibly UDP-GlcNAc, whereas the late step appears to be a block in the PBP-catalyzed peptidoglycan polymerization reaction. This

**Figure 7. Model for the regulation of PG synthesis and B-lactam-induced lysis in amino acid-deprived *E. coli.***

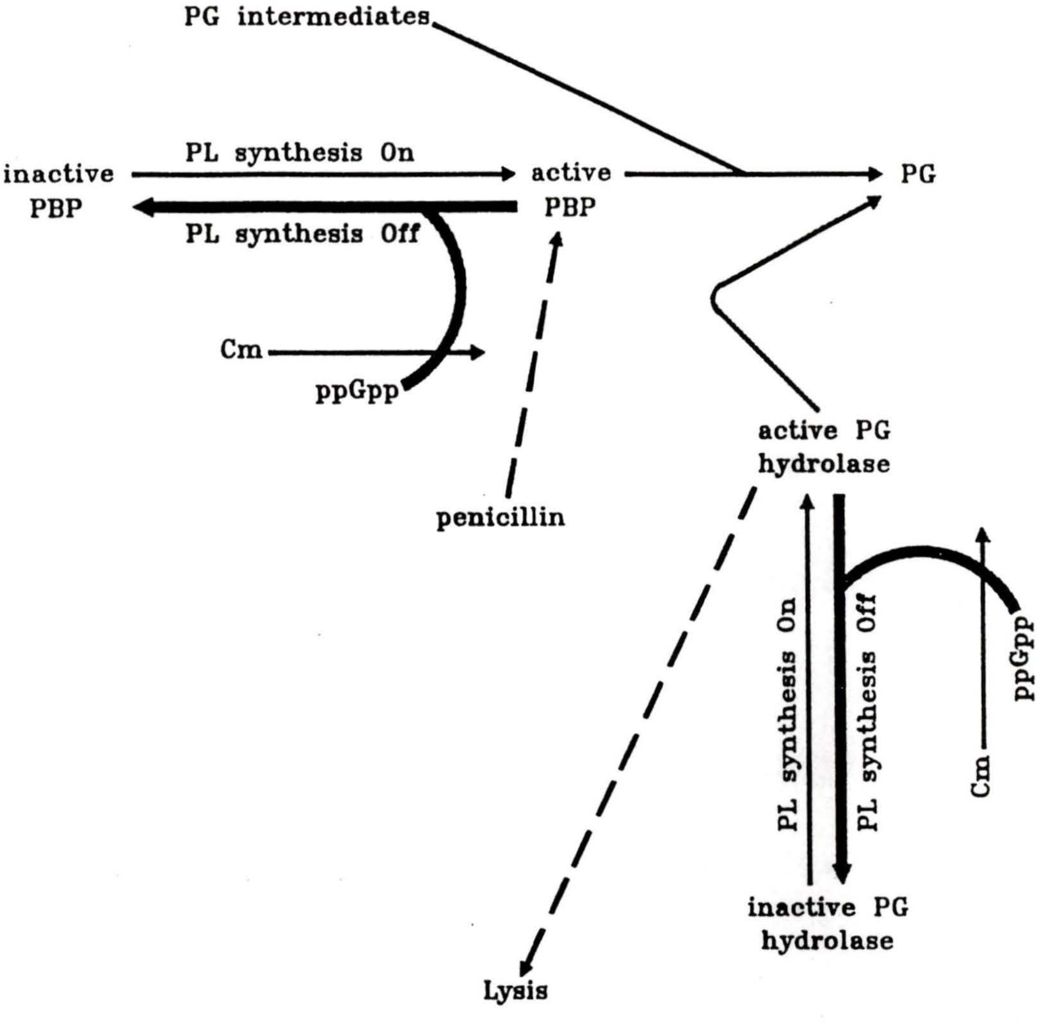
The thick arrows depict the effects of the stringent response. The thin arrows depict the effects of blocking the stringent response, e.g., with chloramphenicol (Cm). The broken arrows indicate the proposed effects of penicillin treatment in the presence of a stringent response inhibitor such as Cm.

1. The penicillin-binding proteins (PBPs) involved in PG polymerization and the PG hydrolases responsible for β-lactam induced lysis undergo reversible transitions from active to inactive forms. The active forms of these enzymes require ongoing phospholipid (PL) synthesis.

2. PL synthesis is known to be inhibited by ppGpp. This apparently accounts for the inactivation of both PBPs and PG hydrolases during the stringent response.

3. Stringent response inhibitors (e.g., Cm) or a mutation in *relA* inhibit ppGpp synthesis during amino acid deprivation. This relaxes PL synthesis, and the consequent activation of PBPs and PG hydrolases accounts for the suppression of penicillin tolerance.

From Reference 63


PG intermediates
inactive PBP
PL synthesis On
PL synthesis Off
active PBP
PG
Cm
ppGpp
penicillin
active PG hydrolase
PL synthesis On
PL synthesis Off
ppGpp
Cm
inactive PG hydrolase
Lysis

peptidoglycan polymerization reaction is apparently dependent on ongoing phospholipid synthesis. When phospholipid synthesis is inhibited by ppGpp, both PBPs and peptidoglycan hydrolase(s) are converted from active to inactive forms. It has therefore been suggested that the inhibition of PL synthesis during the stringent response is reponsible for the inhibition of i) the PBP activity during the peptidoglycan polymerization step, and ii) the peptidoglycan hydrolase activity during the lysis induction stage.

In this model, if the stringent response is relaxed by chloramphenicol, the active forms of PBPs and peptidoglycan hydrolases are present. This allows the binding of the β-lactam antibiotic to the active PBP in what is termed the "priming" stage. After priming, the active hydrolases result in the lysis induction stage. Priming can be dissociated from the lysis induction stage. It is known that early in the period of amino acid deprivation the antibiotic interacts with its target, the PBPs, since bacteria in which excess unbound antibiotic has been removed can be induced to lyse by the addition of chloramphenicol.

Several questions remain concerning this model on the mechanism of penicillin-induced autolysis-tolerance in amino acid-deprived *E. coli* cells. These include: (1) the role played by PL synthesis in, and the molecular basis for the reversible inactivation of PBPs and peptidoglycan hydrolase(s); (ii) if peptidoglycan hydrolases play a role in peptidoglycan synthesis, and what that role may be; and (iii) the relationship between the interaction of PBP and β-lactam, and the subsequent peptidoglycan-mediated autolysis (63).

## 1.5 Purpose and Organization of This Thesis

The objective of this M.Sc. project was to further characterize mutants exhibiting abnormalities in peptidoglycan metabolism. Two strains carrying temperature sensitive mutations, designated *lytA* and *lytB*, have been isolated in this laboratory which exhibit a tolerance to the autolytic effects of penicillin when shifted to the restrictive temperature. Strains VC4217 and VC4218 carry the *lytB1* and *lytB2* mutant alleles, respectively. This project involved confirming the phenotype of VC4218, and identifying the *lytB*$^+$ gene.

# Chapter 2

# MATERIALS AND METHODS

## 2.1 Bacteria and Plasmids

All bacteria used in this study were derivatives of *E. coli* strain K-12. The bacterial strains and plasmids used are listed in Table 1 and Table 2, respectively.

## 2.2 Media and Culture Conditions

Bacteria were cultured in Tryptic Soy Broth (TSB, Difco Laboratories) or Luria Broth (LB, Gibco Laboratories). Solidified versions of these media, designated TSA and LA, respectively, contained 1% agar. In some experiments, 2YT-X-gal agar was used (1.6% Tryptone, 1.0% Yeast Extract, 0.5% NaCl, and 40 µg/ml X-gal).

Cultures in liquid media were incubated in gyrotory waterbath shakers at the indicated temperatures, and growth was monitored with a spectrophotometer at a wavelength of 600 nm. Unless otherwise stated, antibiotics were added at the following concentrations when required: tetracycline, 20 µg/ml; ampicillin, 50 µg/ml; kanamycin, 50 µg/ml;

chloramphenicol, 50 µg/ml; spectinomycin, 50 µg/ml. When IPTG was added, a concentration of 0.1 mM was used.

## 2.3 Maintenance of Bacterial Strains

For long term storage, all strains were prepared from an overnight culture grown at the appropriate temperature with antibiotic selection where relevant. Glycerol was added to a final concentration of 30% v/v, and the suspension stored in 1.5 ml screw-capped plastic tubes at -70$^{0}$C. Fresh cultures were prepared by streaking a loopful of the glycerol suspension onto a TSA plate (with antibiotics as appropriate) followed by overnight incubation just prior to use.

## 2.4 Determination of Temperature Sensitivity

The relative degrees of temperature sensitivity exhibited by different mutant strains were quantified in terms of colony forming capabilities. This procedure, called efficiency of plating (E.O.P.), involved serial dilutions of cultures grown overnight in TSB at 30$^{0}$C were plated in duplicate on TSA. One set of plates was incubated at 30$^{0}$C and the other at 42$^{0}$C. Plate counts were determined after 24 hrs of incubation. Longer periods of incubation did not change the plate counts. The efficiency of colony formation was expressed as a ratio of the plate count in terms of colony-forming units (CFU) per ml obtained at 42$^{0}$C to the plate count obtained at 30$^{0}$C.

## 2.5 General Recombinant DNA Techniques

Plasmid DNA was isolated by the alkaline lysis miniprep procedure (64), with the addition of a phenol/chloroform step before ethanol precipitation. DNA restriction endonuclease digestions, blunt-ending reactions of overhanging single strand DNA ends created from restriction digests, and ligations of either staggered-end or blunt-end DNA were performed as described by Maniatis (65). Transformations were by electroporation (64) or by the procedure of Chung *et al.* (66). Agarose gel electrophoresis of DNA fragments was performed in 0.7% gels following the procedure of Maniatis using the TAE buffer system (0.04 M Tris-acetate; 0.002 M EDTA) (65). For preparative DNA gels, the appropriate band which was stained with ethidium bromide (0.5 µg/ml) and visualized briefly with a UV lightbox, was excised with a razor blade. The DNA was extracted from the agarose by electroelution (IBI Electroeluter).

Restriction endonucleases, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, T4 DNA ligase, and DNA molecular weight standards (λ DNA digested with *Hin*dIII) were purchased from Amersham, Pharmacia, New England Biolabs, or Boehringer Mannheim.

## 2.6 Cloning Methods

### 2.6.1 Deletion mapping of *orf316*.

The strategy used for deletion mapping was based on the pAA-PZ718 vector system first described by Ahmed (67) and further modified by Peng and Wu (68). Plasmid pVW8 and vector pAA-PZ718 were digested with

*Xba*I. The 3.6 kb *Xba*I-*Xba*I fragment of interest from pVW8 was cloned into the *Xba*I site of pAA-PZ718. The colonies were selected on 2YT-Xgal plates containing Cm and Ap, and one clone showing the correct construction by restriction analysis was designated pCG17. Subculturing of strains carrying the pAA-PZ718 plasmid in the absence of Cm allows excision from the plasmid of the transposon Tn9 which encodes $Cm^R$. Plasmid pCG17 was subcultured in the strain DH5α four times with Ap but in the absence of Cm. This resulted in variable portions of adjacent plasmid DNA being deleted with the transposon. In this fashion, random deletions of the insert cloned in plasmid pAA-PZ718 were formed. Colonies from Ap plates were then screened for Cm sensitivity to confirm loss of the transposon. $Cm^S$ colonies were further screened by plasmid isolation, restriction endonuclease digestion, and were sized by agarose gel electrophoresis. The deletion derivatives were analyzed for complementation of the *lytB2* mutation in strain VC4218.

### 2.6.2 Subcloning into pUC18/pUC19 and pT7T318U/pT7T319U

All subcloning procedures were similar to that described above. The fragment to be cloned and the vector were digested with the appropriate restriction enzymes in 10 μl reaction volumes. Completeness of digestion was checked by agarose gel electrophoresis. The enzymes were heat inactivated for 10-15 min at either 65 or 95$^{o}$C and put on ice immediately. At this point, the DNA fragment of interest was usually purified by preparative agarose gel electrophoresis. Vector DNA was added to insert DNA at a ratio of 1:3 and a ligation reaction set up in a 15 μl volume. Cohesive-end ligations using T4 DNA ligase were incubated at 16$^{o}$C overnight. Ligation mixtures were then used to electrotransform the strain DH5α and plated on 2YT-X-gal plates containing appropriate antibiotics. Only 2 µl of the ligation mixtures was added to the cells for electroporation. White DH5α colonies in the presence of X-gal were picked for plasmid isolation, and the DNA analysed by restriction digests and agarose gel electrophoresis. Plasmids which appeared correct after restriction analyses were transformed into VC4218 for complementation analyses. Where stated, strain VC4217 was transformed for complementation analyses.

### 2.6.3 *In vitro* insertion mutagenesis

Plasmid pCG18 to be mutagenized was linearized with the restriction endonuclease, *Bst*EII, a unique site within *orf316*. The single-strand overhang generated from digestion with *Bst*EII was filled in using Klenow fragment of *E. coli* DNA polymerase I. The omega (Ω) insertion element from pHP45Ω (71) was digested with *Sma*I. The Ω element consists of a gene encoding spectinomycin and streptomycin. This gene is flanked by transcription and translation terminators and several restriction endonuclease sites. *Sma*I-digested pHP45Ω was added to *Bst*EII-linearized pCG18 at a ratio of 4:1 in a ligation reaction volume of 15 µl. The blunt-end ligation reaction with T4 DNA ligase was incubated at room temperature overnight. The strain DH5α was electrotransformed with 2 µl of the ligation mixture and incubated on TSA plates containing ampicillin, and spectinomycin to select for mutagenized recombinants. Plasmids were isolated for restriction analyses, and those which appeared correct on the gel were transformed into VC4218 for complementation analyses.

## 2.7 Thin-layer chromatography

In order to demonstrate the chronic accumulation of ppGpp in the mutant strain VC4218 at 42°C, a nucleotide fraction was analysed by thin layer chromatography (72). All cultures were grown in complex media to avoid subjecting the cells to amino acid deprivation, and thus inadvertantly inducing the stringent response. Strains were incubated at either 30°C or 42°C. To one portion of VC4218 at 30°C, 500 µg/ml of serine hydroxamate was added. Chloramphenicol was added to one portion of VC4218 at 42°C. Material from $2 \times 10^6$ cells were pulse-labeled with [$^{32}$P]-phosphate for 15 min. Formic acid extracts of these cells were applied to each lane of a Polygram Cel 300 PEI thin-layer plate (Brinkmann Instruments Canada Ltd., Rexdale, Ontario, Canada). The plate was developed in 1.5 M $KH_2PO_4$ and then subjected to autoradiography.

## 2.8 Expression of Cloned DNA

The bacteriophage T7 RNA polymerase-promoter coupled system described by Tabor and Richardson (70) was used to exclusively express and label a cloned gene and its product. The DNA to be expressed was cloned downstream of the T7 promoter Ø10 in plasmid vectors pT7T318U and pT7T319U. The resulting plasmids were then transformed into strain KX100 (73) carrying pGP1-2 which encodes T7 RNA polymerase. The transformants were grown in LB with selective drugs at 30°C to early exponential phase. A sample of 1 ml culture was centrifuged and the pellet washed twice with M9 medium and then resuspended in 2 ml M9 medium supplemented with glucose (2% final concentration) and 0.2 ml methionine assay medium (Difco Laboratories). Cells were further grown at 30°C for 1 hr and shifted to 42°C for 20 min. Rifampicin was then added to a final concentration of 200 μg/ml to inhibit selectively the host RNA polymerase. After 10 min of rifampicin treatment, the cells were shifted down to 30°C for an additional 40 min. The sample was labeled with 10 μCi [$^{35}$S] methionine (800 Ci/mmol; Amersham Corp.) for 5 min at 30°C. The sample was briefly centrifuged and the cell pellet was suspended in 100 μl of cracking buffer (60mM Tris-HCl pH 6.8; 1% SDS; 1% 2-mercaptoethanol; 10% glycerol; 0.01% bromophenol blue). Samples were heated to 95°C for 3 min and 10 μl loaded on an SDS-polyacrylamide gel as described below.

### 2.9 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE of [$^{35}$S]-methionine labeled proteins was carried out in 12.5% gels (0.75 mm thick) according to the method of Swank and Munkres (74). Protein molecular weight standards were from Pharmacia, and these included bovine serum albumin (66 kD), egg albumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), trypsin inhibitor (20 kD), and o-lactalbumin (14 kD). Samples were run at a constant voltage (100 V for stacking gel and 200 V for the separating gel). The gel was placed in fixative (40% methanol/10% acetic acid) for 30 min. Proteins were stained by gentle agitation for 2 hrs in 0.1% (w/v) Coomassie Brilliant Blue G250 dissolved in fixative. Destaining was accomplished with several changes of a 10% methanol/7.5% acetic acid solution with gentle agitation for 4 hrs. After drying on Whatman 3MM chromatography paper at $60^{0}$C for 2 hrs on a Bio Rad gel drier, the gels were subject to autoradiography. [$^{35}$S]-methionine autoradiography was performed at room temperature for 24-48 hrs using Kodak film.

**Table 1. *E. coli* K-12 strains**

| Strain | Relevant Genotype/Description | Reference/Source |
|---|---|---|
| VC7 | *thi-1 lysA23 rpsL109* | This Laboratory |
| VC8 | *thi-1 lysA23 rpsL109 relA* | This Laboratory |
| VC4217 | *thi-1 lysA23 lytB1* | This Laboratory |
| VC4218 | *thi-1 lysA23 lytB2* | This Laboratory |
| VC550 | *thi-1 lysA23 rpsL109 relA lytB* | This Laboratory |
| DH5α | *thi hsdR17 recA1 relA1 endA1 gyrA96* ∅80d*lac*Z M15 | BRL[a] |
| KX100 | *hsdR514 lacY1 galK2 galT22 metB1 trpR55 recA56 srl*::Tn*1* | (73) |

[a] Bethesda Research Laboratories, Gaithersburg, ML. U.S.A.

## Table 2. Plasmids

| Plasmid | Relevant Genotype/Description | Reference/Source |
|---|---|---|
| pAA-PZ718 | $Ap^R$ $Cm^R$ ISI-Tn*9* *ori* | GBT[a] |
| pUC18 | $Ap^R$ | (69) |
| pUC19 | $Ap^R$ | (69) |
| pT7T318U | $Ap^R$ T7 promoter Ø10 | (70) |
| pT7T319U | $Ap^R$ T7 promoter Ø10 | (70) |
| pGP1-2 | $Km^R$ T7 RNA polymerase | (70) |
| pHP45Ω | $Ap^R$ $Sm^R/Sp^R$ Ω | (71) |
| pGM21 | $Ap^R$ *IleS-lsp* operon and *dapB* on a 7 kb fragment in pBR322 | (75) |
| pVW8 | $Ap^R$ *IleS-lsp* operon and *dapB* on a 8 kb fragment in pUC13 from pGM21 | This Laboratory |
| pCG17 | $Ap^R$ $Cam^R$ *lytB*$^+$ on 3.6 kb *Xba*I fragment in pAA-PZ718 | This study |
| pCG17.7 | $Ap^R$ *lytB*$^+$ deletion derivative | This study |
| pCG17.18 | $Ap^R$ *lytB*$^+$ deletion derivative | This study |
| pCG17.19 | $Ap^R$ *lytB* deletion derivative | This study |
| pCG17.30 | $Ap^R$ *lytB* deletion derivative | This study |

**Table 2 : continued**

| | | |
|---|---|---|
| pCG17.31 | Ap$^R$ *lytB* deletion derivative | This study |
| pCG17.43 | Ap$^R$ *lytB*$^+$ deletion derivative | This study |
| pCG17.44 | Ap$^R$ *lytB* deletion derivative | This study |
| pCG18 | Ap$^R$ *lytB*$^+$ on 3.6 kb *Xba*I fragment in pUC18 | This study |
| pCG19 | Ap$^R$ Sm$^R$/Sp$^R$ Ω-inactivated *lytB* of pCG18 | This study |
| pCG20 | Ap$^R$ *lytB*$^+$ on 1.7 kb *Xba*I-*Pst*I fragment in pT7T319U (+ orientation) | This study |
| pCG21 | Ap$^R$ *lytB*$^+$ on 1.7 kb *Xba*I-*Pst*I fragment in pT7T318U (- orientation) | This study |
| pCG23 | Ap$^R$ Ω-inactivated *lytB* fragment in pT7T318U (- orientation) | This study |
| pCG24 | Ap$^R$ Ω-inactivated *lytB* fragment in pT7T318U (+ orientation) | This study |

---

[a] Gold Biotechnology Inc. St. Lois, MO. U.S.A.

# Chapter 3

# RESULTS

## 3.1 Description of mutant VC4218 phenotype

### 3.1.1 Introduction

Several temperature-sensitive mutants exhibiting β-lactam-induced lysis-tolerance at the restrictive temperature when growing in complex media have been previously isolated (76). One of these mutants, strain VC4218, carries a mutation in a new gene which was designated *lytB* (77). It is shown here that the *lytB2* mutant allele in VC4218 confers a defect in the regulation of the stringent response.

### 3.1.2 Results

Figure 8 illustrates the temperature-dependent ampicillin tolerance conferred by the *lytB2* mutation. A culture of VC4218 (*lytB2*) growing at 30°C was either maintained at 30°C, or shifted to 42°C at 115 min (first arrow). Ampicillin (50 μg/ml) was added at 125 min to both portions of the culture (second arrow). It is clear that shifting the mutant strain to 42°C protected it from the lysis-inducing activity of ampicillin.

The addition of certain ribosome inhibitors suppressed the temperature-dependent ampicillin tolerance of strain VC4218. Figure 9

**Figure 8. Temperature-dependent ampicillin tolerance in strain VC4218 (*lytB2*).**

A culture growing at 30$^{0}$C was either maintained at 30$^{0}$C (●) or shifted to 42$^{0}$C (○) at 115 min (first arrow). Ampicillin (50 µg/ml) was added at 125 min (second arrow) to both portions of the culture. Cell growth was measured at an absorbance of 600nm.

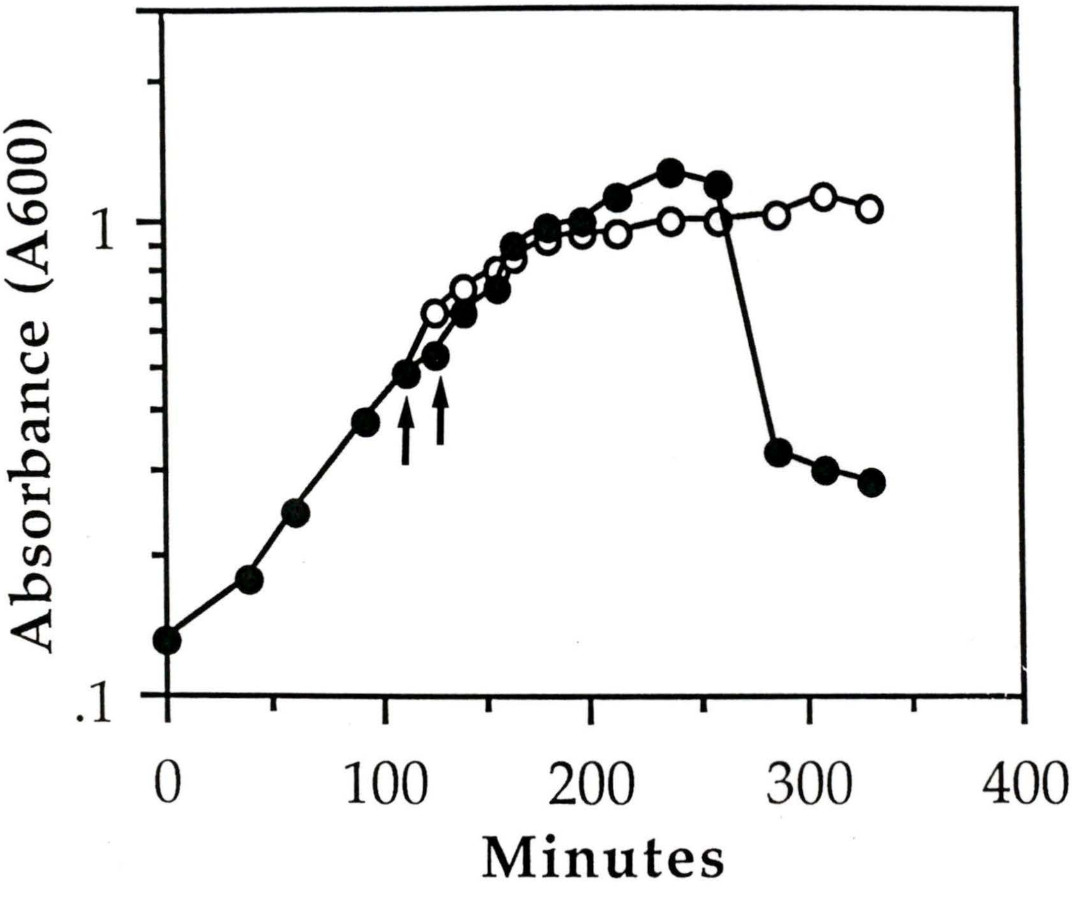
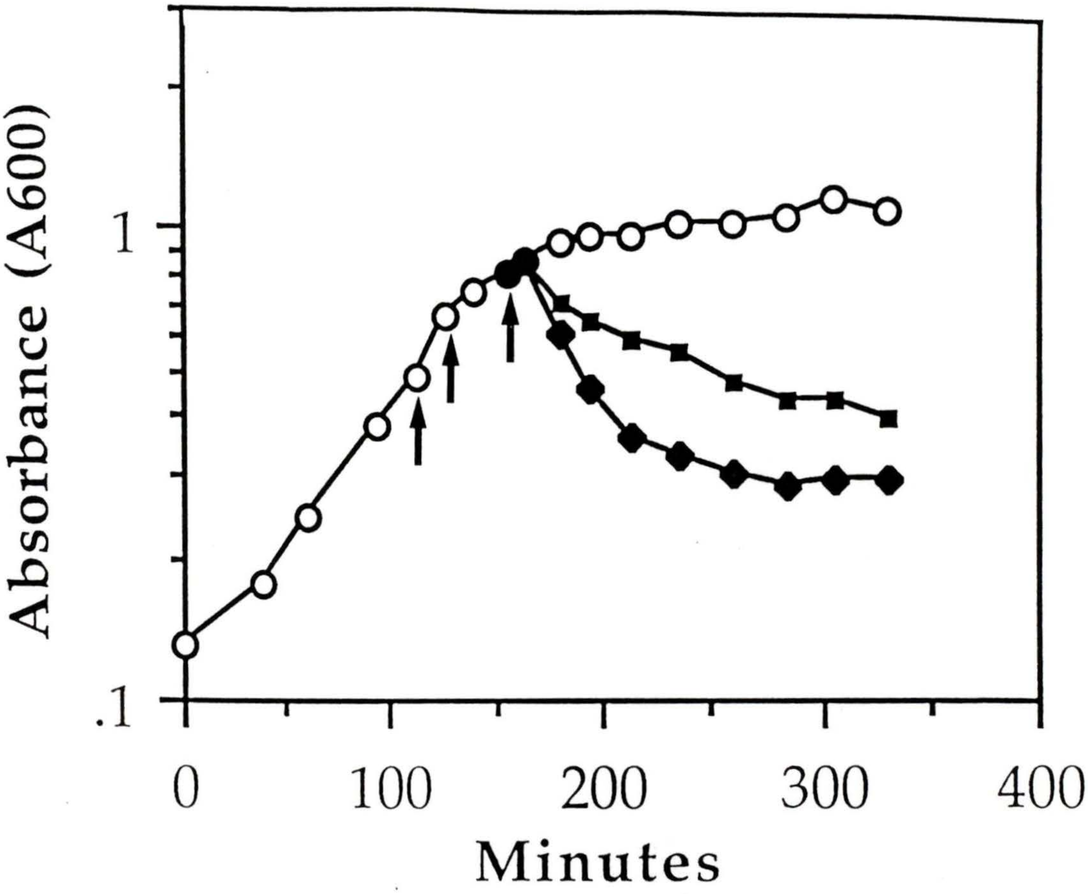

Absorbance (A600)
1
.1
0
100
200
300
400
Minutes

**Figure 9. Suppression of temperature-dependent ampicillin tolerance in strain VC4218 by ribosome inhibitors which are known to block the stringent response.**

A culture (○) growing at 30°C was shifted to 42°C at 115 min (first arrow). Ampicillin (50 µg/ml) was added at 125 min (second arrow). At 155 min (third arrow), kanamycin (100 µg/ml; ■) or chloramphenicol (100 µg/ml; ◆) were added to portions of the ampicillin-treated culture.


Absorbance (A600)
1
.1
0
100
200
300
400
Minutes

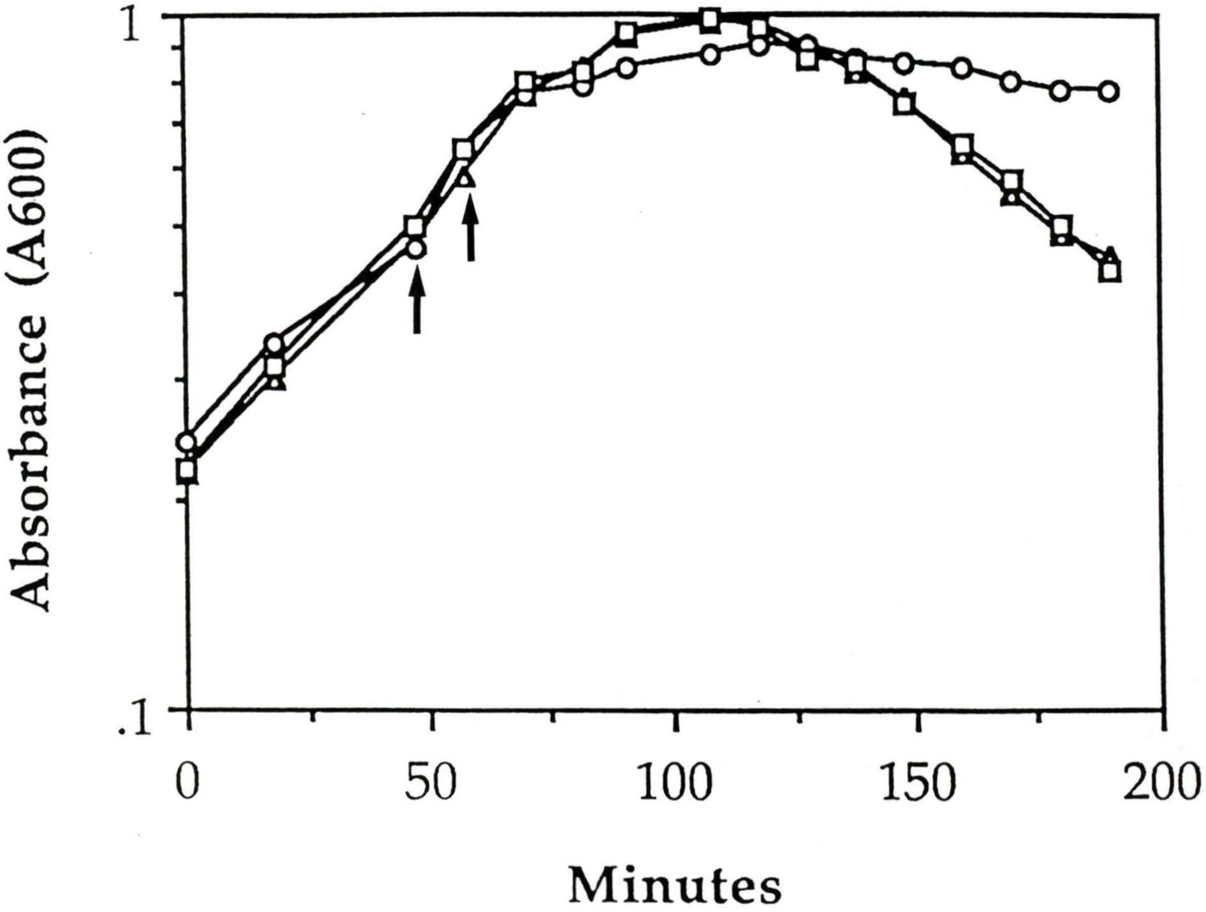
illustrates this suppression by kanamycin and chloramphenicol. A culture of strain VC4218 (*lytB2*) growing at 30$^{0}$C was shifted to 42$^{0}$C at 115 min (first arrow). Ampicillin (50 µg/ml) was added at 125 min (second arrow). At 155 min, kanamycin (100 µg/ml) or chloramphenicol (100 µg/ml) were added to portions of the ampicillin-treated culture (third arrow). The ampicillin-treated culture stopped growing but did not lyse in accordance with what has already been shown in Figure 8. However, lysis occurred shortly after the addition of kanamycin or chloramphenicol to the portions of the ampicillin-treated culture.

The abilities of the ribosome inhibitors to suppress *lytB2* was believed to be related to their abilities to inhibit the stringent response because the ampicillin tolerance associated with *lytB2* was also suppressed by a mutation in *relA*. In Figure 10, cultures of VC4218 (*lytB2*, *relA*$^{+}$), VC550 (*lytB2*, *relA*), and VC8 (*lytB*$^{+}$, *relA*) were grown at 30$^{0}$C, shifted to 42$^{0}$C at 47 min (first arrow), and treated with ampicillin (50 µg/ml) at 57 min (second arrow). As previously illustrated, VC4218 stopped growing but failed to lyse upon the addition of ampicillin at 42$^{0}$C. In contrast, the *lytB*$^{+}$ *relA* strain, VC8, lysed as expected. VC550, the *lytB2 relA* double mutant, also lysed upon the addition of ampicillin. These results indicate that the *relA* mutation suppressed the ampicillin tolerance associated with the *lytB2* mutation. Furthermore, this conclusion suggests that the *lytB2* suppressor activities of chloramphenicol and kanamycin are related to the ability of these agents to inhibit the stringent response.

The ppGpp metabolism of strain VC4218 was studied to further investigate the possible link between the stringent response and the temperature-dependent ampicillin tolerance conferred by *lytB2*. The

**Figure 10. Suppression of temperature-dependent ampicillin tolerance conferred by the *lytB2* allele in a *relA* genetic background.**

Symbols: □ ,strain VC8 (*relA*$^-$ *lytB*$^+$); ○ VC4218 (*relA*$^+$ *lytB2*); △ VC550 (*relA*$^-$ *lytB2*). Cultures growing at 30°C were shifted to 42°C at 47 min (first arrow), and ampicillin (50 µg/ml) was added at 57 min (second arrow).

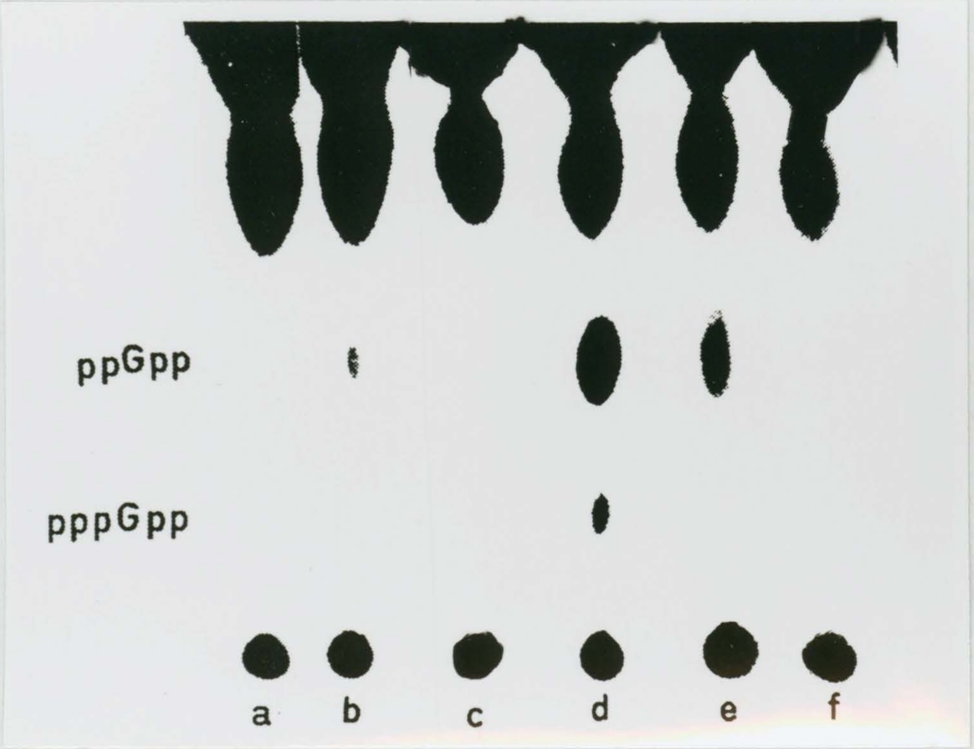

1
.1
Absorbance (A600)
0
50
100
150
200
Minutes

autoradiograph in Figure 11 illustrates thin-layer chromatography of formic acid extracts of [$^{32}$P]-phosphate pulse-labeled cells from cultures subjected to a range of conditions. Lane a is a control from a wild type strain, VC7 (*lytB*$^+$), grown in complex media at 30°C. As expected, there was no accumulation of ppGpp. Lane b is VC7 shifted from 30°C to 42°C. The small amount of ppGpp accumulation is characteristic of heat shock induction of ppGpp synthesis (78). In lane c, the temperature sensitive mutant VC4218 grown at 30°C as a control showed no accumulation of ppGpp. When 500 µg/ml of serine hydroxamate was added to VC4218 grown at 30°C, the stringent response was induced. Thus, there was a substantial accumulation of ppGpp and some pppGpp. Lane e shows the effects of temperature upshift on strain VC4218. Again there was a substantial accumulation of ppGpp, i.e., in excess of the amount expected from heat shock induction alone. This suggests that the mutant LytB protein was somehow involved in inducing the stringent response. In support of this proposal, the ppGpp accumulation in VC4218 at 42°C was suppressed in the presence of the stringent response antagonist, chloramphenicol (lane f).

These results in conjunction with the demonstration of temperature dependent tolerance to β-lactam-induced autolysis indicate that the stringent response is induced in VC4218 (*lytB2*) upon upshift to the restrictive temperature.

**Figure 11. Temperature-dependent ppGpp accumulation in strain VC4218.**

Thin-layer chromatography of formic acid extracts of [$^{32}$P]-labeled cells, showing the effects of temperature upshift from 30°C to 42°C, amino acid deprivation, and chloramphenicol treatment. Material from 2 x $10^6$ cells labeled for 15 min was applied to each lane of a Polygram Cel 300 PEI thin-layer plate (Brinkmann Instruments Canada Ltd., Rexdale, Ontario, Canada). The plate was developed in 1.5 M $KH_2PO_4$ and then subjected to autoradiography. Lanes: a, strain VC7 (*lytB*$^+$) at 30°C, control; b, VC7, 30°C to 42°C upshift; c, VC4218 (*lytB2*) at 30°C, control; d,VC4218 at 30°C with 500 µg of serine hydroxamate per ml; e, VC4218, 30°C to 42°C upshift; f, VC4218, 30°C to 42°C upshift in the presence of chloramphenicol.


ppGpp
pppGpp
a
b
c
d
e
f

## 3.2 Identification of the *lytB* Locus

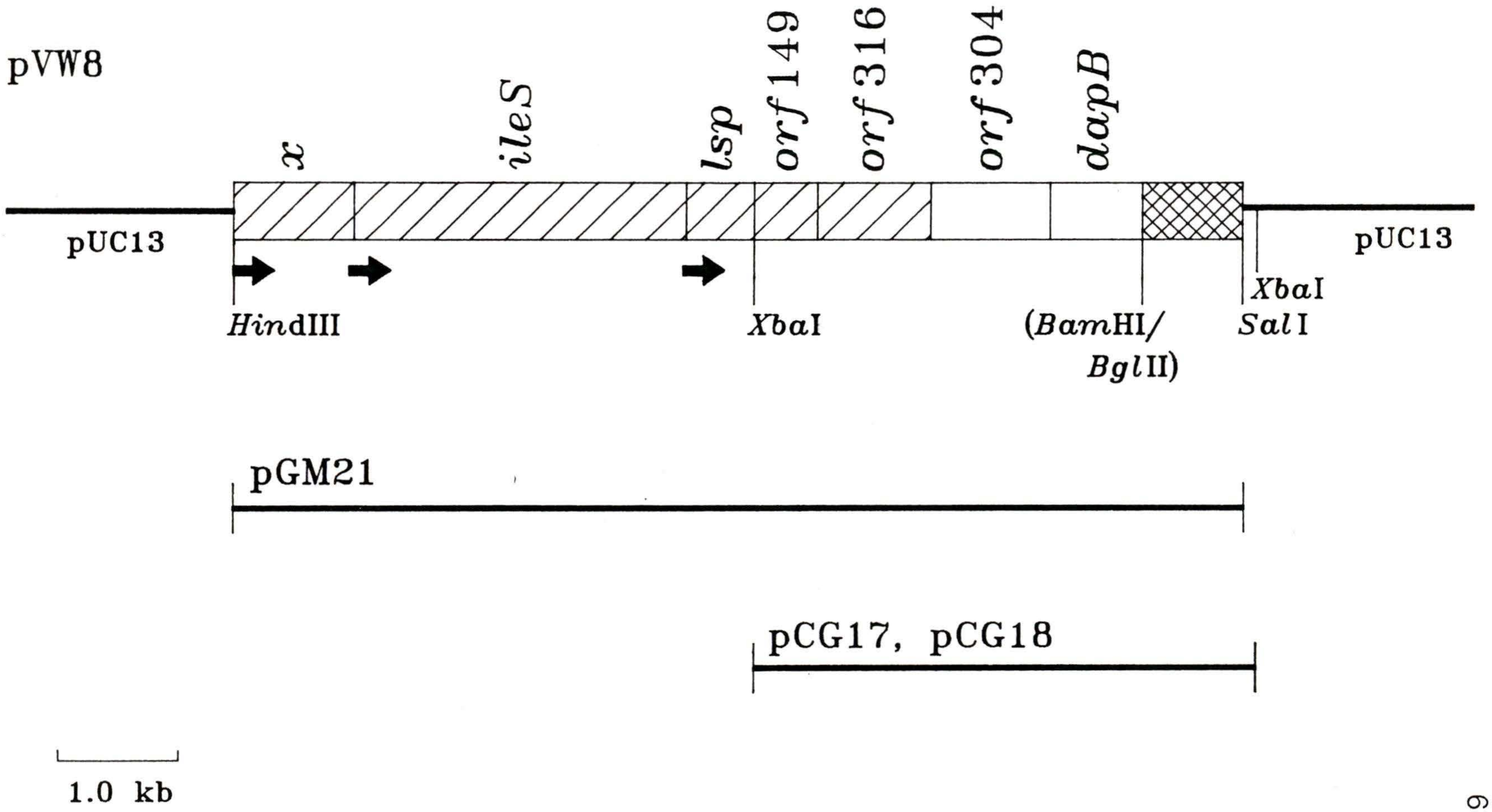
### 3.2.1 Background

The *lytB* gene was mapped at 0.5 min on the *E. coli* genetic linkage map by W. Kusser (submitted for publication). This region of the genome has been cloned and characterized by Mackie (75) and by Miller *et al.* (79). A plasmid, designated pGM21, carrying a 7 kb insert derived from this area was obtained from G. Mackie (University of Western Ontario). The insert from pGM21 was subcloned into the plasmid vector, pUC13, by W. Kusser to create pVW8 (Figure 12). The insert carries the entire *ileS-lsp* operon, an open reading frame designated *orf304*, and a portion of the *dapB* gene (encoding dihydropicolinate reductase). The *ileS-lsp* operon consists of (i) a gene called *x* which encodes a 34 kD soluble protein of unknown function; (ii) *ileS* (isoleucyl-tRNA synthetase); (iii) *lsp* (prolipoprotein signal peptidase); and (iv) two open reading frames designated *orf149* and *orf316*. This unit is believed to represent an operon because transcripts representing the entire unit are initiated from a promoter located upstream of gene *x*. However, there are at least two other promoters, one within gene *x* and another in *ileS* (79). Transcriptional control of the operon may therefore be complex.

Both pGM21 and pVW8 were shown to complement the *lytB2* mutation in strain VC4218 by eliminating penicillin tolerance and temperature sensitive growth at the restrictive temperature (W. Kusser, unpublished data). The main objective of this study was to identify the *lytB* locus on these plasmids. As reported below, this was accomplished by two independent methods: (i) deletion analysis, and (ii) insertion mutagenesis.

**Figure 12. The relevant structural features of plasmid pVW8 and related plasmids**

Plasmid pVW8 is a derivative of pUC13 carrying an 8 kb *Hin*dIII-*Sal*I fragment derived from plasmid pGM21. The 8 kb fragment consists of the 5-gene *ileS-lsp* operon, highlighted by hatching (*x*, *ileS*, *lsp*, *orf149*, and *orf316*), an open reading frame designated *orf304*, a protion of the *dapB* gene, and approximately 300 bp derived from the pBR322 parent vector of pGM21 (crosshatched section). The *ileS-lsp* operon is proposed to contain three promoters which are indicated by arrows. Shown below are the segments of this region carried by three plasmids related to pVW8. As already noted, the *Hin*dIII-*Sal*I fragment in pVW8 was derived from pGM21. Plasmid pCG17 carries the *Xba*I-*Xba*I fragment derived from pVW8 on the vector pAA-PZ718. Plasmid pCG18 carries the same fragment on the vector pUC18.


pVW8
x
ileS
lsp
orf149
orf316
orf304
dapB
pUC13
pUC13
HindIII
XbaI
(BamHI/
BglII)
XbaI
SalI
pGM21
pCG17, pCG18
1.0 kb

### 3.2.2 Identification of *lytB* by deletion analysis

As indicated in Figure 12, plasmid pVW8 was a derivative of plasmid pUC13 carrying an 8 kb *Hin*dIII-*Sal*I insert derived from pGM21. A 3.6 kb *Xba*I-*Xba*I fragment from this insert, which extended from *orf149* of the *ileS-lsp* operon to the *dapB* gene, was subcloned into the vector pAA-PZ718 to yield plasmid pCG17, as summarized in Figure 13. This 3.6 kb fragment complemented *lytB2* thereby ruling out *x*, *ileS*, and *lsp* as possible candidates for the *lytB* gene. The vector pAA-PZ718 contains the transposon Tn9 which confers chloramphenicol resistance and is designed to facilitate the selection of deletions. This system is based on the observation that the excision of Tn9 often results in the removal of variable portions of adjacent DNA. The deletions of interest in this case were those which extended into the cloned insert. Such deletions could be detected by physical mapping of pCG17 with *Pst*I. All deletion derivatives would be expected to retain one of the three *Pst*I sites shown in Figure 13, namely, the site distinguished by the asterisk. The extents of the deletions would determine whether one or both of the other *Pst*I sites were retained. Thus the sizes of the deletions could be measured after separating *Pst*I-digested plasmids by gel electrophoresis as illustrated in Figure 14. Linearized plasmids showing one band had only the one *Pst*I$^*$ site remaining, indicating a deletion past the end of *orf316*, (e.g. lanes a, b, and c, Figure 14). Those showing two bands still had both *Pst*I sites remaining (e.g. lanes d, e, f, and g, Figure 14), and these carried smaller deletions. Figure 15 summarizes these data in the form of a deletion map. The results indicated that deletion of part or all of *orf316* resulted in loss of *lytB2* complementing activity; ie., *lytB* is *orf316*.

**Figure 13. Construction of the plasmid pCG17.**

Plasmid pVW8 and vector pAA-PZ718 were digested with *Xba*I. The 3.6 kb *Xba*I-*Xba*I fragment from pVW8 was cloned into the *Xba*I site of pAA-PZ718. The colonies were selected on 2YT-Xgal-IPTG plates containing Cm and Ap, and one such clone was designated pCG17. Three *Pst*I sites exist on pCG17; two flanking the Tn9 transposon which encodes $Cm^R$ (crosshatched area), and one within the 3.6 kb insert. Selection in the absence of Cm allows excision of Tn9 from the plasmid, leaving behind the *Pst*I site marked with an asterisk. *Orf 149*, *orf316* (highlighted in green), and *orf304* have been labeled for reference. The normal transcription orientations of the inserts are indicated by the short arrows outside the cloned fragment.

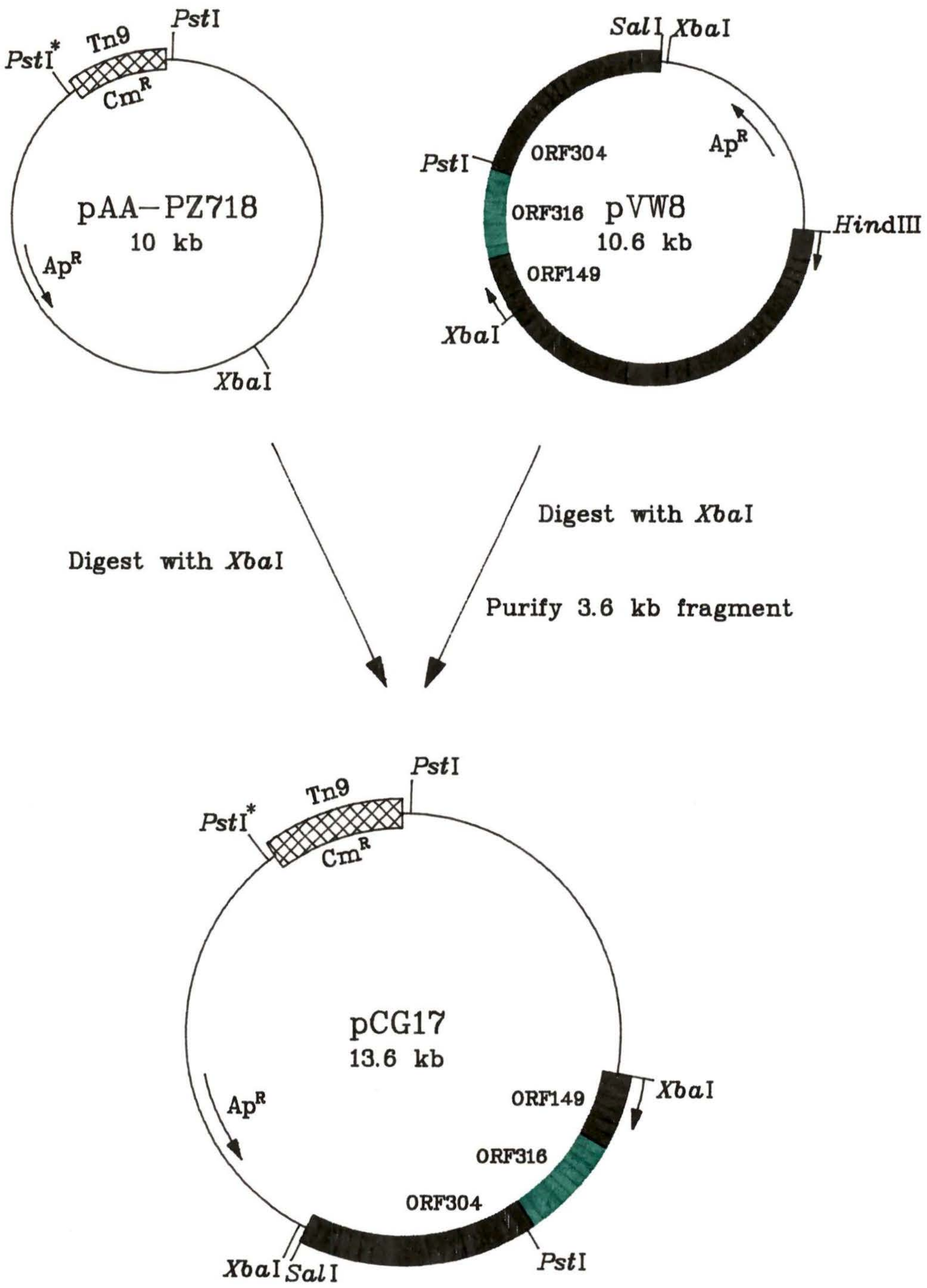

PstI*
Tn9
PstI
CmR
pAA–PZ718
10 kb
ApR
XbaI
SalI
XbaI
ApR
ORF304
PstI
ORF316
pVW8
10.6 kb
HindIII
ORF149
XbaI
Digest with XbaI
Digest with XbaI
Purify 3.6 kb fragment
PstI*
Tn9
PstI
CmR
pCG17
13.6 kb
ApR
ORF149
XbaI
ORF316
ORF304
XbaI
SalI
PstI

**Figure 14. Analysis of pCG17 deletion derivatives by restriction endonuclease digestion.**

Illustrated are deletion derivatives of pCG17 after digestion with *Pst*I and electrophoresis on a 7% agarose gel. The *Hin*dIII-digested λ molecular weight standards are shown in the right lane.

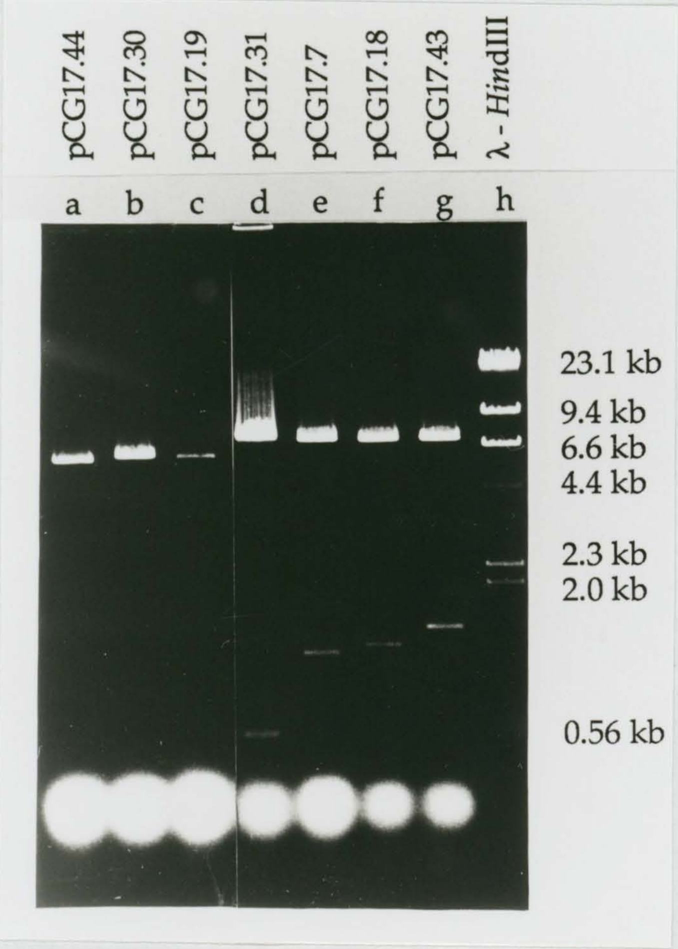
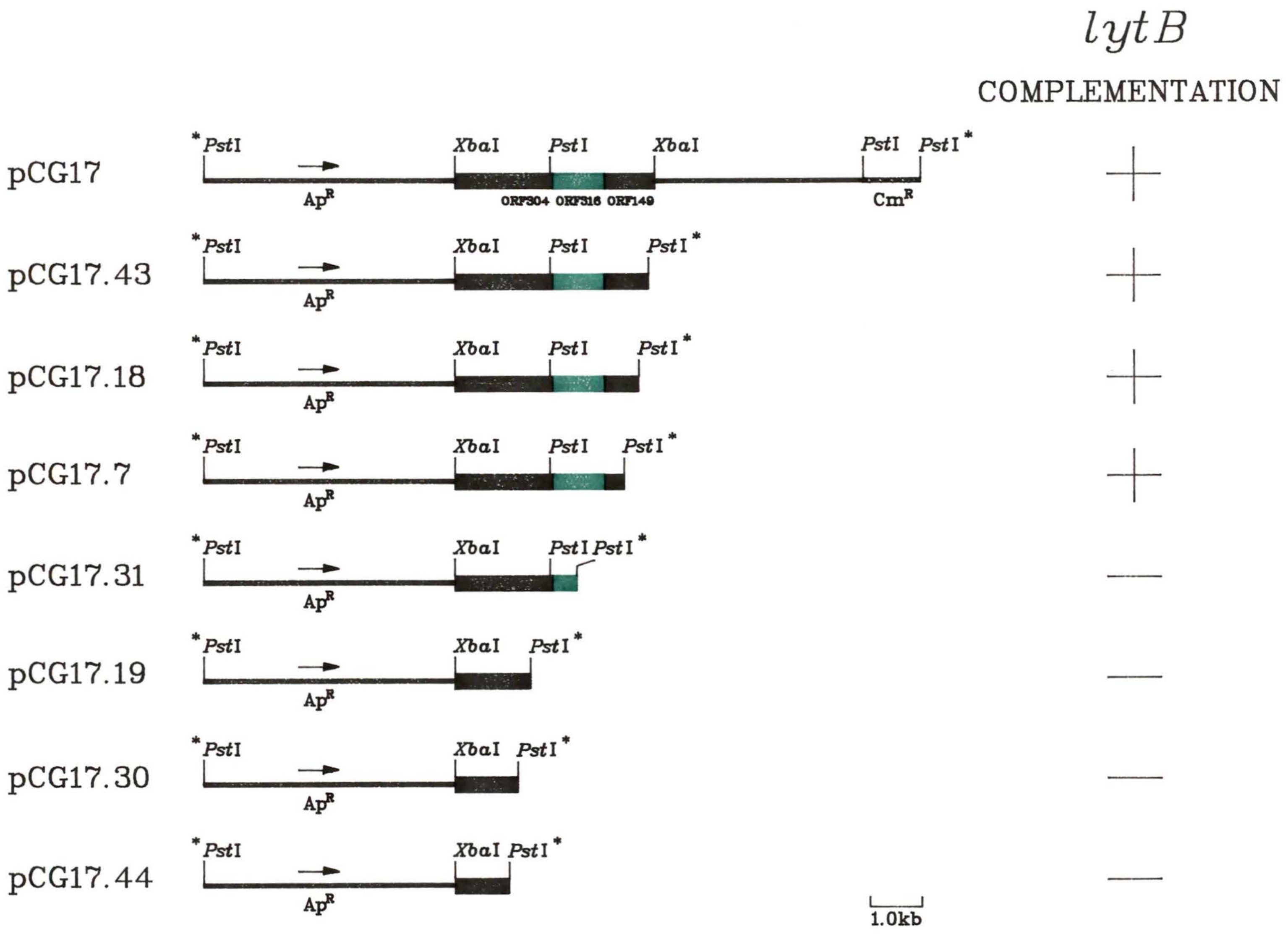

pCG17.44
pCG17.30
pCG17.19
pCG17.31
pCG17.7
pCG17.18
pCG17.43
λ - *Hind*III
a
b
c
d
e
f
g
h
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
0.56 kb

**Figure 15. Formation of deletion derivatives of pCG17.**

Plasmid pCG17 was grown in strain DH5α with Ap but in the absence of Cm. Cm sensitive colonies were chosen for complementation analyses and restriction endonuclease digestion. The maps show linear forms of pCG17 and the deletion plasmids derived from it. *Orf149*, *orf316* (highlighted in green), and *orf304* are labeled in the parent plasmid, pCG17. The *Pst*I sites used for restriction analysis have been noted, and the Tn9-flanking *Pst*I site retained after Tn9 excision is marked with an asterisk. Complementation of the *lytB* mutation by the deletion derivatives is indicated by a + or a - to the right of each plasmid.


lytB
COMPLEMENTATION
pCG17
pCG17.43
pCG17.18
pCG17.7
pCG17.31
pCG17.19
pCG17.30
pCG17.44
*PstI
XbaI
PstI
XbaI
PstI
PstI*
ApR
ORF304 ORF316 ORF149
CmR
+
—
1.0kb

### 3.2.3 Identification of *lytB* by insertion mutagenesis

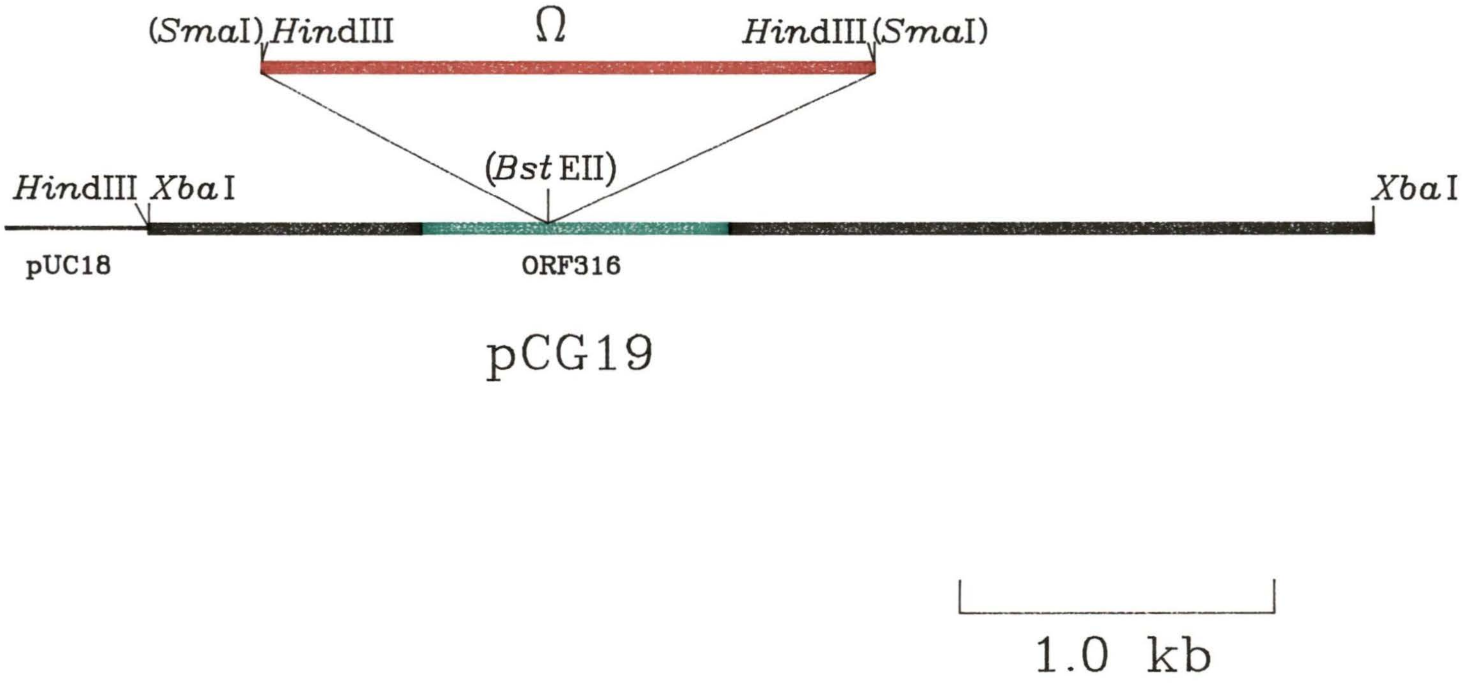
An insertion mutation in *orf316* was constructed in order to confirm that it represented the *lytB* gene. The 3.6 kb *Xba*I-*Xba*I fragment carrying *orf316* in pVW8 (Figure 12) was first subcloned into the unique *Xba*I site of plasmid pUC18 to yield pCG18. The insertion mutagenesis strategy is summarized in Figure 16. Plasmid pCG18 was linearized with *Bst*EII which cuts at a unique site located approximately in the center of *orf316*. The linearized plasmid was then blunt-ended. The insertion element known as omega (Ω) carrying *Sma*I termini was ligated to the blunt-ended pCG18. The Ω element consists of a gene encoding spectinomycin/streptomycin resistance flanked by transcription and translation terminators and a set of useful restriction sites (the *Sma*I and *Hin*dIII sites are relevant here). The structure of the constructed plasmid, designated pCG19, was confirmed by restriction mapping; e.g., by using the symmetrical *Hin*dIII sites at the ends of the Ω element and a *Hin*dIII site at one end of *orf316* (Figure 16). The inactivation of *orf316* by Ω insertion in pCG19 resulted in complete loss of *lytB* complementation. This confirms that the *lytB* complementing activity resided in *orf316*.

### 3.2.4. Proof that the mutations in strains VC4217 and VC4218 are allelic

Strain VC4217 carries a mutation which confers a phenotype indistinguishable from that of VC4218; ie., VC4217 fails to form colonies and is penicillin-tolerant at the restrictive temperature. The mutations in VC4217 and VC4218 were independently isolated. The designation *lytB1* was assigned to the mutation in VC4217 on the basis of (i) phenotypic

**Figure 16. Insertion mutagenesis of *orf316* by Ω.**

Plasmid pCG18 was linearized with *Bst*EII, a unique site within *orf316* (indicated in green). The *Bst*EII site was blunt-ended and the omega insertion element (indicated in red) was inserted by *in vitro* ligation using flanking *Sma*I sites. This resulted in the loss of the *Bst*EII and the *Sma*I restriction sites, as indicated by brackets. Restriction analyses utilized the *Hin*dIII site at the *orf316*-proximal end of the insert and the *Hin*dIII sites flanking the Ω element. One recombinant clone showing the correct construction was designated pCG19.


Ω
(SmaI) HindIII
HindIII (SmaI)
(BstEII)
HindIII XbaI
XbaI
pUC18
ORF316
pCG19
1.0 kb

properties, and (ii) a map position of 0.5 min. However, there was no genetic proof that the penicillin tolerance mutations in VC4217 and VC4218 were allelic. The availability of the insertion-inactivated *orf316* derivative and the deletion derivatives of *orf316* described above permitted the question of allelism to be settled. All plasmids carrying *orf316* complemented the mutation in VC4217. However, the insertion-inactivated and deletion derivatives of *orf316* were all devoid of complementing activity in VC4217. This proves that the mutations in VC4217 and VC4218 are indeed allelic.

## 3.3 Instability of Multicopy Plasmids Carrying *lytB*

### 3.3.1 Introductory comments

During the course of this work, there were indications that multicopy plasmids carrying *lytB* were unstable. The main observations are summarized here. It is suggested below that the apparent instability of these plasmids may reflect a toxic effect associated with overexpression of *lytB*.

### 3.3.2 Spontaneous mutations in *orf316*-containing clones

Early in this work, derivatives of pGM21 and pVW8 carrying deletions which inactivated *lytB* complementation were discovered during routine physical characterizations. These derivatives were not studied further because the implications of such mutations were not realized at that time.

It was later found that a spontaneous mutation or mutations had altered the 3.6 kb insert in pCG18. This resulted in the introduction of two restriction sites which were not present in the original source of this insert, pGM21. As shown in Figure 17, the alterations included the introductions of a *Bgl*II site at the 3'-end of *orf316* and a *Pst*I site at the 5'-end of *orf304*. These changes apparently did not completely inactivate *lytB* since pCG18 still at least partially complemented *lytB* mutant strains.

### 3.3.3. Attempts to subclone *orf316* for gene expression studies

Numerous attempts were made to subclone a portion containing only *orf316* for the purpose of overexpressing the *lytB* gene. In this case, it was necessary to clone *orf316* in the same orientation as the *lac* promoter in either pUC18 or pUC19, or the phage T7 promoter in either pT7T318U or pT7T319U. The initial attempts involved directional cloning employing combinations of two different restriction sites to ensure that the fragment carrying *orf316* would be cloned in only one orientation. The combinations tested were *Xba*I plus *Eco*RV and *Cla*I plus *Hpa*I (Figure 18). These attempts yielded no *lytB* complementing clones. In subsequent attempts, the *Xba*I sites flanking the 3.6 kb fragment carrying *orf316* were utilized (Figure 18). In this case, it should have been equally possible to clone the *orf316* fragment in either of the two possible orientations. Instead, it was found very few of the total recombinant molecules formed carried *orf316* in the same orientation as the expression promoter. Furthermore, none of these derivatives complemented *lytB*. Presumably, these derivatives contained alterations in *orf316* which inactivated *lytB* complementation but such changes were not obvious from restriction analyses. Most of the recombinant molecules (a total of 136 screened from five separate cloning experiments) carried *orf316* in the reverse orientation with respect to the

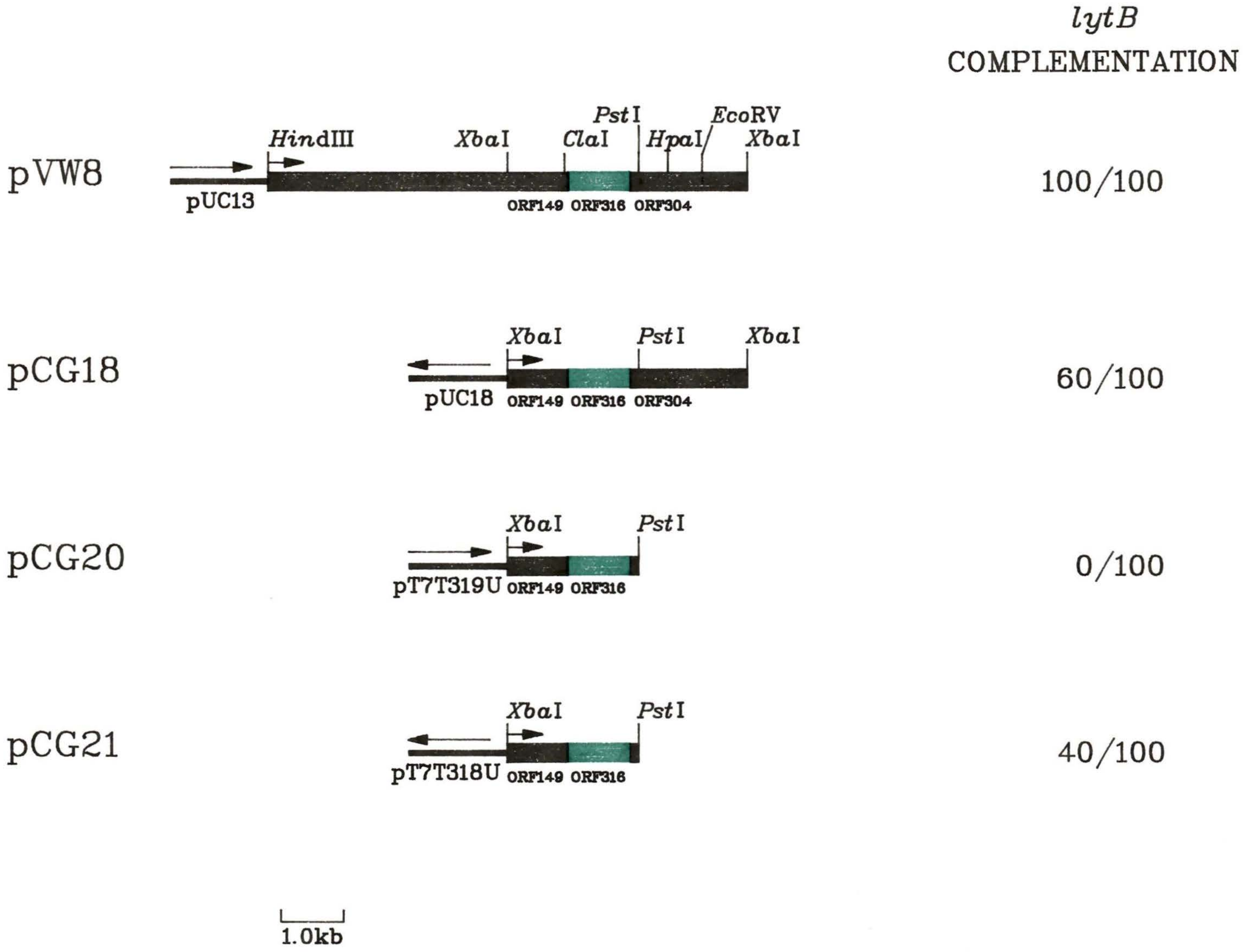
**Figure 17. Mutations altering the 3.6 kb insert carrying *orf316* in pCG18.**

A portion of pCG18 is shown, illustrating the positions of the new *Pst*I and *Bgl*II restriction endonuclease sites (highlighted in magenta) in pCG18 which are not found in pGM21, the original source of this fragment. The dark thin line depicts the cloning vehicle, while the insert is depicted by open boxes. *Orf316* is shown in green.

pCG18

**Figure 18. Construction of plasmids for the purpose of gene expression.**

The maps of pVW8 and three plasmids which were derived from it showing relevant restriction sites are represented. The vectors of each plasmid are identified below the thin lines, with the directions of transcription from the expression promoters indicated above by long arrows. The cloned inserts are depicted by thick lines, and *orf149*, *orf316* (highlighted in green), and *orf304* are labeled. The normal transcription orientations of the cloned inserts are indicated by the short arrows above the thick lines. Complementation of the *lytB* mutation by the plasmids is indicated to the right of each plasmid.


*lytB*
COMPLEMENTATION
pVW8
*Hind*III
*Xba*I
*Cla*I
*Pst*I
*Hpa*I
*Eco*RV
*Xba*I
pUC13
ORF149
ORF316
ORF304
100/100
pCG18
*Xba*I
*Pst*I
*Xba*I
pUC18
ORF149
ORF316
ORF304
60/100
pCG20
*Xba*I
*Pst*I
pT7T319U
ORF149
ORF316
0/100
pCG21
*Xba*I
*Pst*I
pT7T318U
ORF149
ORF316
40/100
1.0kb

expression promoter. Furthermore, although these derivatives exhibited *lytB* complementation, the degree of complementation was only partial; i.e., temperature resistance was only partially restored.

The following experiments are typical of these initial attempts to subclone *orf316* (Figure 18). The 8 kb fragment containing the entire *ileS-lsp* operon in pGM21 was readily subcloned in the orientation of the expression promoter in pUC13 (e.g., pVW8). Such subclones exhibited full *lytB* complementing activity. However, the 3.6 kb *Xba*I-*Xba*I fragment (pCG18) which removed the upstream promoters of the *ileS-lsp* operon (see Figure 12) could only be cloned efficiently in the opposite orientation with respect to the expression promoter, and this derivative showed only partial *lytB* complementation. In further experiments, a 1.7 kb *Xba*I-*Pst*I fragment containing only *orf149* and *orf316* was subcloned in the opposite orientation to the expression promoter in pT7T318U (pCG21) much more frequently than in the same orientation in pT7T319U (pCG20). Although pCG21 exhibited partial *lytB* complementing activity, pCG20 was completely devoid of such activity. The derivatives pCG20 and pCG21 were overexpressed from the T7 promoter and the results are shown in Figure 19. Both plasmids exhibited the three β-lactamase bands encoded by the vector (labeled in Figure 19). These were the only products in the case of pCG21 (lane a) and this confirms that *orf149* and *orf316* were in the opposite orientation with respect to the T7 promoter. Lane b, representing cells carrying pCG20, shows the β-lactamase bands as well as a band at approximately 53 kD. The expected gene product sizes of *orf149* and *orf316* are 17 kD and 36 kD, respectively. Therefore, the possibility exists that the translated 53 kD protein encoded by pCG20 corresponds to a fusion protein

**Figure 19. Overexpression of pCG20 and pCG21 by the T7 RNA polymerase and promoter system.**

Plasmids pCG20 and pCG21 carrying *orf149* and *orf316* cloned in both orientations in front of the *T7* promoter were overexpressed by the method of Tabor and Richardson (73). The plasmid proteins were labeled with [$^{35}$S]-methionine and an autoradiograph of the proteins run on a 12.5% SDS-PAGE. Labeled are the three proteins produced by the β-lactamase gene, the one marked by an asterisk derived from translation of the opposite strand. Furthermore, a 53 kD protein from pCG20 is also marked.


pCG21
pCG20
a
b
← 53 kD
} β - lactamase
*

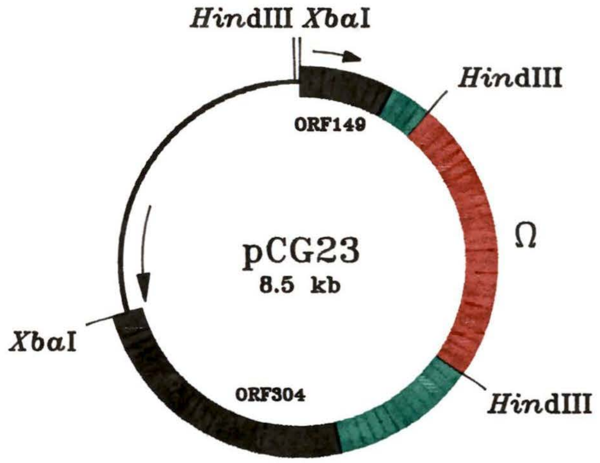
composed of the *orf149* and *orf316* gene products. This also presumably explains the inability of pCG20 to complement *lytB*.

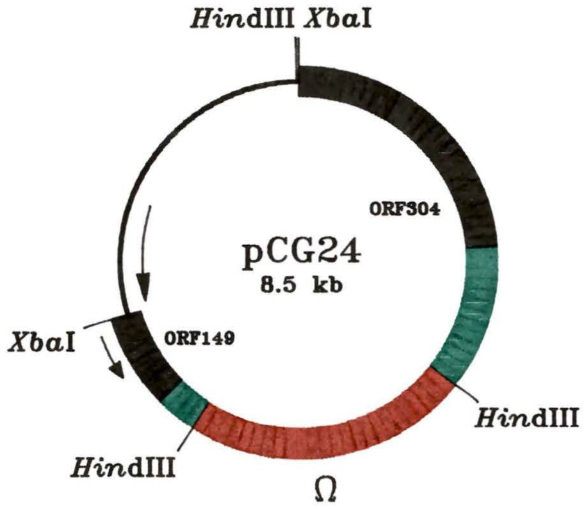
### 3.3.4. Subcloning of inactivated *orf316*

The results described in the preceding section suggest that fragments carrying only *orf149*, *orf316*, and *orf304* cannot be cloned in multicopy vectors in the same orientation as the expression promoter. As discussed below, this may be a consequence of the toxic effects of overexpression of one of the open reading frames from the *lac* promoter of these high-copy vectors. If this was so, and if the toxicity was due to overexpression of *orf316* (i.e., *lytB*), it was reasoned that it should be possible to efficiently subclone fragments carrying the insertion-inactivated *orf316* in both orientations, i.e., because the proposed toxicity of *orf316* should be nullified by the insertion element. To test this, the *Xba*I fragment from pCG19 containing the omega element-inactivated *orf316* (Figure 16) was subcloned into the unique *Xba*I site in the vector pT7T318U. The strategy of this experiment is summarized in Figure 20. If the toxicity of this fragment was eliminated by the Ω insertion, it was expected that it would be equally possible to clone the fragment in either orientation. Figure 21 shows a *Hin*dIII restriction analysis of twelve randomly selected subclones. It can be seen that six subclones contained the *Xba*I insert in the opposite orientation of the expression promoter (lanes b, c, e, h, j, and k), and the other six had the insert in the same orientation (lanes a, d, f, g, i, and l). These results support the proposal that overexpression of *orf316* originating from the expression promoter on the vector is responsible for the instability of multicopy plasmids carrying *orf316*.

**Figure 20. Subcloning of inactivated *orf316* in both orientations**

The 5.6 kb *Xba*I-*Xba*I fragment from pCG19 (see Figure 16), carrying *orf316* (shown in green) inactivated by insertion of the Ω element (shown in red), was subcloned into the unique *Xba*I site of the expression vector, pT7T318U. The vector sequences in the illustrations are represented by the thin lines, and the positions of the phage T7 promoters in the vectors are indicated by the long arrows, with the arrows pointing in the direction of transcription from these promoters. The transcriptional orientation of *orf316* is indicated by the short arrows. As documented in Figure 21, the 5.6 kb *Xba*I-*Xba*I fragment could be cloned with equal efficiency in either orientation with respect to the T7 promoter. Plasmid pCG23 is an example of a derivative in which *orf316* is in the opposite orientation to the T7 promoter, and pCG24 is an example where *orf316* and the T7 promoter are in the same orientation. The accompanying table summarizes the fragments derived from pCG23 (left side) and pCG24 (right side) upon digestion with *Hin*dIII.





| Fragment Sizes Generated by *Hin*dIII Digest | |
|---|---|
| 0.9 kb | 2.7 kb |
| 2.0 kb (Ω) | 2.0 kb (Ω) |
| 5.6 kb | 3.8 kb |

### Figure 21. Subcloning of inactivated *orf316* : *Hin*dIII restriction analysis of recombinant plasmids

Twelve recombinant plasmids constructed as described in Figure 20 were selected at random for *Hin*dIII restriction analysis. Lane m represents the molecular weight standards. The plasmids analyzed in lanes b, c, e, h, j, and k gave rise to sets of *Hin*dIII fragments corresponding to those expected if *orf316* was cloned in the opposite direction as the T7 promoter (e.g. pCG23, Figure 20). In comparison, the *Hin*dIII digests of plasmids in lanes a, d, f, g, i, and l were compatible with constructs in which *orf316* was cloned in the same orientation to the T7 promoter (e.g. pCG24, Figure 20).


a
b
c
d
e
f
g
h
i
j
k
l
m
5.6 kb
3.8 kb
2.7 kb
Ω 2.0 kb
0.9 kb
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
0.56 kb

# Chapter 4

# DISCUSSION

## 4.1 Phenotype of *lytB2* and Proposed Function of LytB

It was confirmed that the temperature sensitive mutant, VC4218, was tolerant to ampicillin-induced lysis at the restrictive temperature, i.e., ampicillin inhibited growth but did not induce lysis at $42^{\circ}$C (Figure 8). The ampicillin tolerance was suppressed by known inhibitors of the stringent response, or by a *relA* mutation. These results were further correlated with the accumulation of ppGpp at $42^{\circ}$C, above levels which could be attributed to heat shock caused by temperature upshift. Thus, *lytB2*, like the various *lytA* and *lytB1* alleles, results in the induction of the stringent response at the restrictive temperature.

Temperature sensitive colony formation exhibited by the mutant, VC4218, is proposed to be due to the inhibitory effects of ppGpp on macromolecular synthesis and other metabolic processes. The tolerance to lysis by ampicillin may therefore be due to an inhibition of PG hydrolase activities by the stringent response.

The phenotype of VC4218 suggests that the function of the wild type LytB protein may be to maintain RelA in its inactive state during normal growth as previously proposed by Kusser and Ishiguro (77) and as summarized in Figure 22. A schematic representation of the proposed role of LytB in the stringent response is outlined in Figure 23. Although this model is currently speculative and undoubtedly incomplete, it provides the basis of a working hypothesis for future experiments. It should be noted that both LytA and LytB proteins have been found to exhibit similar properties, and the component designated "Lyt" in Figure 23 could therefore conceivably represent either of the two proteins.

Figure 23A depicts the role of LytB during normal growth. In accordance with known facts, the RelA enzyme is shown associated with a ribosome, and its inactivity is dependent on active translation. We propose here that the maintenance of RelA in its inactive form is also dependent on the function of LytB (and LytA). Although the model shows this being achieved through a direct interaction between Lyt and RelA, it is possible that it is effected by an indirect interaction. Furthermore, it should be emphasized that there is no evidence for the direct association between Lyt and the ribosome depicted in the model, and it is possible that Lyt is associated, either directly of indirectly, with RelA instead.

Figure 23B diagrammatically represents the stringent response. As discussed previously, it has been proposed (25) that the binding of an uncharged tRNA molecule to its respective codon over the acceptor of the ribosome idles translation and causes a conformational change in the ribosome. This conformational change apparently activates RelA and results in the synthesis of a single molecule of pppGpp. We suggest here that, in order for this to occur, the Lyt-RelA interaction must first be

**Figure 22. Effect of LytB on the RelA pppGpp synthetic activity.**

The cycle of ppGpp metabolism is as described in Figure 3. In this figure, wild type cells are grown in complex medium, and the LytB protein is proposed to inhibit the pppGpp synthetic activity of the RelA enzyme (indicated in green).

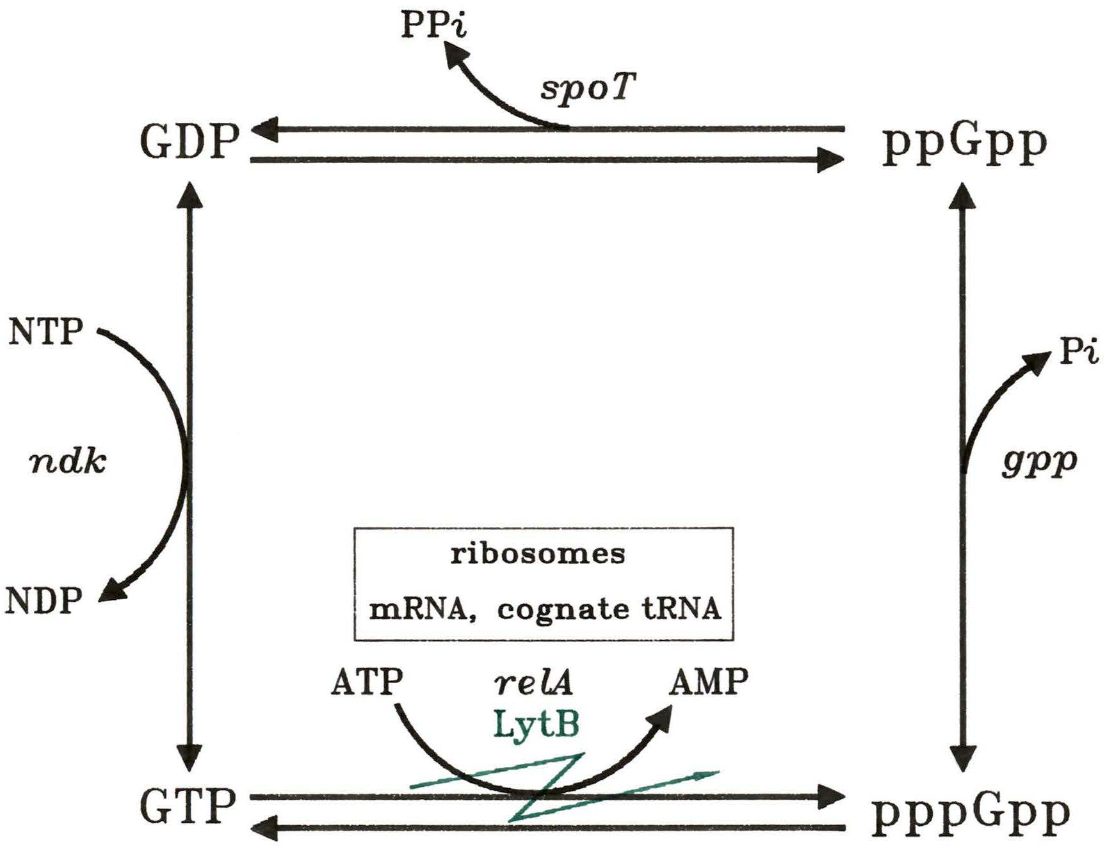
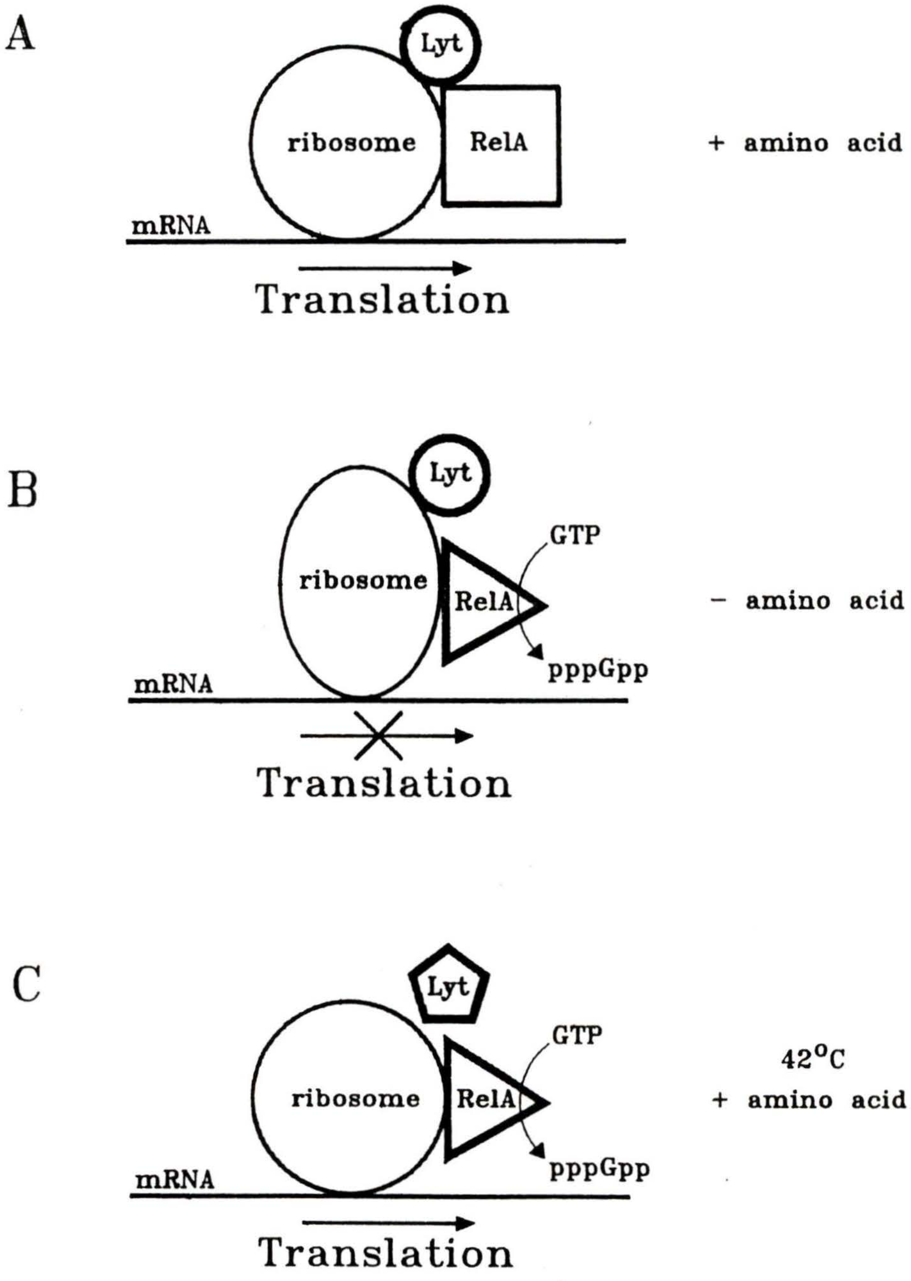

PPi
spoT
GDP
ppGpp
NTP
ndk
NDP
Pi
gpp
ribosomes
mRNA, cognate tRNA
ATP
relA
LytB
AMP
GTP
pppGpp

**Figure 23. Model illustrating the proposed interaction of the Lyt protein with RelA.**

**Panel A** represents normal growing cells.
**Panel B** represents the normal stringent response.
**Panel C** represents the induction of the stringent response in *lytB* or *(lytA)* mutants. See text for discussion of the model.


A
Lyt
ribosome
RelA
+ amino acid
mRNA
Translation
B
Lyt
GTP
ribosome
RelA
– amino acid
pppGpp
mRNA
Translation
C
Lyt
GTP
42°C
ribosome
RelA
+ amino acid
pppGpp
mRNA
Translation

dissociated. It is possible that the conformational change in the ribosome could account for this dissociation.

Figure 23C attempts to account for the phenotype(s) of the *lytB* (and *lytA*) mutant(s). Growing cultures of these mutants which are subjected to a temperature upshift to 42$^0$C overproduce ppGpp through the RelA-dependent pathway. Since the temperature upshift has no immediate effects on translation in the mutants, it is unlikely that the activation of RelA can be attributed to a ribosomal conformational change. Our data suggest that this step of the stringent response is bypassed at the restrictive temperature in these mutants, and RelA activation results when the Lyt-RelA interaction is dissociated upon thermal denaturation of the temperature sensitive mutant Lyt proteins.

## 4.2 Identification of the *lytB* Locus

Two independent methods, deletion mapping and insertion mutagenesis, were used to demonstrate that the *lytB* gene was identical to *orf316* of the *ileS-lsp* operon. Furthermore, the two independently isolated mutants, VC4217 and VC4218, were shown to carry mutations in the same gene by complementation analyses. Previously, the mutations in VC4217 and VC4218 had been assigned the designations *lytB1* and *lytB2*, respectively, solely on the basis of genetic mapping data.

The sequence of the *lytB* gene was published while this work was in progress (80). Computer assisted analyses of the sequence showed no obvious function for the LytB protein. The sequence was not significantly homologous to other reported genes of known function. In addition, a hydropathy index computation using an interval of nine amino acids indicated that LytB does not appear to be a membrane spanning protein since there are no extensively hydrophobic regions, and is therefore probably cytoplasmic or membrane associated.

Miller *et al.* (79) analyzed the polycistronic mRNAs transcribed from the *ileS-lsp* operon. They identified three promoters, two of which reside upstream of or within gene *x*, and the third one located at the end of the *ileS* gene, upstream of *lsp* (Figure 12). Furthermore, they showed that transcripts of *orf149* and *orf316* were initiated from either of the two promoters located upstream of *ileS* but not from the promoter located at the end of *ileS*. Thus, the existence of multiple promoters and mRNA transcript lengths within this operon suggests that expression of these genes is regulated in a complex way.

The five genes comprising the *ileS-lsp* operon are also found in *Pseudomonas fluorescens* and *Enterobacter aerogenes* in the same order and with the same transcriptional polarity as in *E. coli* (81, 82). It remains to be determined how much further widespread the conservation of this unit is. Nevertheless, the small number of examples known to date still suggests a significance in the grouping of these genes together in an operon and possibly the importance of the gene products.

## 4.3 Instability of DNA Fragments Containing *orf316*

Examples cited in Chapter 3 of spontaneous mutations affecting *orf316* may reflect an inherent instability of DNA fragments carrying *orf316*, especially in multicopy. This became apparent during unsuccessful attempts to subclone the small DNA fragment carrying only *orf149*, *orf316*, and *orf304* in both orientations in an expression vector. It was possible to clone this DNA fragment only in the opposite orientation to the expression promoter, suggesting that the overexpression of one of the three open reading frames on the DNA fragment had a toxic effect. In this respect, the following factors are noteworthy. It is known that the levels of *lac* repressor protein produced in host strains such as those employed in this study are insufficient to completely repress expression from the *lac* promoters of the high copy number vectors employed, i.e., expression from the *lac* promoter of these vectors occurs even in the absence of an inducer such as IPTG (83). Consequently, genes cloned behind the *lac* promoter and in the same orientation as the promoter would be overexpressed if one considers the high copy number of the vectors. Our data indicate that the overexpression of *orf316* but not *orf149* or *orf304* was toxic. For example, the same DNA fragment carrying an inactivated *orf316* was readily cloned in both orientations in the same expression vector.

There are other examples in the literature of genes which have been impossible to clone in multicopy vectors, presumably due to a toxic effect resulting from overexpression of the gene product. These include *ompA* (outer membrane protein) (84), *dacA* (PBP5) (85), *pbpA* (PBP2) (85), and *lpp* (lipoprotein) (86). Interestingly, the *lytA* gene has been impossible to clone in multicopy (unpublished data from this laboratory).

In the original cloning procedures by Mackie (75), the occurrence of spontaneous deletions were observed to occur. In fact, the formation of these deletions was utilized for mapping the genes on the plasmid. Mackie (75) noted that some plasmids which were variations of the parent plasmid did not give rise to deletions, leading to the conclusion that the probability of a deletion occurring depended on the particular fragment of DNA being cloned. This conclusion correlates with the observations on plasmid instability encountered in this work.

The degree of instabiltiy of DNA fragments carrying *orf316* apparently depended on the structure of the fragment. The fragment carrying the entire *ileS-lsp* operon was more stable than the smaller fragments carrying only the *orf149* and *orf316* loci in multicopy vectors. For example, the entire *ileS-lsp* operon could be cloned in the same direction as the expression promoter, although we did note changes in the restriction map which presumably represented a spontaneous mutation(s) (see Figure 17 and corresponding text). This derivative still complemented the *lytB* mutation implying that the mutation(s) did not completely destroy the activity of *orf316* . It is possible that the mutation(s) reduced the toxic effect of the overexpressed protein. In contrast, attempts to clone fragments containing only the last two open reading frames of the *ileS-lsp* operon, *orf149* and *orf316* , in both orientations were unsuccessful as already noted. The derivatives carrying the fragment in the direction of the expression promoter were mutant derivatives with completely inactivated *orf316* (e.g., the fusion derivative in Figure 19).

The amount of *lsp* gene expression is apparently low in normal cells (87), and since *lytB* resides downstream of*lsp*, it may be inferred that the expression of *lytB* is similarly low. Furthermore, it has been proposed that one purpose of polycistronic mRNAs in an operon is to maintain gene expression of closely related genes in stoichiometric amounts (88). Varying the 3' endpoints of transcripts is one way in which the levels of gene expression within an operon can be modulated (89). As seen by the variable 3' termini of the *ileS-lsp* operon mRNAs (79), the transcripts of the operon did not always include *orf149* and *orf316*, indicating that these two genes are transcribed to a lesser degree in the cell than the *ileS* and *lsp* genes. The basis for the down-regulation of the terminal *orf149* and *orf316* components is of interest since this might explain the toxicity of *orf316* when it is overexpressed.

The nature of some of the expression signals within the *ileS-lsp* operon which may play a role in regulating downstream gene expression include a pair of repetitive extragenic palindromic (REP) sequences located between *lsp* and *orf149* (80). REP sequences are highly conserved inverted repeats present in about 1,000 copies on the *E. coli* chromosome which are capable of forming stem-loop structures in mRNA. They are present at various locations, frequently between genes which are differentially expressed. The exact purpose of these REP sequences is uncertain but two observations are relevant here. It was reported (90,91) that the segment of an mRNA molecule located on the 5' end of a REP sequence is stabilized apparently because REP blocks the processive action of 3' to 5' exonucleases. Thus, the presence of REP sequences within a polycistronic mRNA , e.g., between *lsp* and *orf149*, may cause differential gene expression within an operon with the control being at a post-transcriptional level. In this case, the internal REP sequence may serve to increase the half-life of the promoter-proximal portion of the mRNA and thereby increase the probability of translation of this region. If the tandem REP sequences in the *ileS-lsp* operon function in this way, the result should be a reduction in *orf149* and *orf316* gene expression.

The second observation relevant to REP sequences is that of Yang *et al.* (92). They showed that DNA gyrase binds to REP sequences. Since the supercoiling of DNA by DNA gyrase affects gene transcription (93) either in a positive or a negative fashion, it is possible that the regulation of gene expression by REP sequences may be mediated by DNA gyrase.

### 4.4 Future work

Future work on this project should involve a more detailed characterization of the *lytB* gene product. The cellular localization and ultimately, the purification and characterization of LytB will be essential. Certainly, one of the primary questions concerns the normal function of LytB. However, in view of the toxicity of the gene product in high copy number vectors, an approach such as the construction of gene fusions, e.g., with *lacZ* may be useful in meeting these ends.

The isolation of extragenic mutations which would suppress *lytB* may provide information on the functional interactions of LytB with the products of other genes.

This project covered some preliminary work on LytB and its role in the stringent control of autolysis in *E. coli*. A great deal of work obviously lies ahead since much is still unknown regarding the *lytB* gene product, and about the stringent response itself. Reports in the literature do not always agree concerning several aspects of the stringent response, and determining the role of LytB may provide insight into the mechanism of action of the stringent response.

# REFERENCES

1. **Cashel, M., and Gallant, J.** (1969) Two compounds implicated in the function of the *RC* gene of *Escherichia coli*. *Nature (London)* **221:**838-841
2. **Schreiber, G., Metzger, S., Aizenman, E., Roza, S., Cashel, M., and Glaser, G.** (1991) Overexpression of the *relA* gene in *Escherichia coli*. *J. Biol. Chem.* **266:**3760-3767
3. **Furano, A.V., and Wittel, F.P.** (1976) Effect of the *relA* gene on the synthesis of individual proteins in vivo. *Cell* **8:**115-122
4. **Reeh, S., Pedersen, S., and Friesen, J.D.** (1976) Biosynthetic regulation of individual proteins in *relA*$^+$ and *relA* strains of *Escherichia coli* during amino acid starvation. *Mol. Gen. Genet.* **149:**279-289
5. **Cashel, M.** (1969) The control of ribonucleic acid synthesis in *Escherichia coli*: Relevance of unusual phosphorylated compounds from amino acid-starved stringent strains. *J. Biol. Chem.* **244:**3133-3141
6. **Fiil, N.P., Willumsen, B.M., Friesen, J.D., and von Meyenberg, K.** (1977) Interaction of alleles of the *relA, relC,* and *spoT* genes in *Escherichia coli*: analysis of the interconversion of GTP, ppGpp and pppGpp. *Mol. Gen. Genet.* **150:**87-101

7. **Haseltine, W.A., Block, R., Gilbert, W., and Weber, K.** (1972) MSI and MSII made on ribosome in idling step of protein synthesis. *Nature (London)* **238:**381-384

8. **Gallant, J., Margason, G., Finch, B.** (1972) On the turnover of ppGpp in *Escherichia coli. J. Biol. Chem.* **247:**6055-6058

9. **Heinemeyer, E.A., and Richter, D.** (1978) Mechanism of the *in vitro* breakdown of guanosine 5'-diphosphate 3'-diphosphate in *Escherichia coli. Proc. Natl. Acad. Sci. U.S.A.* **75:**4180-4183

10. **Sy, J.** (1980) Activation of ppGpp-3'-pyrophosphohydrolase by a supernatant factor and ATP. *J. Biol. Chem.* **255:**10056-10059

11. **Stamminger, G. and Lazzarini, R.A.** (1974) Altered metabolism of the guanosine tetraphosphate, ppGpp, in mutants of *E. coli. Cell* **1:**85-90

12. **Cashel, M. and Rudd. K.E.** (1987) The stringent response. In: *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology. (Neidhardt, C.F., ed) pp. 1410-1483, American Society for Microbiology, Washington, D.C.

13. **Fiil, N., and Friesen, J.D.** (1968) Isolation of relaxed mutants of *Escherichia coli. J. Bacteriol.* **95:**729-731

14. **Metzger, S., Ben-Dror, I.B., Aizenman, E., Schrieber, G., Toone, M., Friesen, J.D., Cashel, M., and Glaser, G.** (1988) The nucleotide sequence and characterization of the *relA* gene of *Escherichia coli. J. Biol. Chem.* **263:**15699-15704

15. **Sarubbi, E., Rudd, K.R., and Cashel, M.** (1988) Basal ppGpp level adjustment shown by new *spoT* mutants affect steady state growth rates and *rrnA* ribosomal promoter regulation in *Escherichia coli. Mol. Gen. Genet.* **213:**214-222

16. **Metzger, S., Schrieber, G., Aizenman, E., Cashel, M., and Glaser, G.** (1989) Characterization of the *relA1* mutation and comparison of *relA1* with new *relA* null alleles in *Escherichia coli. J. Biol. Chem.* **264:**21146-21152

17. **Christiansen, L., and Nierhaus, K.H.** (1976) Ribosomal proteins of *Escherichia coli* that stimulate stringent-factor-mediated pyrophosphoro transfer *in vitro. Proc. Natl. Acad. Sci. U.S.A.* **73:**1839-1843

18. **Sy, J., Ogawa, Y., and Lipmann, F.** (1973) Nonribosomal synthesis of guanosine 5', 3'-polyphosphates by the ribosomal wash of stringent *Escherichia coli. Proc. Natl. Acad. Sci. U.S.A.* **70:**2145-2148

19. **Friesen, J.D., Fiil, N.P., Parker, J.M., and Haseltine, W.A.** (1974) A new relaxed mutant of *Escherichia coli* with an altered 50S ribosomal subunit. *Proc. Natl. Acad. Sci. U.S.A.* **71:**3465-3469

20. **Ramagopal, S., and Davis, B.D.** (1974) Localization of the stringent protein of *Escherichia coli* on the 50S ribosomal subunit. *Proc. Natl. Acad. Sci. U.S.A.* **71:**820-824

21. **Rojiani, M.V., Jakubowski, H., and Goldman, E.** (1989) Effect of variation of charged and uncharged tRNA$^{Trp}$ levels on ppGpp synthesis in *Escherichia coli. J. Bacteriol.* **171:**6493-6502

22. **Richter, D.** (1976) Stringent factor from *Escherichia coli* directs ribosomal binding and release of uncharged tRNA. *Proc. Natl. Acad. Sci. U.S.A.* **73:**707-711

23. **Sy, J.** (1977) *In vitro* degradation of guanosine 5'-diphosphate, 3'-diphosphate. *Proc. Natl. Acad. Sci. U.S.A.* **74:**5529-5533

24. **Gourse, R.L., de Boer, H.A., and Nomura, M.** (1986) DNA determinants of rRNA synthesis in *E. coli*: growth rate dependent regulation, feedback inhibition, upstream activation, antitermination. *Cell* **44:**197-205

25. **Igarashi, K., Fujita, N., and Ishihama, A.** (1989) Promoter selectivity of *Escherichia coli* RNA polymerase: omega factor is responsible for the ppGpp sensitivity. *Nucl. Acids Res.* **17:**8755-8765

26. **Gentry, D., Xiao, H., Burgess, R., and Cashel, M.** (1991) The omega subunit of *Escherichia coli* K-12 RNA polymerase is not required for stringent RNA control *in vivo*. *J. Bacteriol.* **173:**3901-3913

27. **Schleifer, K.H., and Kandler, O.** (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.* **36:**407-477

28. **Fiil, N.** (1969) A functional analysis of the *rel* gene in *Escherichia coli*. *J. Mol. Biol.* **45:**195-203

29. **Xiao, H., Kalman, M., Ikehara, K., Zemel, S., Glaser, G., and Cashel, M.** (1991) Residual guanosine 3', 5'-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. *J. Biol. Chem.* **266:**5980-5990

30. **Hernandez, V.J., and Bremer, H.** (1991) *Escherichia coli* ppGpp synthetase II activity requires *spoT*. *J. Biol. Chem.* **266:**5991-5999

31. **Chaloner-Larsson, G., and Yamazaki, H.** (1978) Effects of the *spoT* and *relA* mutations on the synthesis and accumulation of ppGpp and RNA during glucose starvation. *Can. J. Biochem.* **56:**264-272

32. **Brock, T.D., and Madigan, M.T.** (1988) In: Biology of Microorganisms 5th edition. pp. 72-81. Prentice Hall, Englewood Cliffs, New Jersey.

33. **Murray, R.G.E., Steed, P., and Elson, H.E.** (1965) The location of the muropeptide in sections of the cell wall of *Escherichia coli* and other gram-negative bacteria. *Can. J. Microbiol.* **11:**547-560

34. **Schindler, M., Mirelman, D., and Schwarz, U.** (1976) Quantitative determination N-acetylglucosamine residues at the non-reducing ends of peptidoglycan chains by enzymic attachment of [$^{14}$C]-D-galactose. *Eur. J. Biochem.* **71:**131-134

35. **Driehuis, F., and Wouters, J.T.M.** (1987) Effect of growth rate and cell shape on the peptidoglycan composition in *Escherichia coli. J. Bacteriol.* **169:**97-101

36. **Holtje, J-V., and Schwarz, U.** (1985) Biosynthesis and growth of the murein sacculus. In: Molecular cytology of *Escherichia coli* (Nanninga, N. ed.) pp. 77-114. Academic Press, London.

37. **Glauner, B., Holtje, J.-B., and Schwarz, U.** (1988) The composition of the murein of *Escherichia coli. J. Biol. Chem.* **263:**188-195

38. **Holtje, J.-B., and Tuomanen, E.I.** (1991) The murein hydrolases of *Escherichia coli*: properties, functions and impact on the course of infections *in vivo*. *J. Gen. Microbiol.* **137:**441-454

39. **Labischinski, H., Goodell, E.W., Goodell, A., and Hochberg, M.L.** (1991) Direct proof of a "More-than-single-layered" peptidoglycan architecture of *Escherichia coli* W7: a neutron small-angle scattering study. *J. Bacteriol.* **173:**751-756

40. **Leduc, M., Frehel, C., Siegel, E., and van Heijenoort, J.** (1989) Multi-layered distribution of peptidoglycan in the periplasmic space of *Escherichia coli*. *J. Gen. Microbiol.* **135:**1243-1254

41. **Park, J.T.** (1987) In: *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology. (Neidhardt, C.F., ed) pp. 663-671. American Society for Microbiology, Washington D.C.

42. **Matsuhashi, M., Nakagawa, J., Tomioka, S., Ishino, F., and Tamaki, S.** (1982) In: Drug resistance in bacteria - genetics, biochemistry and molecular biology. (Mitsuhashi, S., ed) pp..297-301. Japan Scientific Societies Press, Tokyo

43. **Tamaki, S., Nakajima, S., and Matsuhashi, M.** (1977) Thermosensitive mutation in *Escherichia coli* simultaneously causing defects in penicillin-binding protein IBs and in enzyme activity for peptidoglycan synthesis *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **74:**5472-5476

44. **Suzuki, H., Nishimura, Y., and Hirota, Y.** (1978) On the process of cellular division in *Escherichia coli*, a series of mutants of *Escherichia coli* altered in the penicillin binding proteins. *Proc. Natl. Acad. Sci. U.S.A.* **75:**664-668

45. **Spratt, B.G.** (1975) Distinct penicillin-binding proteins involved in the division, elongation, and shape of *Escherichia coli* K-12. *Proc. Natl. Acad. Sci. U.S.A.* **72:**2999-3003

46. **Goodell, E.W., and Schwarz, U.** (1985) Release of cell wall peptides into culture medium by exponentially growing *Escherichia coli*. *J. Bacteriol.* **162:**391-397

47. **Goodell, E.W., and Schwarz, U.** (1983) Cleavage and resynthesis of peptide cross bridges in *Escherichia coli*. *J. Bacteriol.* **156:**136-140.

48. **Glauner, B., and Holtje, J-V.** (1990) Growth pattern of the murein sacculus of *Escherichia coli*. *J. Biol. Chem.* **265:**18988-18996

49. **Holtje, J-V., Mirelman, D., Sharon, N., and Schwarz, U.** (1975) Novel type of murein transglycosylase in *Escherichia coli*. *J. Bacteriol.* **124:**1067-1076

50. **Betzner, A.S., and Keck, W.** (1989) Molecular cloning, overexpression and mapping of the *slt* gene encoding the soluble lytic transglycosylase of *Escherichia coli*. *Mol. Gen. Genet.* **219:**489-491

51. **Holtje, J-V., and Keck, W.** (1988) Organization of the major autolysin in the envelope of *Escherichia coli*. In: Antibiotic inhibition of bacterial cell surface assembly and function. (Actor, P., ed) pp..181-188. American Society for Microbiology, Washington, D.C.

52. **Yem, D.W., and Wu, H.C.** (1976) Isolation of *Escherichia coli* K-12 mutants with altered levels of β-N-acetylglucosaminidase. *J. Bacteriol.* **125:**372-373

53. **Korat, B., and Keck, W.** (1988) Expression of *dacB*, the structural gene of penicillin-binding protein 4, in *Escherichia coli*. In: Antibiotic inhibition of bacterial cell surface assembly and function (Actor, P., ed) pp. 306-311. American Society for Microbiology, Washington, D.C.

54. **Beck, B.D., and Park, J.T.** (1976) Activity of three murein hydrolases during the cell division cycle of *Escherichia coli* K-12 as measured in toluene-treated cells. *J. Bacteriol.* **126:**1250-1260

55. **Spratt, B.G., and Strominger, J.L.** (1976) Identification of the major penicillin binding proteins of *Escherichia coli* as D-alanine carboxypeptidase 1A. *J. Bacteriol.* **127:**660-663

56. **Pratt, J.M., Jackson, M.E. and Holland, I.B.** (1986) The C terminus of penicillin-binding protein 5 is essential for localisation to the *E. coli* inner membrane. *EMBO J.* **5:**2399-2405

57. **Hackenbeck, R., and Messer, W.** (1977) Activity of murein hydrolases in synchronized cultures of *Escherichia coli. J. Bacteriol.* **129:**1239-1244

58. **Tuomanen, E., Markiewicz, Z., and Tomasz, A.** (1988) Autolysis-resistant peptidoglycan of anomalous composition is amino-acid-starved *Escherichia coli. J. Bacteriol.* **170:**1373-1376

59. **Tuomanen, E., Cozens, R., Tosch, W., Zak, O., and Tomasz, A.** (1986) The rate of killing of *Escherichia coli* by β-lactam antibiotics is strictly proportional to the rate of bacterial growth. *J. Gen. Microbiol.* **132:**1297-1304

60. **McQuillen, K.** (1958) Lysis resulting from metabolic disturbance. *J. Gen. Microbiol.* **18:**498-512

61. **Kitano, K., Tuomanen, E., and Tomasz, A.** (1986) Transglycosylase and endopeptidase participate in the degradation of murein during autolysis of *Escherichia coli. J. Bacteriol.* **167:**759-765

62. **Tomasz, A.** (1979) The mechanism of irreversible antimicrobial effects of penicillins: how the betalactam antibiotics kill and lyse bacteria. *Ann. Rev. Microbiol.* **33:**113-137

63. **Ishiguro, E.E., Pisabarro, A.G., De Pedro, M.A., and Kusser, W.** (1991) An updated model for the regulation of peptidoglycan biosynthesis and β-lactam-induced autolysis in amino acid-deprived *Escherichia coli.* manuscript in preparation

64. **Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, and J.A., Struhl, K.** (eds.) (1989) Current Protocols in Molecular Biology. Wiley and Sons, Inc. New York

65. **Maniatis T., Fritsch, E.F., and Sambrook, J.** (1982) Molecular cloning. Cold Spring Harbor, New York

66. **Chung, C.T., Niemela, S.L., and Miller, R.H.** (1989) One step preparation of competent *Escherichia coli* transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. U.S.A.* **86:**2172-2175

67. **Ahmed, A.** (1985) A rapid procedure for DNA sequencing using transposon-promoted deletions in *Escherichia coli. Gene* **39:**305-310

68. **Peng, Z., and Wu, R.** (1986) A simple and rapid nucleotide sequencing strategy and its application in analysing a rice histone 3 gene. *Gene* **45:**247-252

69. **Vieira, J., and Messing, J.** (1982) The pUC plasmids, a M13mp-7 derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19:**259-268

70. **Tabor, S., and Richardson, C.C.** (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. U.S.A.* **82:**1074-1078

71. **Prentki, P., and Krisch, H.M.** (1984) *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* **29:**303-313

72. **Cashel, M.** (1975) Regulation of bacterial ppGpp and pppGpp. *Annu. Rev. Microbiol.* **29:**301-318

73. **Muller, K.H., Trust, T.J., and Kay, W.W.** (1989) Fimbriation genes of *Salmonella enteritidios. J. Bacteriol.* **171:**4648-4654

74. **Swank, R.T., and Munkres, K.D.** (1971) Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide-gels with sodium dodecyl sulfate. *Anal. Biochem.* **39:**462-477

75. **Mackie, G.** (1980) Cloning of fragments of λdapB2 DNA and identification of the *dapB* gene product. *J. Biol. Chem.* **255:**8928-8935

76. **Harkness, R.E., and Ishiguro, E.E.** (1983) Temperature-sensitive autolysis-defective mutants of *Escherichia coli. J. Bacteriol.* **155:**15-21

77. **Kusser, W., and Ishiguro, E.E.** (1987) Suppression of mutations conferring penicillin tolerance by interference with the stringent control mechanism of *Escherichia coli. J. Bacteriol.* **169:**4396-4398

78. **Van Bogelen, R.A., Kelley, P.M., and Neidhardt, F.C.** (1987) Differential induction of heat shock, SOS, and oxidation stress regulons and accumulation of nucleotides in *Escherichia coli. J. Bacteriol.* **169:**26-32

79. **Miller, K.W., Bouvier, J., Stragier, P., and Wu, H.C.** (1987) Identification of the genes in the *Escherichia coli ileS-lsp* operon: analysis of multiple polycistronic mRNA's made *in vivo. J. Biol. Chem.* **262:**7391-7397

80. **Bouvier, J., and Stragier, P.** (1990) Nucleotide sequence of the *lsp-dapB* interval in *Escherichia coli. Nucl. Acids Res.* **19:**180

81. **Isaki, L., Beers, R., and Wu, H.C.** (1990) Nucleotide sequence of the *Pseudomonas fluorescens* signal peptidase II gene (*lsp*) and flanking genes. *J. Bacteriol.* **172:**6512-6517

82. **Isaki, L., Kawakami, M., Beers, R., Hom, R., and Wu, H.C.** (1990) Cloning and nucleotide sequence of the *Enterobacter aerogens* signal peptidase II *(lsp)* gene. *J. Bacteriol.* **172:**469-472

83. **Stark, M.J.R.** (1987) Multicopy vectors carrying the *lac* repressor gene for regulated high-level expression of genes in *E. coli. Gene* **51:**255-267

84. **Beck, E., and Bremer, E.** (1980) Nucleotide sequence of the gene *ompA* coding the outer membrane protein II* of *Escherichia coli* K-12. *Nucl. Acids Res.* **8:**3011-3024

85. **Stoker, N.G., Broome-Smith, J.K., Edelman, A., and Spratt, B.G.** (1983) Organization and subcloning of the *dacA-rodA-pbpA* cluster of cell shape genes in *Escherichia coli. J. Bacteriol.* **155:**847-853

86. **Lee, N., Nakamura, K., and Inouye, M.** (1981) Expression of the *Serratia marcescens* lipoprotein gene in *Escherichia coli. J. Bacteriol.* **146:**861-866

87. **Tokunaga, M., Loranger, J.M., Chang, S.-Y., Regue, M., Chang, S., and Wu, H.C.** (1985) Identification of prolipoprotein signal peptidase and genomic organization of the *lsp* gene in *Escherichia coli*. *J. Biol. Chem.* **260:**5610-5615

88. **Lewin, B.** (1983) In: Genes (Cell, ed.) pp. 219-255. John Wiley & Sons, Inc., New York

89. **Kennell, D.E.** (1986) In: Maximizing Gene Expression (Reznikoff, W., and Gold, L. eds.). pp. 103-139. Butterworth Publishers, U.S.A.

90. **Gilson, E., Clement, J.-M., Brutlag, D., and Hofnung, M.** (1984) A family of dispersed repetitive extragenic palindromic DNA sequences in *E. coli*. *EMBO J.* **3:**1417-1421

91. **Higgins, C.F., McLaren, R.S., and Newbury, S.F.** (1988) Repetitive extragenic palindromic sequences, mRNA stability and gene expression: evolution by gene conversion? - a review. *Gene* **72:**3-14.

92. **Yang, Y., and Ames, G.F-L.** (1988) DNA gyrase binds to the family of prokaryotic repetitive extragenic palindromic DNA sequences *Proc. Natl. Acad. Sci. U.S.A.* **85:**8850-8854

93. **Pruss, G.J. and Drlica, K.** (1989) DNA supercoiling and prokaryotic transcription. *Cell* **56:**521-523

# VITA

| | | | |
|---|---|---|---|
| **Surname:** | Gustafson | **Given names:** | Corinne Elaine |
| **Place of birth:** | Edmonton, Alberta | **Date of birth:** | 1st June 1965 |

**Educational Institutions attended:**

| | |
|---|---|
| Northwest Community College | 1983 to 1984 |
| University of Victoria | 1984 to 1991 |

**Degrees awarded:**

| | | |
|---|---|---|
| B. Sc. | University of Victoria | 1987 |

**Honours and Awards:**

| | |
|---|---|
| B. C. Post-Secondary Scholarship | 1986, 1987 |

**Publications and Communications:**

**Gustafson, C. G., Kusser, W., and Ishiguro, E. E.** (1991) Characterization of the *lytB* mutants of *Escherichia coli.* Canadian Society of Microbiology.

**Gustafson, C. G., and Ishiguro, E. E.** (1991) Cloning and identification of the *lytB* gene in *Escherichia coli.* Manuscript in preparation.

## Partial Copyright License

I hereby grant the right to lend my thesis to users of the University of Victoria Library, and to make single copies only for such users or in response to a request from the Library of any other university, or similar institution, on its behalf or for one of its users. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by me or a member of the University designated by me. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

**Title of thesis:** Cloning and Identification of the *lytB* Locus in *Escherichia coli.*

Author: [redacted]

Corinne Elaine Gustafson

17th September 1991