NUCLEOTIDE SEQUENCE OF murE GENE OF ESCHERICHIA COLI AND PRELIMINARY STUDIES ON EXTRAGENIC SUPPRESSOR MUTATIONS OF murE

by

Jing-Song Tao

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We accept this thesis as conforming to the required standard

____

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

____

Dr. Paul. J. Romaniuk, Departmental Member (Department of Biochemistry & Microbiology)

____

Dr. Louis. A. Hobson, Outside Member (Department of Biology)

____

Dr. Robert. D. Burke, External Examiner (Department of Biology)

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University of Victoria

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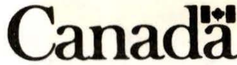

Supervisor: Dr. Edward. E. Ishiguro

ABSTRACT

Two approaches were used to study the murE gene encoding the diaminopimelic acid adding enzyme of Escherichia coli. In the first approach, the cloning and nucleotide sequencing of the murE gene has been performed. The coding region of murE consisted of 1,413 base pairs. The open reading frame of murE gene was separated from the ftsI (penicillin-binding protein 3) gene by 61 base pairs, and it overlapped the initiation codon of the murF (D-Ala-D-Ala adding enzyme) gene by one base pair. The deduced primary structure of MurE comprised 417 amino acid residues with a molecular mass of 50.6 kilodaltons. Amino acid sequences corresponding to the domains A and B of proposed ATP binding sites were identified in the deduced amino acid sequence of MurE. The close linkage of the murE gene to its upstream and downstream neighbouring genes was similar to the close linkage of the other genes in 2-min region of E. coli genetic linkage map. The interesting physical arrangement of the genes in this region and the fact that they are all involved in cell envelope metabolism or cell division has prompted the speculation of some novel form of regulation. As a second approach, extragenic suppressor mutations, which suppress the temperature- sensitive lysis phenotype of the murE16 allele, have been isolated and preliminarily characterized. Growth studies indicated that the murE16 mutation was at least partially osmoremedial, Further more, the two suppressor mutations characterized in this study functioned as suppressors of

murE16 only in high osmolarity medium. The attempt to map the suppressor mutations were unsuccessful, and the products of the suppressor genes have not been identified.

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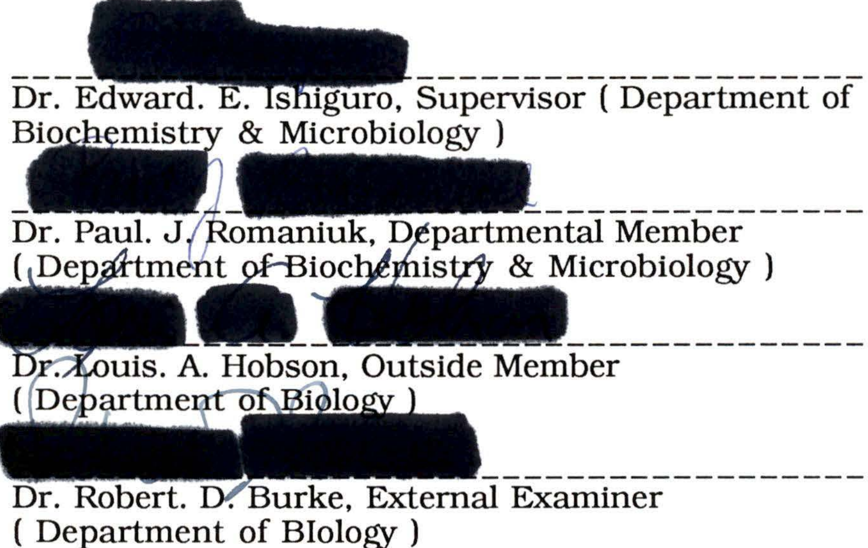

Dr. Edward. E. Ishiguro, Supervisor (Department of Biochemistry & Microbiology)

Dr. Paul. J. Romaniuk, Departmental Member
(Department of Biochemistry & Microbiology)

Dr. Louis. A. Hobson, Outside Member
(Department of Biology)

Dr. Robert. D. Burke, External Examiner
(Department of BIology)

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LIST OF ABBREVIATIONS

Amp	ampicillin
D-Ala	D-alanine
DAP	meso-diaminopimelic acid
D-Glu	D-glutamic acid
DMA	Davis minimal agar
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis(ß-aminoethyl ether) tetraacetic acid
GlcNAc	N-acetylglucosamine
IL-1	interleukin-1
Kb	kilobase pairs
KDal	kilodalton
L-Ala	L-alanine
LP	lipoprotein
MDP	muramyldipeptide
MurNAc	N-acetylmuramic acid
MurNAc-pentapeptide	N-acetylmuramyl-L-alanyl-D-glutamyl-meso-DAP-D-alanyl-D-alanine
NA	nutrient agar
ORF	open reading frame
PBP	penicillin-binding protein
ppGpp	guanosine 5'-diphosphate 3'-diphosphate
pppGpp	guanosine 5'-triphosphate 3'-diphosphate
SDS	sodium dodecyl sulfate

Tris	tris(hydroxymethyl)aminomethane
TSA	tryptic soy agar
TSB	tryptic soy broth
UDP	uridine 5'-diphosphate

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CHAPTER 1. INTRODUCTION: STRUCTURE, PROPERTIES AND SYNTHESIS OF E. coli PEPTIDOGLYCAN

I. Structure of E. coli Peptidoglycan

1. General Structure of E. coli Peptidoglycan

All eubacteria have cell walls containing a rigid sac-like macromolecule called peptidoglycan. Peptidoglycan is a net-like macromolecule composed of long glycan chains interlinked by peptide side chains. The glycan chains are composed of alternating residues of N-acetylglucosamine and N-acetylmuramic acid. The sugars are linked by ß(1-4) glycosidic bonds. In E. coli, the estimated length of the glycan chain is about 35 disaccharide units, (1, 2, 3,) with a nonreducing 1,6-anhydromuramic acid at the end of each strand (4). Each N-acetylmuramic acid residue carries a short peptide substitution. There are variations in the amino acid composition and sequence of the peptide side chains among different bacterial species (5). These variations are of taxonomic importance. Thus peptidoglycan can be structurally viewed as being composed of small basic subunits which are disaccharide-peptides. These units are called muropeptides, and the structure of the basic E. coli muropeptide is shown in Fig. 1. The net-like structure in Fig. 2 is an schematic representation of E. coli peptidoglycan. The peptidoglycan in Fig. 2 is represented as a monolayer with apparent ordered structural features. It must be emphasized that this structure is almost certainly oversimplified as discussed below in sections 2 and 3. Recent studies suggest that

Fig. 1. Disaccharide-pentapeptide unit (muropeptide) of E. coli peptidoglycan.

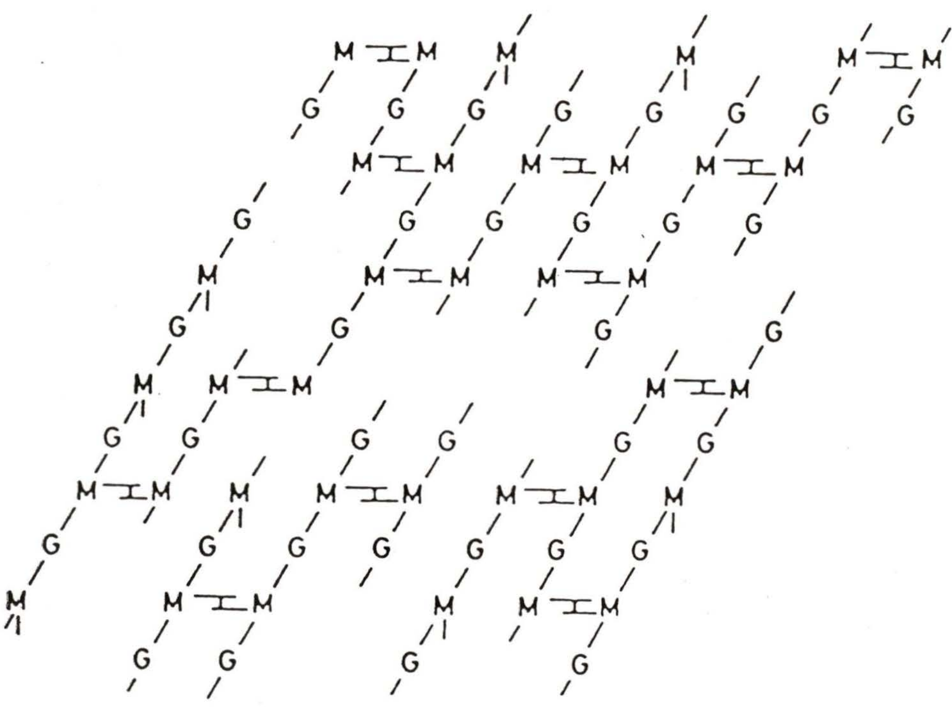


Fig. 2. Schematic representation of a section of E. coli peptidoglycan. M: N-acetylmuramic acid. G: N-acetylglucosamine. Bars between G and M represent glycosidic bonds. Bars between two M residues represent crosslinked peptides. Bars extending from M residues represent peptide side chains which are not crosslinked.

E. coli peptidoglycan is multilayered and contains minor structural modifications which will undoubtedly detract from the ordered appearance depicted in Fig. 2. The amino acid sequence of the peptide substitution is L-Ala-D-Glu-meso-2,6-diaminopimelic acid (DAP)-D-Ala-D-Ala. The occurrence of D-amino acid isomers and the novel amino acid, DAP, make peptidoglycan a unique biological polymer. It is also important to note that the amino sugar, N-acetylmuramic acid, does not occur in any other known substance.

The individual peptidoglycan chains are crosslinked to each other through adjacent peptides to yield the net-like structure shown diagrammatically in Fig. 2. In E. coli about 40 to 60% of the peptides are involved in these crosslinkages. About 50% of the crosslinkages involve peptide bonds between the carboxyl group of the D-alanine of one muropeptide and the free amino group of DAP of an adjacent muropeptide. More rarely, crosslinkage may involve peptide bonds between the DAP residues of neighbouring chains (2).

A unique lipoprotein is covalently linked to 10% of the peptide chains (6, 7). The linkage occurs between the amino terminal lysine residues of the lipoprotein and the diaminopimelic acid residues of the muropeptide. The carboxy terminus of the lipoprotein is substituted with a lipid which in turn is anchored in the cell wall outer membrane. This arrangement provides a physical connection between peptidoglycan and the outer membrane. This association may be necessary in regulating the coordinated growth of the two separate cell wall layers. This aspect is discussed further below.

2. Composition of E. coli Peptidoglycan

It was thought until recently that the detailed chemical structure of peptidoglycan was reasonably well understood. Our understanding of this structure was based on the determination of the structure of soluble peptidoglycan fragments generated either by enzymatic degradation or by controlled acid hydrolysis of peptidoglycan (4, 8). These soluble peptidoglycan derivatives were separated by paper chromatography or by thin layer chromatography, and the structure of each derivative was subsequently determined. However recent studies involving the superior separation power of high performance liquid chromatography (HPLC) coupled with more sensitive detection methods indicate that the structure of peptidoglycan is far more complex than originally thought (2, 9). For example, the standard paper chromatographic separation of a lysozyme digest of E. coli peptidoglycan permits the identification of 6 soluble products. In contrast, HPLC separation applied to the same digest permits the resolution and identification of about 80 different soluble fragments. The use of HPLC separation has also forced us to modify and expand our views on the synthesis of peptidoglycan. Some of the main findings in these recent studies are as follows. (i) A novel type of interpeptide crosslinkage between 2 diaminopimelic acid residues has been discovered. (ii) Peptides containing glycine instead of D-alanine occur. (iii) Newly synthesized peptidoglycan is structurally different from "older" or mature peptidoglycan in several ways. For example, new peptidoglycan is less crosslinked and contains fewer covalently linked lipoprotein than old peptidoglycan. These findings raise the question of whether the structural variants are simply

accidental or whether they actually serve an important structural or regulatory role. The fact that they are consistently encountered suggests the latter.

3. Three Dimensional Structure of E. coli Peptidoglycan

For a long time, a chitin-like monolayer structure had been accepted as the model of peptidoglcan. According to this model, the peptidoglycan sacculus was described as a quasicrystalline arrangement of glycan strands interlinked by peptide bonds in a β-sheet (7, 10, 11). But this model has lost favour due to recent physical studies. These studies involve X-ray diffraction, infrared spectroscopy and NMR analyses (12, 13, 14). A comparative study between peptidoglycan and chitin using these techniques clearly demonstrated that the crystalline arrangement were unlikely structures for bacterial peptidoglycan.

A new model for E. coli peptidoglycan has been proposed by Barnickl et al. (12). They suggest that peptidoglycan is a multilayered structure rather than a simple monolayer. In their model, the glycan strands of peptidoglycan are arranged parallel to each other and run perpendicular of the cell cylinder.The consequence of this arrangement is that the peptidoglycan strand exists in the form of helix with each peptide side chain tilted 90 degrees around the helix relative to its neighbours. With this arrangement, about one of every four peptide side chains are positioned adjacent to each other within one plane and can be crosslinked. The rest of peptide side chains which are not positioned correctly cannot be crosslinked. Some of them, especially those positioned perpendicular to the plane of

peptidoglycan layer they belong to, may however be involved in the crosslinks which connect other planes of peptidoglycan layer. Since the average length of individual glycan strand is 30 nm, about 80 strands aligned end to end will provide enough length to cover the cell circumference once (15, 16). This model is consistent with many experimental data. According to this model, peptidoglycan is flexible and rigid enough to carry out its biological function. In a recent electron microscopic study of cell envelope, a thick gel-like E. coli peptidoglycan structure with high water content was demonstrated. This result provides further structural evidence for a multilayer peptidoglycan model (17).

4. Relationship of the Peptidoglycan to the Outer Membrane and Cytoplasmic Membrane

The cell envelope of Gram negative bacteria such as E. coli is composed of three layers, the cytoplasmic membrane, the peptidoglycan and the outer membrane. The peptidoglycan lies between the cytoplasmic membrane and the outer membrane. The peptidoglycan sacculus is associated with the outer membrane and the cytoplasmic membrane. The anchoring of peptidoglycan to the outer membrane by the covalently linked lipoprotein molecules is the primary force which connects these two layers together (6, 7). The outer membrane proteins called porin, e. g., the ompC and ompF gene products, are also involved in maintaining a connection between peptidoglycan and outer membrane. The porins are noncovalently associated with peptidoglycan (18). The basis for the interaction between the porins and peptidoglycan is unknown. An apparent

connection between the cytoplasmic membrane and peptidoglycan has been observed in ultrastructural studies, but the actual fusion between the two structures has never been directly demonstrated (19). It has been proposed that the so called fusion site may actually represent a site where the outer membrane components are being synthesized and new peptidoglycan strands are being inserted into the wall sacculus. Such sites may represent localized fusions between the cytoplasmic membrane and the outer membrane.

II. Biological Functions and Activities of Peptidoglycan

1. Introductory Comments

It is well known that peptidoglycan is a rigid polymer which determines both the shape and the size of bacterial cells. Furthermore, it performs an essential function as an exoskeleton which provides mechanical support for the underlying cytoplasmic membrane. Its destruction invariably results in the osmotically induced lysis of bacteria. In recent years, it has become apparent that peptidoglycan also exhibits an array of biological activities with relevance to an animal host. Some of these activities are summarized below. It should be emphasized that the activities described are those elicited by small, soluble fragments of peptidoglycan and not the insoluble, intact wall peptidoglycan.

2. Immunogenicity

Peptidoglycan is a very good immunogen. It can induce B cell-mediated as well as T cell-mediated immune responses (20, 21). There are four immuno-determinant groups which are responsible for the immunogenicity of peptidoglycan. They are N-acetylglucosamine, N-acetylmuramic acid, the interpeptide bridge structure, and D-Ala-D-Ala residues (22, 23). Since these four groups are part of the basic structural component of peptidoglycan in all bacterial species, an extensive immunological cross reaction occurs.

3. Adjuvant Function

Peptidoglycan can act as immunoadjuvant to modulate the immune response (24). The modulation can represent either an enhancement or a suppression of the immune response depending on the host, the dose of peptidoglycan, and the time of injection relative to that of antigen (25). The minimum structure required for the immuno-modulation activity is N-acetylmuramyl-L-alanyl-D-glutamic acid (muramyldipeptide or MDP) (25, 26). Thus, muramyldipeptide or derivatives of muramyldipeptide have been added to vaccines to boost the immune response (27).

4. Complement Activation

Peptidoglycan from both gram positive and gram negative bacteria can activate both the classical and the alternate pathways of complement in vitro (28). The expression of the complement activation property is dependent on the size of the peptidoglycan fragment and the degree to which it is structurally modified. In

general, the higher the molecular weight of peptidoglycan fragment, the stronger the activity in complement activation (28). It has been reported that the minimum structure required for the activation of both complement pathways is a glycan chain with the length of 10 to 11 disaccharide units (29). The activation of both complement pathways is believed to be mediated by anti-peptidoglycan antibodies and direct activation of the C1 component (30). The significance of complement activation by peptidoglycan in vivo requires further study.

5. Inflammatory Reaction

Four hours after injection of peptidoglycan fragments, there is an intensive infiltration of polymorphonuclear neutrophil leukocytes and disruption of collagen bundles at the injection site. These histological changes are believed to be induced by the acute toxicity of peptidoglycan. Soluble peptidoglycan fragments induce fever and other aspects of the inflammatory reaction (31). When peptidoglycan is modified by covalently bound polysaccharide, it becomes even more toxic. Polysaccharide modification can apparently protect peptidoglycan from tissue enzymes resulting in an enhanced persistence of peptidoglycan in tissue, and this results in chronic inflammation (32). Certain autoimmune diseases have be proposed to be caused by persistence of soluble peptidoglycan in tissues (33, 34).

6. Somnogenicity

A group of soluble compounds that can induce slow wave sleep in experimental animals have been purified from human brain and urine. Structural analysis of these compounds indicated that they were

muramylpeptides, presumably derived from bacterial cell walls. Authentic muramylpeptides prepared from bacteria were subsequently shown to be active in a sleep induction assay. It has been proposed that the soluble somnogens found in urine and brain were derived from the cell walls of bacteria in the normal human flora (35).

7. A Possible Mechanism to Account for the Biological Activities of Peptidoglycan

From above information, it is clear that peptidoglycan can exhibit a wide variety of biological activities. It can function as an immunogen, an immunoadjuvant, an inflammatory factor, and a somnogen. The search for a unified mechanism which could account for such a wide array of activies has resulted in an interesting possibility (36, 37, 38, 39). Recent studies indicate that muropeptides induce the synthesis of interleukin-1 (IL-1) and other cytokines in macrophages and lymphocytes (40). It is possible that the various biological activities attributed to peptidoglycan are actually directly mediated by cytokines such as IL-1. For example, IL-1 has a well-documented role in immune regulation and in the inflammatory response. Furthermore, IL-1 is a potent somnogen (41). Macrophages probably routinely process peptidoglycan derived from either the normal bacterial flora or invading bacteria. The processed peptidoglycan could serve as an inducer of cytokine synthesis. The ultimate activity exhibited (e. g., inflammation, immune regulation, or somnogenicity), according to this proposal, will be determined by what cytokines are produced and by what type of cells the cytokines interact with.

III. Synthesis of E. coli Peptidoglycan

1. Introductory Comments

The synthesis of peptidoglycan is a very complex process involving many precisely programmed reactions and specific enzymes. The biosynthetic reactions occur both in the cytoplasm and on the cell membrane. The cytoplasmic reactions are a series of reactions catalyzed by a group of soluble enzymes to produce UDP-activated peptidoglycan precursors. The membrane- bound reactions result in the incorporation of precursors into the existing wall peptidoglycan. Some of the main steps in peptidoglycan synthesis are shown in Fig. 3.

2. Cytoplasmic Reactions: Synthesis of UDP-activated Precursors

A group of soluble enzymes catalyze the synthesis of the 2 main soluble precursors of peptidoglycan, UDP-N-acetylglucosamine (UDP-GlcNAc) and UDP-N-acetylmuramyl-L-Ala-D-Glu-meso-DAP-D-Ala-D-Ala (UDP-MurNAc-pentapeptide)(42, 43). The synthesis of UDP-GlcNAc is obviously an important process in E. coli since this compound serves as a source of GlcNAc for peptidoglycan as well as for cell wall lipopolysaccharide synthesis. It is therefore interesting that the reactions involved have not been characterized in detail in E. coli and are therefore omitted from Fig. 3. As shown in Fig. 3, UDP-GlcNAc is also the precursor for UDP-MurNAc (reactions 1 and 2). This is followed by the synthesis of the pentapeptide substitution on UDP-MurNAc. This involves the sequential addition of L-Ala, D-Glu, meso-DAP, and D-ALa-D-Ala (reactions 3 to 8). Each addition requires a specific ATP-dependent enzyme. This thesis is primarily on the meso-DAP-adding enzyme (reactions 5, Fig. 3).

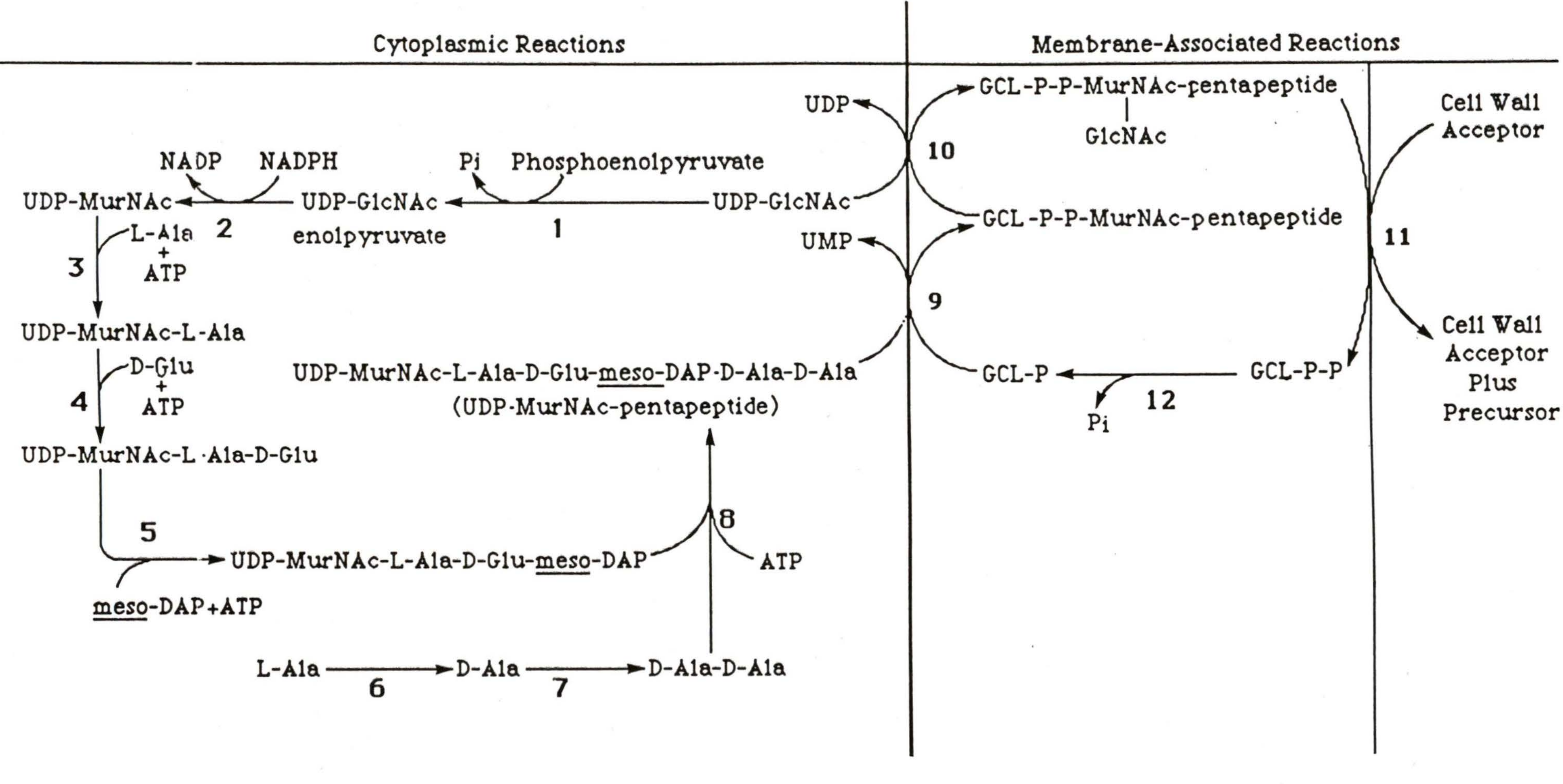


Fig. 3. The major steps in the biosynthesis of E. coli peptidoglycan.

Fig. 3. The major steps in the biosynthesis of E. coli peptidoglycan. The enzymes involved in each of the steps are: 1. phosphoenol-pyruvate: UDP-GlcNAc-pyrophosphorylase; 2. UDP-GlcNAc-enolpyruvate reductase; 3. L-alanine adding enzyme; 4. D-glutamate adding enzyme; 5. meso-DAP adding enzyme; 6. alanine racemase; 7. D-alanyl-D-alanine synthetase; 8. D-ala-D-ala adding enzyme; 9. UDP-MurNAc-pentapeptide translocase; 10. GlcNAc-translocase; 11. glycan polymerase and/or transpeptidase; 12. pyrophosphatase.

3. Membrane-Bound Reactions: Synthesis of Lipid-linked Intermediates and Peptidoglycan Polymerization

The membrane-bound reactions can be subdivided into those reactions which occur on the cytoplasmic side of the membrane and those reactions which occur on the periplasmic side of the membrane. As shown in Fig. 3, the reactions occuring on the cytoplasmic side of the membrane involve the sequential translocation of P-MurNAc-pentapeptide and GlcNAc to undecaprenylphosphate (labeled "GCL-P" in fig. 3 for "Glycosyl Carrier Lipid Phosphate"; reactions 9 and 10). Undecaprenyl-phosphate is a minor membrane lipid which serves as a glycosyl carrier to mediate transport of the hydrophilic disaccharide-pentapeptide peptidoglycan subunit across the cell membrane barrier (44, 45, 46, 47).

On the periplasmic side of the membrane, the disaccharide-pentapeptide units are translocated from the lipid carrier to the acceptor sites on the existing cell wall peptidoglycan (reaction 11). The biochemical and genetic evidence currently favors the view that this polymerization reaction is catalyzed exclusively by one of several membrane-bound enzymes refered to as penicillin-binding proteins (PBPs) (48, 49, 50). In E. coli, there are at least seven major PBPs, and these are designated PBP 1A, 1B, 2, 3, 4, 5, and 6. The four high molecular weight PBPs (1A through 3) are the peptidoglycan polymerases. The three low molecular weight PBPs appear to act as peptidoglycan hydrolases (See below). Finally, a pyrophosphatase acts on undecaprenylpyrophosphate to recycle the lipid carrier (reaction 12).

Peptidoglycan polymerization by PBPs may occur in two ways. One way is by a transglycosylation, or glycan chain extension, reaction which links the disaccharide portion of the precursor to an existing glycan chain in the cell wall. The second way is through a transpeptidation reaction which creates the DAP to D-Ala interpeptide cross-linkage. Thus, the PBPs 1A,1B and 3 of E. coli are actually bifunctional enzymes, i. e., they possess 2 active sites and are capable of catalyzing either transglycosylation or transpeptidation (51). On the other hand, PBP 2 is exclusively a transpeptidase (52). The transglycosylase activity of the PBPs is inhibited by the antibiotic moenomycin and the transpeptidase activity by penicillin and cephalosporin derivatives (53). The name given to these enzymes is obviously derived from the latter characteristic. At present, there is no information regarding when and where each of these 2 reactions occur during the growth of the peptidoglycan sac. Further details on the specific functions of the individual PBPs is presented below.

IV. Regulation of Peptidoglycan Synthesis

1. Introductory Comments

The mechanism of the regulation of peptidoglycan biosynthesis is poorly understood. However, there are reports of stringent response (54) and feedback inhibition (55) involved in the regulation of both cytoplasmic stage and reactions in the outer phase of the cytoplasmic membrane of peptidoglycan synthesis.

2. Stringent Response

The stringent response is a regulatory mechanism by which the synthesis of key cellular macromolecules such as stable RNA species, phospholipids and cell wall peptidoglycan is shut down due to a rapid accumulation of guanosine 5'-triphosphate 3'-diphosphate (pppGpp) and guanosine 5'-diphosphate 3'-diphosphate (ppGpp). The accumulation of these componds occurs during the growth arrest of relA+ strains of E.coli induced by amino acid deprivation. The inhibition of peptidoglycan synthesis during the stringent response apparently occurs at both an early step in the synthesis of UDP-MurNAc-pentapeptide (56) and a late step in the polymerization of peptidoglycan (57). The mechanism of stringent response of the peptidoglycan polymerization reactions appears to be due to the inhibition of phospholipid synthesis by ppGpp. It has been shown that peptidoglycan polymerization, presumably by PBPs, is dependent on ongoing phospholipid synthesis (54). Therefore, the inhibition of phospholipid synthesis results in the reversible inhibition of peptidoglycan polymerization.

3. Feedback Inhibition

For the reactions of the peptidoglycan synthetic pathway, feed back inhibition by a soluble precursor is a control mechanism one can expect to encounter. Attempts have been made to prove this expectation (57). When E. coli is treated with a ß-lactam antibiotic, i. e., to inhibit PBP activity, UDP-MurNAc-pentapeptide does not accumulate, suggesting that its synthesis may be controlled by feedback inhibition. However, if E. coli is treated with D-cycloserine, a

drug capable of blocking the synthesis of D-Ala-D-Ala and therefore of UDP-MurNac-pentapeptide, an apparently uncontrolled accumulation of UDP-MurNAc-tripeptide occurs. The accumulation of UDP-MurNAc-tripeptide in the absence of UDP-MurNAc-pentapeptide suggests that the latter feedback inhibits an early step in its own synthesis (58, 59). The exact site of this regulation has not been determined.

V. Growth of Peptidoglycan Sacculus

1. Introductory Comments

As already noted, peptidoglycan serves as a cellular exoskeleton which is the determinant of both the characteristic shape and the size of a bacterium. The morphogenetic events involved in peptidoglycan synthesis which ensure the species-specific uniformity of these characteristics must clearly depend on an exquisite topological and temporal control system. This process is still far from being understood, but it very likely will involve the activities of the PBPs and a group of enzymes known as peptidoglycan hydrolases.

2. The Role of PBPs in E. coli

All bacterial species examined to date have multiple PBPs. For example, as mentioned above, E. coli possesses at least 7 major PBPs (60, 61). The functions of these PBPs in E. coli have been elucidated by a combination of biochemical and genetic analysis, and it is reasonably clear that each individual PBP has its own function.

The growth of an E. coli cell involves the elongation of a cylindrically shaped peptidoglycan sac. The enzymes responsible for this process are PBP 1A and PBP 1B. The progress of cell elongation is interfered with by a mutation affecting either PBP 1A or PBP 1B. Although such mutants are viable, strains carrying mutations affecting both PBP 1A and PBP 1B are inviable. Similar conclusions can be drawn from experiments involving treatment of wild type bacteria with penicillin derivatives which have high affinity for PBPs 1A and 1B. Under conditions where such antibiotics specifically inhibit only PBPs 1A and 1B, cell elongation is inhibited, and the cells eventually undergo lysis (62, 63).

The blockage of PBP 3 activity either by a mutation in the ftsI gene (which encodes PBP 3; see Chapter 2) or by treatment with penicillin derivatives which specifically inhibit PBP 3 results in the formation of filamentous cells. These filaments result because cell division is blocked, and it is therefore clear that PBP 3 is responsible for the synthesis of septal peptidoglycan during cell division. The septum, which will eventually represent a cell pole upon completion of division, can be viewed as a hemispherically shaped form of peptidoglycan. It now appears that the PBP 3 activity is turned on at a specific time during the cell division cycle to initiate the formation of septa. The signal for activating this activity appears to be the completion of chromosome replication but the details of this process are unkown (62, 64, 65).

The function of BPB 2 can also be blocked either by a mutation in the PBP 2 structural gene or by treatment of cells with a PBP 2-specific penicillin derivative. In both cases, the bacteria lose their

characteristic rod-shaped morphology and become spherical. It has therefore been proposed that PBP 2 activity is necessary for the synthesis of cylindrical peptidoglycan; i.e., when PBP 2 activity is blocked, cells continually synthesize only hemispherical peptidoglycan, and this results in their spherical morphology (64, 66).

3. Involvement of Hydrolase System

The peptidoglycan hydrolases are a group of enzymes capable of hydrolyzing interglycan, interpeptide or glycan-peptide bonds in peptidoglycan. In E. coli there are eight known hydrolase activities which include some of the PBPs (55, 67). The cleavage pattern of each hydrolase activity is illustrated in fig. 4.

Although peptidoglycan hydrolases appear to be ubiquitous in bacteria, their functions are, at best, poorly understood. They are presumably involved in some aspect of peptidoglycan morphogenesis (1, 68, 69). They are undoubtedly involved in the process known as turnover in which soluble fragments of peptidoglycan are generated from the existing cell wall peptidoglycan during normal growth (70). Turnover has been observed in many bacterial species and may very well be universal. Because it is so commom, turnover is thought to be an essential event during normal growth which serves a morphogenetic function. It has also been suggested that peptidoglycan hydrolases play an essential role in cell division (71, 72). A current popular hypothesis is that the synthesis of peptidoglycan requires the coordinated activities of the enzymes belonging to the biosynthetic pathway and one or more of the peptidoglycan hydrolases. It is not yet known how the activities of peptidoglycan hydrolases are regulated

```
    D-Ala                                D-Ala
      |——— 6                               |
    D-Ala                                D-Ala         D-Ala
      |                                    |      5 ———|
  meso-DAP                             meso-DAP     meso-DAP
      |                                    |            |
    D-Glu                                D-Glu        D-Glu
      |             2         1            |            |
    L-Ala           |         |          L-Ala        L-Ala
3 ————|             |         |            |            |
GlcNAc-MurNAc-GlcNAc-MurNAc-GlcNAc-MurNAc-GlcNAc-MurNAc
                          \
                         L-Ala
                            \
                           D-Glu
                              \
                         meso-DAP-D-Ala
                                \——— 4        D-Ala
                               D-Ala             \
                                  \             D-Ala
 meso-DAP                      meso-DAP            \
     \                              \           meso-DAP
    D-Glu                          D-Glu             \
       \                              \             D-Glu
      L-Ala                          L-Ala             \
         \                              \             L-Ala
       GlcNAc-MurNAc-GlcNAc-MurNAc-GlcNAc-MurNAc
```

Fig. 4. Cleavage sites of peptidoglycan hydrolases within the E. coli peptidoglycan net work. 1. lysozyme and transglycosylase. 2. N-acetyl-glucosaminidase. 3. amidase. 4. carboxypeptidase 1B and penicillin-insensitive endopeptidase. 5. carboxypeptidase II. 6. carboxypeptidase 1A, 1B.

during normal growth and how this control is dissociated under the various conditions which result in cellular lysis, e. g., during penicillin treatment.

VI. Genetics of Peptidoglycan Biosynthesis and Metabolism in E. coli

In E. coli, at least twenty-five genes which are directly involved in or closely related to peptidoglycan synthesis and metabolism have been mapped. These genetic studies are based on temperature sensitive mutants which have a common phenotype. These mutants either lyse or undergo a morphological change at the nonpermissive temperature (usually 42° C).

A summary of peptidoglycan genetics is shown in Table 1. The genes encoding eight of the soluble enzymes involved in the first step of biosynthesis have been identified and mapped. Among these, the murF (112, 113), alr (85), and ddl (114) genes have been cloned and sequenced. The dapF gene coding for DAP epimerase has also been cloned recently (78). The genes encoding D-glutamic acid synthetase and D-glutamic acid adding enzyme have yet to be identified. The genes encoding penicillin-binding proteins 1A, 1B, 3, 4, 5 have been mapped, cloned, and sequenced (84, 115, 116, 117). The gene encoding PBP 6 has not been mapped on the E. coli genetic linkage map due to the fact that a mutation apparently yields no detectable phenotypic change. However, the gene designated dacC which encodes PBP6 has been cloned and sequenced (59). Six genes

coding for the peptidoglycan hydrolases have been identified.

In addition to these 25 genes, there are at least 43 genes involved in the cell division cycle. The discussion of these genes is not the purpose of this thesis. But their function and regulation must be closely related to the peptidoglycan synthesis and metabolic process. Uncovering the relationship between the two will help us understand the mechanism of the growth of sacculus, septation, and cooperations of these processes.

Table 1. Genes and their protein products involved in peptidoglycan metabolism in E. coli

	Gene	Map Location	Gene Product	Reference
Soluble Biosynthetic Enzymes	mrbA	90 min	UDP-N-acetylglucosaminyl -3- enolpyruvate reductase	73
	murA	90 min	UDP-GluNAc:phosphoenol-pyruvate transferase	74
	murC	2 min	L-alanine adding enzyme	58,75,76,77
	dapF	85 min	DAP epimarase	78
	murE	2 min	DAP adding enzyme	58,73,79,80
	alr	93 min	alanine racemase	81
	ddl	2 min	D-Ala-D-Ala ligase	75,82,83,84
	murF	2 min	D-Ala-D-Ala adding enzyme	58.77.85
Membrane-bound Biosynthetic Enzymes	mrcA	75 min	PBP 1A	62
	mrcB	4 min	PBP 1B	62,63
	pbpA	15 min	PBP 2	86,87,88,89
	ftsI	2 min	PBP 3	62,77,89,90
Peptidoglycan Hydrolases	dacB	69 min	PBP 4	90,91,92
	dacA	15 min	PBP 5	86,91,93,94
	dacC	unknown	PBP 6	59
	amiA	51 min	N-acetylmuramyl-L-alanine amidase	95
	mepA	51 min	Penicillin-insensitive peptidoglycan DD-endopeptidase structure gene	96
	mepB	92 min	Penicillin-insensitive peptidoglycan DD-endopeptidase	96
	mraA	2 min	D-alanine carboxypeptidase	73

	Gene	Location	Product	Reference
Peptido-glycan-lipo-protein	lpp	36 min	peptidoglycan lipoprotein structural gene	97-105
Unknown Activity	rodA	15 min	Required for growth with rod shaped sacculus	62,106-109
	murG	2 min	Unknown	110
	mraB	2 min	Unknown	73
	mrbB	90 min	Unknown	73
	murH	99min	Unknown	111

VII. Purpose of This Thesis

As discussed above, the synthesis of peptidoglycan is a complex process involving the coordinated activities of many enzymes. Little is known about the regulation of this process. The original objective of this thesis was to clone and sequence the murE and murF genes which appeared to be directly joined to each other. Furthermore, as discussed in Chapter 2, these genes are located in a region of chromosome which is enriched in genes encoding proteins involved in peptidoglycan synthesis, cell division, or other aspects of cell envelope function. The murE and murF genes encode the meso-diaminopimelic acid adding enzyme (uridine diphosphate-N-acetylmuramyl-L-Ala-D-Glu-meso-2,6-diaminopimelate ligase; EC.6, 3, 2, 13) and the D-alanyl-D-alanine ligase (EC 6, 3, 2, 10), respectively. These enzymes are involved in the synthesis of UDP-N-acetylpentapeptide. It was thought that the cloning and sequencing of murE and murF would yield information concerning the regulation of gene expression as well as information on the primary structures of their protein products (neither enzyme has been purified). However, while this work was in progress, we learned that the work on the murF sequence was almost completed in another laboratory (cited by Walsh in ref. 85). We therefore decided to complete the murE sequence and to terminate the attempts to sequence murF. The murF sequence was reported (113) while the writing of this thesis was in progress. The murE sequence is reported in Chapter 2. The thesis objective was modified to include preliminary studies on extragenic mutations which suppress a mutation in the murE gene. It was thought that such studies would reveal a protein(s) which interact directly or

indirectly with the murE gene product. These studies are reported in Chapter 3.

CHAPTER 2. NUCLEOTIDE SEQUENCE OF THE murE GENE OF E. coli

I. Introduction

A total of 13 genes involved in functions related to cell division, peptidoglycan synthesis or cell envelope function have been mapped in the 2-minute region of the Escherichia coli genetic linkage map (118). There is considerable interest in this region because, as shown in Fig. 5, these 13 genes appear to be arranged contiguously. Takeda et al. (90) first demonstrated that at least 3 of these genes, ftsI (also known as pbpB), murE and murF, were present along with the leuA locus on plasmid pLC26-6 from the Clarke and Carbon gene bank (119). The ftsI gene which encodes penicillin-binding protein 3 (PBP 3) was subsequently cloned and sequenced by Nakamura et al. (116).

As mentioned in Chapter 1, the murE and murF genes encode the ATP-dependent diaminopimelic acid and D-alanyl-D-alanine adding enzymes, respectively (Fig. 3). Maruyama et al (112) have recently reported the localization of the murE and murF genes on the restriction map. They also identified the murE and murF gene products as 56-KDal and 52-KDal proteins, respectively, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after expression of the cloned genes in maxicells. We report here the nucleotide sequence of murE gene.

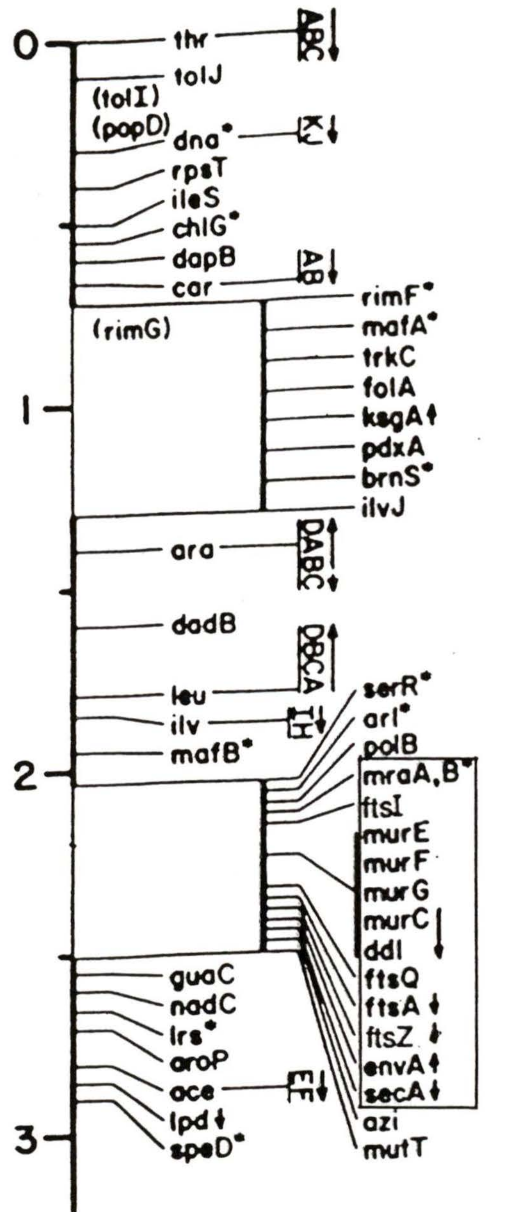


Fig. 5. The 2-min region of E. coli genetic linkage map with the cell envelope gene cluster in the bracketed area.

II. Material and Methods

1. Bacteria and Plasmids

The E. coli K-12 strains and the plasmids used in this study are presented in Table 2 and Table 3.

2. Enzymes and Biochemicals

The restriction endonucleases KpnI, EcoRI, PstI were purchased from Boehringer Mannheim Canada, and HindIII, PvuI, EcoRV were from Pharmacia LKB Biotechnology AB. The lambda DNA, lambda DNA/Hind III standard were from Bio-RAD. DNA T4 ligase was from Boehringer Mannheim Canada. The Sequenase DNA sequencing kit was from Bio-RAD, and the T7 polymerase DNA sequencing kit was from Phamacia LKB Biotechnology AB. [^{35} S]-dATP (specific activity 1000 Ci/mmol) was purchased from Amersham.

3. Bacterial Growth Conditions

a) Media

In most cases, Tryptic Soy Broth (TSB, from Difco laboratories) or Tryptic Soy Agar (TSA. from Difco laboratories) were used as rich medium. Thymine and uracil (100 μg per ml of each) were added to the medium when it was used to grow strain TKL-11 and TKL-46. In a few cases, LB medium and 2YT medium were used for specific purposes. M9 medium or Davis minimal agar (DMA, from Difco laboratories) were used as minimal media for the selection of auxotrophic mutants. Minimal media were supplemented with

Table 2. E. coli K-12 strains

Strain	Sex	Genotype[a]	Source
DH5- alpha	F⁻	thi hsdR17$r^{-}_{k}m^{+}_{k}$ recA relA	Laboratory Stock
TKL-11	F⁻	thr1 leuB6 murE16(ts) codA1 pyrF101 thyA6 argG66 his-108 iluA634 thi-1 deoC1 lacY1 tonA21 tsx-95 supE44	B.J.Bachmann[b]
AA102-F'lac	F'	[recA pro thi supE endA $hsdR_{K}$ 1 (gal-che-pgl-att) (F':traD36 proAB $lacI^{q}z\Delta M15$)]	Gold Biotechnology Inc. St louis. MO. USA
TKL46	F⁻	thr1 leuB6 murF2(ts) codA1 pyrF101 thyA6 argG66 his-108 iluA634 thi-1 deoC1 lacY1 tonA21 tsx-95 supE44	B.J.Bachmann[b]
JM107	F'	Δ(lac proA,B) thi pyrA96 endA1 hsdR17 relA1 supE44($r^{-}_{k}m_{k}^{+}$) lambda⁻/F'traD36 proAB lac $Iqz\Delta M15$	Laboratory Stock

a) For genetic symbols see Bachmann (118)

b) Address of B.J.Bachmann: E.coli Genetic Stock Centre, Department of Human Genetics, Yale University School of Medicine. New Haven. CT. U.S.A.

Table 3. Plasmids

Plasmid	Relevant Characteristics	Source
pLC26-6	ftsI+, murE+, murF+	Laboratory Stock
pUC18	Amp^R	Laboratory Stock
pUC19	AmpR	Laboratory Stock
pAA-PZ618	Amp^R, ISI-Tn9, M13-origin	Gold Biotechnology Inc. St. louis. MO. USA
pAA-PZ619	Amp^R, ISI-Tn9, M13-origin	Gold Biotechnology Inc. St. louis. MO. USA
pJS718	pUC18 carrying 4.0 Kb EcoRI-KpnI murE and murF clone	this study
pJS719	pUC19 carrying 4.0 Kb EcoRI-KpnI murE and murF clone	this study
pJS800	pAA-PZ618 carrying 4.0 Kb EcoRI-KpnI murE and murF clone	this study
pJS900	pAA-PZ619 carrying 4.0 Kb EcoRI-KpnI murE and murF clone	this study
pJS801-pJS804	deletion derivatives of plasmid pJS800	this study
pJS901-pJS906	deletion derivatives of plasmid pJS900	this study

0.2% carbon source, 0.5 μg of thiamine per ml, and 100 μg per ml of required amino acids and nucleotides.

b) Bacterial Growth

Bacterial cultures were incubated in gyrotory water bath shakers at 30° C or 37° C depending on the specific strain used. Incubation at 42° C was used as the nonpermissive growth condition for temperature-sensitive mutants. Culture turbidity was followed with a Klett-summerson colorimeter using a green filter (540 nm) for rich media or a blue filter (420 nm) for minimal media.

4. Recombinant DNA Technology

a) Small Scale Plasmid Isolation

Plasmid purification procedure was based on the modified alkaline lysis method of Birnboim and Doly (120).

b) Determination of the Concentration, Size and Purity of DNA Preparation

The concentration of DNA was determined by reading the absorption of DNA solution at 260 nm (A_{260}) (121). One A_{260} unit equals a DNA concentration of 50 μg per ml. DNA concentration was also determined by agarose gel electrophoresis of serially diluted DNA samples using lambda DNA as the standard. The size of plasmid DNA was determined by agarose gel electophoresis of linearized DNA sample using lambda/Hind III DNA as the standard.

c) Agarose Gel Electrophoresis Analysis

Tris-acetate solution (TAE, 40 mM Tris, 10 mM sodium acetate and 1 mM EDTA. pH 7.8)(121) was used as the running buffer in agarose gel electophoresis. DNA samples were heated to 65°C for 7 minutes and mixed with a solution of 5% sucrose and 0.025% bromophenol blue. The mixture was loaded on the gel. The gel concentration used was between 0.5-1.5% depending on the size of DNA fragment to be analyzed. After electrophoresis, the gel was stained with ethidium bromide (2 µg per ml) for 20 minutes. DNA bands were visualized by illumination with ultraviolet light.

d) Extraction of DNA from Agarose

DNA bands required were recovered either by electrophoretic elution at 100 volts for 3 hours or by the freezing method. In the freezing method, the agarose gel was cut into pieces and deep frozen (70° C) in the presence of phenol. After removal of the agarose by centrifugation, the DNA was recovered by a combination of phenol-chloroform extraction and ethanol precipitation (121).

e) Restriction Endonuclease Digestion

Restriction endonuclease digestions were carried out using the procedure of Maniatis et al (121). Restriction mixtures were incubated at 37° C for 60 min. Reactions were stopped by heat denaturation at 70° C for 10 minutes.

f) Formation of Recombinant DNA

Both vector and insert DNA were linearized, purified, and mixed in a ratio of 1:10 respectively. The ligation reactions were catalyzed by T4 DNA ligase. The ligation reaction mixtures were incubated overnight at 12° C. The reactions were stopped by 70° C heat denaturation (121).

g) Transformation

A modification of Hanahan's (122) procedure was used for transformation.

(i). Preparation of Competent Cells

An overnight culture was diluted 100 fold in TSB medium and grown to a density of 5×10^7 cells per ml (20 Klett units). The culture was chilled on ice for 15 minutes. This was followed by centrifugation at 5,000 rpm for 5 minutes at 4° C. The supernatant was discarded, the pellet was resuspended in half of the original culture volume of ice cold solution of 50 mM $CaCl_2$ and 10 mM Tris-HCl (pH 8.0). The suspension was incubated on ice for 15 min. After incubation, the suspension was centrifuged at 2500 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended in one tenth of the original culture volume of ice cold 50 mM CaCl, 10 mM Tris-HCl (pH 8.0).

(ii). Storage of Competent Cells

Competent cells from above steps were stored at -70° C in 10% sterile glycerol.

(iii). Transformation

1 μl DNA (0.5 μg per μl) was added to 0.2 ml of competent cells. The mixture was then incubated in ice for 60 minutes. At the end of 60 minutes incubation, the sample was quickly moved to the 42° C and incubated for 75 seconds. 1 ml of TSB was added to the heat treated mixture. The mixture was incubated at the appropriate temperature for 90 minutes to allow expression of antibiotic resistance mark. After expression, 0.1 ml cell suspension was plated on selective medium and incubated at appropriate temperature for 1-2 days.

5. Complementation Assay

Complementation assays were carried out by transforming recombinant DNA carrying required gene into temperature sensitive mutant cells. The transformation mixture was plated onto the rich medium containing antibiotic. The plates were incubated at 30° C to select drug resistant marker. The drug resistant transformants were further screened for temperature resistant complementation.

6. Construction of Overlapping Deletion Derivatives

The DNA to be sequenced was cloned into either plasmid pAA-PZ618 or pAA-PZ619 (123). The in vivo deletion strategy is based on the fact that the vectors pAA-PZ618 and pAA-PZ619 contain a modified transposon Tn9 element close to the cloning sites, and the spontaneous excision of this element promotes deletions which could extend into the cloned fragment. To construct random deletion derivatives, plasmids pJS800 and pJS900 (Fig. 6) were transformed

into strain AA102F'lac. The transformants were plated onto the TSA medium containing ampicillin (Amp). Amp resistant colonies were picked and grown overnight. During growth, the random deletions occurred. The deletion derivatives were selected using restriction endonuclease digestion and gel electrophoresis for those with required size.

7. Sequencing Strategy

Plasmids containing deletions derivatives of murE were transformed into E. coli K-12 strain JM107. The transformants were infected with helper phage M13K07. Upon infection, the M13 origin presented on the plasmids permits phage-directed plasmid replication and results in high yields of single-stranded plasmid DNA. The single stranded DNA of deletion derivatives were used to sequence both strands in their entirety by the dideoxy chain termination method of Sanger et al (124) in the presence of [^{35} S] dATP. The DNA sequence data were compiled and analysed with a computer program.

III. Results

1. Genetic Complementation Tests and Identification of DNA Fragment Containing murE and murF Genes.

A 4.0 kilobase-pair (Kb) fragment was obtained from the plasmid pLC26-6 by digestion with the restriction endonucleases KpnI and EcoRI. This fragment was subcloned in both orientations into the KpnI-EcoRI-digested polylinker sites of plasmids pUC18 and pUC19 to yield the recombinant plasmids pJS718 and pJS719, respectively. Both pJS718 and pJS719 were found to complement the temperature sensitive phenotype of strains TKL11 (murE) and TKL46 (murF) indicating that the 4.0 Kb KpnI-EcoRI fragment carried both the murE and the murF genes.

2. Determination of the Nucleotide Sequence of murE

The DNA sequencing strategy is summarized in Fig. 6. The first step was to prepare sets of overlapping deletions of murE DNA. For this purpose, the 4.0 Kb KpnI-EcoRI fragment was cloned in both orientations into the vectors pAA-PZ618 and pAA-PZ619 to yield the plasmids pJS800 and pJS900. Fig. 6 also shows a restriction map of the cloned fragment. The vectors pAA-PZ618 and pAA-PZ619 contain a modified transposon Tn9 element which promotes deletions which could extend into the cloned fragments. Two sets of over-lapping deletions were prepared in this way. Plasmids pJS801 through pJS804 were deletion derivatives of pJS800, and pJS901 through pJS906 were derived from pJS900 Fig. 6. The polarity of murE is as shown in Fig. 6 with the 5'-end of the gene close to the KpnI site of the cloned DNA fragment. The 5'-end was not on plasmid pJS801

which carried the smallest deletion of the KpnI-end of the cloned DNA. Thus, the 5'-end of murE must lies within 500 base pairs of the KpnI site. We were unable to obtain deletions in this area and were consequently unable to more accurately localize the 5'-end of murE. The 3'-end of murE was present on pJS901 but not on pJS902 indicating that it was located close to the EcoRV site.

It should be noted that our data do not allow us to accurately localize the murF gene. What we can say is that the deletion derivatives pJS901 through pJS906 do not carry murF whereas pJS801 through pJS804 all carry murF.

The deletion derivatives were used to sequence both strands of the murE gene in their entirety as summarized in Fig. 6. The sequence obtained is shown in Fig. 7.

3. murE Gene Sequence Analysis

The first 487 bases of the sequence shown in Fig. 7 have been previously reported (137). The initial 128 base pairs represent the 3'-end of the ftsI gene which encode the C-terminal 38 amino acids of PBP 3. I now extend this sequence to the unique EcoRV site shown in Fig. 6. An analysis of this sequence revealed only a single large open reading frame (ORF). This ORF could start 62 base pairs from the end of ftsI at position 179 to encode a 50.6 KDal polypeptide consisting of 471 amino acids. Alternatively, the GTG codon at position 146 is also in frame with this ORF. This would extend the N-terminus by 11 amino acids and increase the molecular weight of the deduced polypeptide to 51.8 KDal. The GTG was considered the least likely of the two possible initiation sites because it was not preceded by a

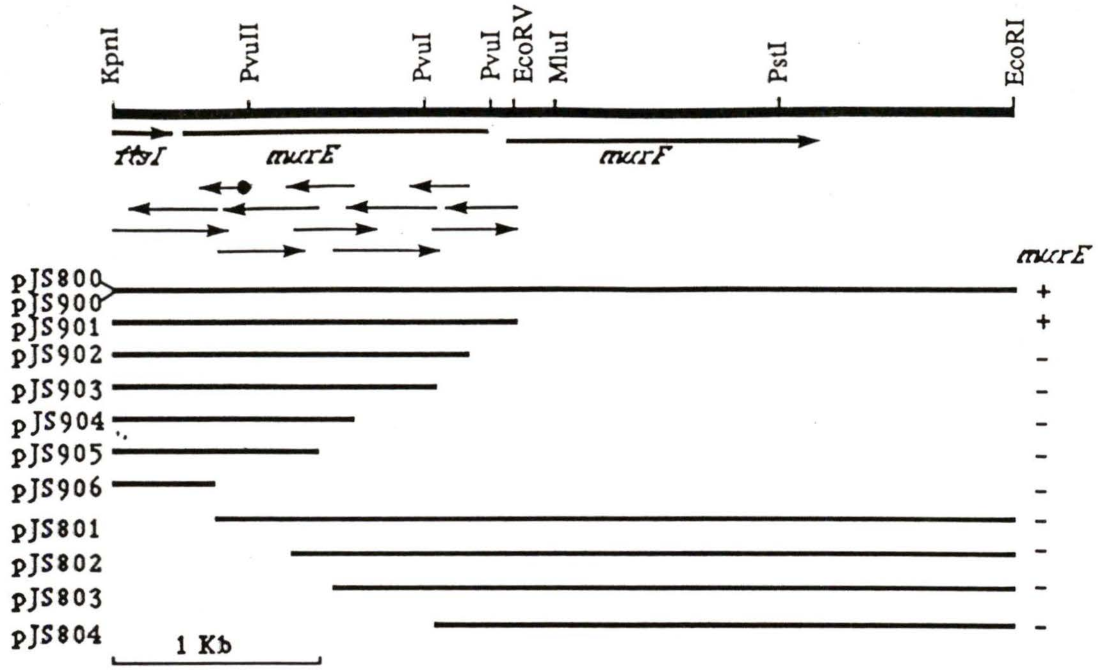


Fig. 6. Sequencing strategy. The larger arrows below the restriction map indicate the positions and orientations of the ftsI, murE, and murF genes determined by restriction mapping and complementation analysis. The smaller arrows indicate the direction and extent of each sequence determination. The closed circle on one of these arrows indicates a reaction which was primed with a synthetic oligonucleotide. The DNA fragments used for sequencing are represented by the bars, and their murE complementation characteristics are indicated.

1 TTTGGTGCCATCATGGGCGGCGTATTGCGTACCATGAACATCGAGCCGGATGCGCTGACA
PheGlyAlaIleMetGlyGlyValLeuArgThrMetAsnIleGluProAspAlaLeuThr

end ftsI

61 ACGGGCGATAAAAATGAATTTGTGATTAATCAAGGCGAGGGGACAGGTGGCAGATCGTAA
ThrGlyAspLysAsnGluPheValIleAsnGlnGlyGluGlyThrGlyGlyArgSer

121 TTTGCCCGACCTTCTTGCTCCGTGGGTGCCAGACGCACCTTCGCGAGCACTGCGAGAG

179 ATGACACTCGACAGCCGTGTGGCTGCGGCGGGCGATCTCTTTGTAGCTGTAGTAGGTCAT
MetThrLeuAspSerArgValAlaAlaAlaGlyAspLeuPheValAlaValValGlyHis

239 CAGGCGGACGGGCGTCGATATATCCCGCAGGCGATAGCGCAAGGTGTGGCTGCCATTATT
GlnAlaAspGlyArgArgTyrIleProGlnAlaIleAlaGlnGlyValAlaAlaIleIle

299 GCAGAGGCGAAAGATGAGGCGACCGATGGTGAAATCCGTGAAATGCACGGCGTACCGGTC
AlaGluAlaLysAspGluAlaThrAspGlyGluIleArgGluMetHisGlyValProVal

359 ATCTATCTCAGCCAGCTCAACGAGCGTTTATCTGCACTGGCGGGCCGCTTTTACCATGAA
IleTyrLeuSerGlnLeuAsnGluArgLeuSerAlaLeuAlaGlyArgPheTyrHisGlu

419 CCCTCTGACAATTTACGTCTCGTGGGCGTAACGGGCACCAACGGCAAAACCACGACTACC
ProSerAspAsnLeuArgLeuValGlyValThrGlyThrAsnGlyLysThrThrThrThr

479 CAGCTGTTGGCGCAGTGGAGCCAACTGCTTGGCGAAATCAGCGCGGTAATGGGCACCGTT
GlnLeuLeuAlaGlnTrpSerGlnLeuLeuGlyGluIleSerAlaValMetGlyThrVal

539 GGTAACGGCCTGCTGGGGAAAGTGATCCCGACAGAAAATACAACCGGTTCGGCAGTCGAT
GlyAsnGlyLeuLeuGlyLysValIleProThrGluAsnThrThrGlySerAlaValAsp

599 GTTCAGCATGAGCTGGCGGGGCTGGTGGATCAGGGCGCGACGTTTTGCGCAATGGAAGTT
ValGlnHisGluLeuAlaGlyLeuValAspGlnGlyAlaThrPheCysAlaMetGluVal

659 TCCTCCCACGGGCTGGTACAGCACCGTGTGGCGGCATTGAAATTTGCGGCGTCGGTCTTT
SerSerHisGlyLeuValGlnHisArgValAlaAlaLeuLysPheAlaAlaSerValPhe

719 ACCAACTTAAGCCGCGATCACCTTGATTATCATGGTGATATGGAACACTACGAAGCCGCG
ThrAsnLeuSerArgAspHisLeuAspTyrHisGlyAspMetGluHisTyrGluAlaAla

779 AAATGGCTGCTTTATTCTGAGCATCATTGCGGTCAGGCGATTATTAACGCCGACGATGAA
LysTrpLeuLeuTyrSerGluHisHisCysGlyGlnAlaIleIleAsnAlaAspAspGlu

839 GTGGGCCGCCGCTGGCTGGCAAAACTGCCGGACGCGGTTGCGGTATCAATGGAAGATCAT
ValGlyArgArgTrpLeuAlaLysLeuProAspAlaValAlaValSerMetGluAspHis

899 ATTAATCCGAACTGTCACGGACGCTGGTTGAAAGCGACCGAAGTGAACTATCACGACAGC
IleAsnProAsnCysHisGlyArgTrpLeuLysAlaThrGluValAsnTyrHisAspSer

959 GGTGCGACGATTCGCTTTAGCTCAAGTTGGGGCGATGGCGAAATTGAAAGCCATCTGATG
GlyAlaThrIleArgPheSerSerSerTrpGlyAspGlyGluIleGluSerHisLeuMet

1019 GGCGCTTTTAACGTCAGCAACCTGCTGCTCGCGCTGGCGACACTGTTGGCACTCGGCTAT
GlyAlaPheAsnValSerAsnLeuLeuLeuAlaLeuAlaThrLeuLeuAlaLeuGlyTyr

1089 CCACTGGCTGATCTGCTGAAAACCGCCGCGCGTCTGCAACCGGTTAGCGGACGTATGGAA
ProLeuAlaAspLeuLeuLysThrAlaAlaArgLeuGlnProValCysGlyArgMetGlu

1139 GTGTTCACTGCGCCAGGCAAACCGACGGTGGTGGTGGATTACGCGCATACGCCGGATGCA
ValPheThrAlaProGlyLysProThrValValValAspTyrAlaHisThrProAspAla

1199 CTGGAAAAAGCCTTACAGGCGGCGCGTCTGCACTGTGCGGGCAAGCTGTGGTGTGTCTTT
LeuGluLysAlaLeuGlnAlaAlaArgLeuHisCysAlaGlyLysLeuTrpCysValPhe

1259 GGCTGTGGTGGCGATCGCGATAAAGGTAAGCGTCCACTGATGGGCGCAATTGCCGAAGAG
GlyCysGlyGlyAspArgAspLysGlyLysArgProLeuMetGlyAlaIleAlaGluGlu

1319 TTTGCTGACGTGGCGGTGGTGACGGACGATAACCCCGCGTACCGAAGAACCGCGTGCCATC
PheAlaAspValAlaValValThrAspAspAsnProArgThrGluGluProArgAlaIle

1379 ATCAACGATATTCTGGCGGGAATGTTAGATGCCGGACATGCCAAAGTGATGGAAGGCCGT
IleAsnAspIleLeuAlaGlyMetLeuAspAlaGlyHisAlaLysValMetGluGlyArg

1439 GCTGAAGCGGTGACTTGCGCCGTTATGCAGGCTAAAGAGAATGATGTGGTACTGGTCGCG
AlaGluAlaValThrCysAlaValMetGlnAlaLysGluAsnAspValValLeuValAla

1499 GGCAAAGGCCATGAAGATTACCAGATTGTTGGCAATCAGCGTCTGGACTACTCCGATCGC
GlyLysGlyHisGluAspTyrGlnIleValGlyAsnGlnArgLeuAspTyrSerAspArg

start murF

1559 GTCACGGTGGCGCGTCTGCTGGGGGTGATTGCATGATTAGCGTAACCCTTAGCCAACTTA
ValThrValAlaArgLeuLeuGlyValIleAla

1619 CCGACATTCTCAACGGTGAACTGCAAGGTGCAGATATC
EcoRV

Fig. 7. Nucleotide sequence and deduced amino acid sequence of murE. The sequence covers the 3'-end of the ftsI gene to the unique EcoRV site located past the 3'-end of the murE gene. Boxed sequence may represent the promoter of murE gene. Underlined amino acid residues are putative ATP-binding site of murE gene product. Underlined ATG at position 1,591 is the start codon of murF gene.

ribosome-binding sequence. On the other hand, the GAG at position 165 to 167 may represent a ribosome-binding site, albeit a weak one, for the proposed initiation site. In either case, the deduced molecular masses of the deduced polypeptides are smaller than the reported 56 KDal value for the murE gene product (130).

The murE gene was expressed in the absence of the complete ftsI gene when it was cloned in either orientation into the polylinker region derived from plasmids pUC18 and pUC19. e.g; pJS800 and pJS 900. It is therefore possible that promoter activity resided either within the ftsI gene or in the short interval between ftsI and murE.The only possible -35 and -10 sequence found in these regions were TTGGTG beginning at position 2 and TATTGC beginning at position 23, respectively (the underlined bases correspond to the consensus sequences), with a spacing of 15 bases.

The codon usage in murE summarized in Table 4 was generally characteristic of genes which are weakly expressed. Thus, codon usage was relatively random, and only 4 of the 61 codons were not used. Furthermore, in most cases, NNU was favoured over NNC when N was either U or A, whereas NNC was favoured over NNU when N was either G or C, and these seem to be the rules followed by weakly expressed genes (147).

As indicated in Chapter 1, the enzymes involved in the synthesis of the peptide portion of UDP-N-acetylmuramyl-pentapeptide are ATP-dependent (72, 94). It has been previously reported that enzymes capable of catalyzing either ATP synthesis or ATP hydrolysis exhibit 2 conserved regions designated domains A and B which are proposed to represent ATP-binding sites (148, 149, 150). We analyzed the murE

sequence for these regions. As shown in Fig. 5 (underlined areas) and Table 5, residues 82 through 105 and 366 through 389 from the deduced amino acid sequence of murE appear to represent domains A and B, respectively.

IV. Discussion

In this work, we cloned and sequenced the murE gene encoding the meso-DAP-adding enzyme which is involved in the cytoplasmic stage of peptidoglycan biosynthesis. The murE gene was present on a 4.0 Kb fragment obtained from Clark-Carbon plasmid pLC26-6 (140) by KpnI and EcoRI digestion. This fragment also carried the murF gene. The restriction map for this region Fig. 6 was in agreement with that reported previously (130) except that we found an additional PvuI site within the murE coding region. The nucleotide sequence of the murE gene presented in Fig. 7 shows an ORF of 1,413 base pairs which would encode a 50.6 Kd protein with 471 amino acid residues. I think this is reasonably close to the 56 Kd value reported by Maruyama et al (130). Their value was obtained by sodium dodecyl sulfate-polyacrylamide gel electophoresis after in vitro expression of the cloned murE gene. The codon usage of murE suggests that the gene is weakly expressed. The codon usage of genes for the high molecular weight PBPs 1A, 1B, 2, and 3 have also been reported to have characteristics of weakly expressed genes (151). However, it is not known whether these observations are relevant. It is worth noting that the ftsI gene which lies immediately upstream of murE encodes the PBP 3 protein (137).

Table 4. Codon usage in murE

UUU	Phe	9	UCU	Ser	3	UAU	Tyr	6	UGU	Cys	4
UUC	Phe	1	UCC	Ser	3	UAC	Tyr	5	UGC	Cys	4
UUA	Leu	5	UCA	Ser	2	UAA	---	0	UGA	---	1
UUG	Leu	4	UCG	Ser	2	UAG	---	0	UGG	Trp	6
CUU	Leu	3	CCU	Pro	0	CAU	His	11	CGU	Arg	15
CUC	Leu	7	CCC	Pro	1	CAC	His	8	CGC	Arg	8
CUA	Leu	0	CCA	Pro	3	CAA	Gln	3	CGA	Arg	1
CUG	Leu	29	CCG	Pro	10	CAG	Gln	13	CGG	Arg	0
AUU	Ile	11	ACU	Thr	3	AAU	Asn	5	AGU	Ser	1
AUC	Ile	7	ACC	Thr	10	AAC	Asn	11	AGC	Ser	9
AUA	Ile	1	ACA	Thr	4	AAA	Lys	14	AGA	Arg	0
AUG	Met	12	ACG	Thr	8	AAG	Lys	2	AGG	Arg	0
GUU	Val	7	GCU	Ala	8	GAU	Asp	22	GGU	Gly	10
GUC	Val	7	GCC	Ala	10	GAC	Asp	9	GGC	Gly	24
GUA	Val	9	GCA	Ala	10	GAA	Glu	21	GGA	Gly	4
GUG	Val	20	GCG	Ala	34	GAG	Glu	7	GGG	Gly	5

Table 5. Comparison of putative ATP binding domains of murE gene product and other ATP binding proteins[a]

Protein	Residue	Sequence	Ref.
Domain A			
E. coli murE gene product	82~105	S D N L R L V G V T G - T N G K T T T T Q L L A Q	
E. coli murF gene product	97~120	Q V P A R V V A L T G - S S G K T S V K E M T A A	113
E. coli ATPase β	142~165	K G G K V G L F - G G A. G V G K T V N M M E L I R	138
E. coli ATPase alpha	171~184	R G G R E L I I - G D R G T G K T A L A I D A I I	139
Bovin ATPase β	149~173	k G G K I G L F - G G A G V G K T V F I M E L I N	126
Adenylate kinase	6-30	K K S K I I F V V G G P G S G K G T Q C E K I V Q	140
Consensus sequence[b]		**G** X X X X **G K T** X X X X X X **I/V**	126
Domain B			
E. coli murE gene product	366-389	R D - K G K R P L M G A I A E E F A D V A V V T D	
E. coli murF gene product	348-372	A E M P G Y R V L V V G D M A E L G A E S E A C G	113
E. coli ATPase β	232-255	R D - E G R D V L L F V D N I Y R Y T L A G T E V	138
E. coli ATPase alpha	270-293	R D - R G E D A L I I Y D D L S K Q A V A T R Q I	139
Bovin ATPaseβ	246-270	R D Q E G Q D V L L F I D N I F R F T Q A G S E V	126
Adenylate kinase	107-130	R K - I G Q P T L L L T V D A G P E T M T K R L L	140
Consensus sequence[b]		**R/K** X X X **G** X X X **L** (hydrophobic)4	126

a) The sequences have been aligned to achieve maximum homology. The " - " represent insertions made during optimization. Conserved residues are boxed.

b) The " X " designations in the consensus sequences refer to variable amino acid residues.

The murE gene product is expected to be an ATP binding protein. It has been proposed (148) that ATP binding proteins contain two conserved domains which may represent the actual ATP binding sites. Amino acid sequences coresponding to domains A and B were located in murE, and these are compared in Table 5 with similar sequences previously reported in other ATP binding proteins. The list also includes the recently published murF sequence (131). The G X X X X G consensus sequence in domain A is believed to form a flexible loop structure which may interact with the adenine nucleotide (148). The lysine which follows G X X X X G is considered essential for the interaction with phosphate (152). The consensus sequence of domain B apparently represents a hydrophobic ß-sheet which is located at the back of the nucleotide binding loop (148).

As noted above, the nucleotide sequence of the murF gene has been recently published (131). The last 144 bases of our sequences shown in Fig. 7 are identical to the first 144 bases of the reported murF sequence. Furthermore, the proposed murF ORF starts within the murE ORF at position 1,591 (Fig. 7). The murF gene thus overlaps with the preceding murE gene by one base pair.

It should be noted that we were unable to accurately locate the 5'-end of murE by deletion analysis. Furthermore, the nucleotide sequence does not appear to contain typical E. coli promoter and ribosome binding site. The only possible -35 and -10 promoter sequences found were TTGGTG beginning at position 2 and TATTGC beginning at position 23, respectively (the underlined bases corespond to the consensus sequences), with a spacing of 15 bases. It should also be noted that the murF gene which is located downstream

of murE also appears to be devoid of obvious promoter and ribosome binding sequences. It seems possible that murF and murE are cotranscribed and cotranslated in view of the sequence data discussed above. This is discussed further below.

As mentioned in Chapter 1, there are eight genes involved in peptidoglycan biosynthesis, three genes involved in the cell division process and two cell envelope related genes in the 2-min region of the E. coli genetic linkage map (139). The order of these genes is mraA, mraB, ftsI, murE, murF, murG, murC, ddl, ftsQ, ftsA, ftsZ, envA and secA . In general the average coding capacity of the E. coli chromosome is utilized to the extent of about 70% (153). But considering the number of reported genes, the capacity of the 2-min region is close to being 100% utilized. Many of these genes have now been sequenced. It was reported that the translational terminator of ddl gene is one base pair away from the initiation codon of ftsQ gene (133). Furthermore, the termination codon of ftsQ gene overlaps the initiation codon of ftsA gene by one base pair (133, 154). Note that these arrangements are identical to the murE and murF situation described above. The terminator of ftsA gene is 60 base pairs away from the initiation codon of ftsZ gene (89) and as we have reported above, the murE and ftsI genes are separated by 61 pase pairs. In addition, all of the genes that have been sequenced so far are found transcribed in the clockwise direction. The same polarity and physical proximity of these genes strongly suggest the existence of translational coupling which may guarantee sequential translation of these genes (133). The possible translational coupling between the ddl gene and the ftsQ gene further suggests the translational coregulation of genes

involved in peptidoglycan synthesis and cell division (133). This may not be unexpected since peptidoglycan metabolism is an important part of cell division. Thus, the unusual clustering of these genes in the 2 min region has prompted the speculation that one or more operons may exist in this region (90, 133, 137, 130, 131).

An operon usually contains genes which are functional related. They are usually coordinately regulated through a single promoter at the 5' end of the operon. Although it is far from proven, it is possible that the genes in the 2-min region are arranged as a series of operons. Furthermore, it is possible that some of these operons may contain alternative internal promoters. This suggestion reflects the fact that most of the cloned genes in this region appear to have their own promoters and to be able to be expressed independent of the presence of upstream genes. These internal promoters could be activated under certain conditions to facilitate differential regulation of gene expression within an operon (155). Examples of this form of regulation have been described (156, 157, 158). Of course, it is also possible that there are no operons in the 2-min region. Nevertheless, the arrangement of the individual genes suggests some form of novel regulation.

The operon concept is supported by preliminary data in several reports. For example, the ddl gene cloned into a phage lambda single copy vector required an upstream sequence from the murG-murC region for biologically effective levels of transcription, although it was adequately expressed without the presence of this upstream sequence when it was cloned in either direction into multicopy vectors (90). Scanning the sequence of ftsI, murE, murF region we found no

apparent transcriptional terminator structure after the translational termination codon of each of the three genes. It was also reported that there is no transcriptional terminator within the ddl, ftsQ, ftsA, ftsZ region (159,133). This indicates that transcription initiated upstream of the ddl gene could continue through to ftsZ. Similarly, we did not find transcriptional terminators in the ftsI, murE, and murF region suggesting that transcription initiated upstream of the ftsI gene could continue through to at least murF. Based on the above discussion, it is clear that the control of the genes in the 2-min region is poorly understood, and this area deserves further study.

CHAPTER 3. ISOLATION AND PRELIMINARY CHARACTERIZATION OF EXTRAGENIC SUPPRESSORS OF THE murE MUTATION

I. Introduction

A suppressor mutation is a second site mutation which can correct the phenotypic defect caused by an initial mutation (141). Intragenic suppressors are those second site mutations which occur within the same mutant gene that they suppress. An intragenic suppressor mutation either causes a frame shift of the open reading frame of the gene or creates an amino acid substitution in the gene product to restore the function of the protein. Extragenic suppressors are those second site mutations which occur outside of the mutant gene. There are several possible mechanisms for extragenic suppression. Extragenic suppression can be achieved by: (i) altering the protein synthesis machinery through a mutation in tRNA; (ii) activating a new pathway to bypass the blockage caused by the original mutation; (iii) inducing overproduction of the mutant gene product; or (iv) inducing a change in a protein which interacts with the original mutant protein (142).

As noted in the preceding chapters, the murE gene encodes an essential enzyme involved in cytoplasmic step of peptidoglycan biosynthesis. Although the enzyme and its coding gene have been known for almost two decades (58), the regulation of the gene expression and the enzymatic activity are still not understood. In this

chapter, I will describe preliminary studies on extragenic suppressor mutations which suppress the temperature-sensitive phenotype of the murE16 mutation.

II. Material and Methods

1. Bacteria

The E. coli K-12 strains used in this study are described in Table 6.

2. Preparation of P1 Phage Lysate

A stationary phase bacterial culture was diluted 1:100 in 5 ml of Tryptic Soy Broth (TSB) medium and incubated at 30° C or 37° C depending on the bacterial host strain used. After about one doubling time (60 min at 30° C, 30 min at 37° C), 0.05 ml of phage P1 vir preparation (10^{10} phage per ml), along with 0.03 ml of 1 M CaCl were added. The culture was further incubated for another 3 hours until the cells were lysed. Then the culture was centrifuged at 6000 rpm for 10 min. The supernatant was collected and vortexed in the presence of a few drops of chloroform.

3. P1 Transduction

The procedure for P1 transduction was a modified version of Miller's method(143). Four ml of overnight recipient cell culture was centrifuged at 10,000 rpm for 3 min. The pellet was suspended in half volume of MC solution (10mM MgSO and 5 mM CaCl). Recipient cell suspension (0.5 ml) was mixed with one tenth volume of P1 lysate.

Table 6 E. coli K-12 strains.

Strain	**Genotype** [a]	**Source**
CP78	leuA arg his thr strep^R thi	Laboratory Stock
VC1025	leu:Tn10 arg his thr thi strep^R	This study
VC1026	leu:Tn10	This study
VC1027	leu$^+$ murE16(ts) Tn10	This study
VC1047	leu$^+$ murE16(ts)	This study
VC1062	leu$^+$ murE16(ts) sup?	This study
VC1063	leu$^+$ murE16(ts) sup?	This study

a) For genetic symbols see Bachmann (118).

The mixture was then incubated at 37° C for 20 min. The infection was terminated by adding 0.025 ml of 0.5 M EGTA to the mixture. Finally 0.1 ml of transduction mixture was plated onto selective medium.

4. Plating Efficiency Assay

Stationary phase cultures grown in TSB were serially diluted in saline. Dilutions were plated in duplicate onto Tryptic Soy Agar (TSA), Nutrient Agar (NA), and NA+1% NaCl medium. The plates were divided into two sets. One set was incubated at 42° C, and the other was incubated at 30° C. After 48 hours, the colonies on the individual plates were counted. The ratio of the plate count at 42° C to the plate count at 30° C was used to express plating efficiency. The figure represents the relative ability of the bacterial strains to form colonies at 42° C; i.e., their relative temperature sensitivity.

5. Growth Studies of Temperature-sensitive Mutants

An overnight culture grown in TSB at 30° C was diluted 100 fold in fresh TSB. The culture was incubated in a 30° C waterbath shaker. Growth was followed in a Klett-Summerson colorimeter with a green filter. When the culture density reached about 5×10^8 cells per ml, a portion was shifted to 42° C water bath shaker.

6. Transposon Random Insertion

Phage lambda 849 carries the modified transposon designated mini-Tn10 (144). Strain VC1062 was infected with lambda 840 at a multiplicity of infection of 0.2 as described previously (144). The

mixture was incubated at 37° C for 1 hr and then plated on LB agar containing tetracycline and 0.0025 M sodium pyrophosphate to select cells which gained transposon insertions in their chromosomes. The tetracycline-resistant colonies were washed off the plate with a small volume of saline. The cell suspension was then heavily inoculated into TSB medium. The cell culture was grown overnight for the P1 lysate preparation next day.

III. Results

1. Characterization of the murE Mutant

Strains VC1026 ($murE^+$) and VC1047 (murE16 ts) are isogenic except for the allelic state of the murE locus. Fig. 8-A shows that the murE16 ts allele had no apparent effect on growth at 30° C in TSB, and strain VC1026 and VC1047 both exhibited identical doubling times of 60 min. On the other hand, the doubling time of VC1026 in TSB decreased to 45 min at 42° C whereas VC1047 underwent lysis within 1 doubling time under the same conditions (Fig. 8-B).

VC1026 plated efficiently on all media tested (Table 7). VC1047 was clearly temperature-sensitive on NA but exhibited a relatively high plating efficiency (1.5×10^{-4}) on TSA (Table 7). The difference was very likely due to the higher salt concentration of TSA since the addition of 1% NaCl to NA resulted in a 10^6-fold increase in the plating efficiency. Therefore, the temperature-sensitive phenotype associated with the murE16 allele could be partially suppressed by increasing the osmolarity of the growth medium.

2. Isolation and Preliminary Characterization of Extragenic Suppressors of murE

As indicated in Table 7, VC1047 exhibited a relatively high efficiency of plating on TSA, i.e., higher than expected to account simply for spontaneous reversion frequency (this would be in the order of about 10^{-6} at best). It was possible that this could be at least partly due to the occurrence of double mutants carrying extragenic suppressor mutations which suppressed the murE16 allele. Colonies from TSA plates incubated at 42° C were therefore tested for the

occurrence of such suppressor mutations. Two temperature-resistant isolates, designated strains VC1062 and VC1063, were characterized. The original murE16 allele could be demonstrated in both strains by cotransduction with the closely linked leu^+ locus serving as a directly selectable marker. For this purpose, strain CP78 (leu^- $murE^+$) was used as a recipient. With VC1062 and VC1063 as donors, 40% of the leu^+ transductants were shown to acquire the temperature-sensitive lysis phenotype of the murE16 allele. The cotransduction frequency correlates well with the reported 0.5 min linkage between the leu and murE loci. Thus, the temperature-resistance of VC1062 and VC1063 was apparently due to extragenic suppression.

Strains VC1062 and VC1063 grew normally in TSB at 30° C (Fig. 8-A). At 42° C, the 2 strains had different phenotypes (Fig. 8-B). VC1062 grew with a reduced doubling time of 60 min but did not apparently lyse. VC1063 showed an initial 30 min of apparent normal growth which was then followed by a decrease in optical density. This was apparently due to partial lysis of the culture. Eventually, the culture attained steady state growth at a doubling time of longer than 120 min. These results indicate that the extragenic suppressor mutation in VC1062 completely suppresses the lysis phenotype of the murE16 allele but strain VC1063 carried a mutation which conferred only partial suppression of murE16.

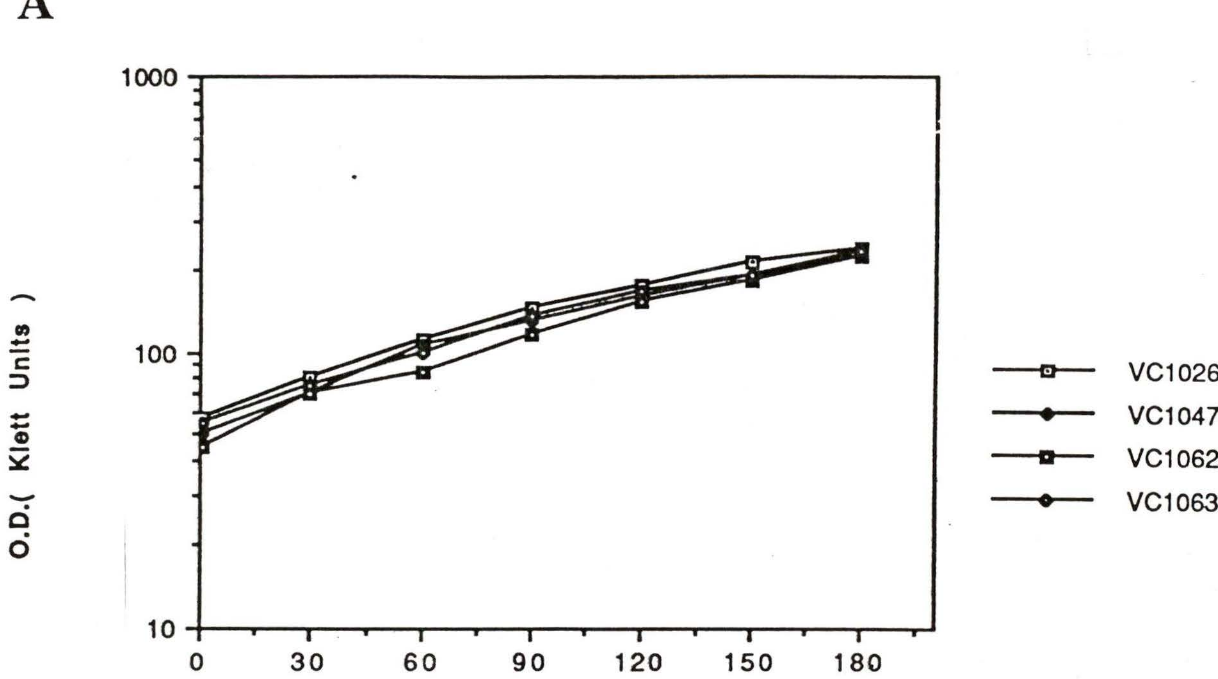
The plating efficiencies of strains VC1062 and VC1063 are shown in Table 7. VC1062 was clearly temperature-resistant on TSA and NA containing 1% NaCl but was temperature-sensitive on NA. VC1063 was markedly, but not completely, temperature-resistant on

Table 7. Plating efficiency study[a] of the murE$^+$ strain VC1026, the murE mutant strain VC1047, and the murE suppressor strains VC1062 and VC1063

	TSA Plate	NA Plate	NA +1% NaCl
VC1026	1.0	1.0	1.0
VC1047	1.5×10^{-4}	1.4×10^{-8}	2.0×10^{-2}
VC1062	1.0	4.7×10^{-8}	1.1
VC1063	5.0×10^{-2}	1.1×10^{-9}	9.4×10^{-1}

a) Plating efficiency assays were performed by plating serially diluted overnight TSB culture onto specific medium. The plates were divided into two sets, one set were incubated at 30° C, and the other were incubated at 42° C for 48 hr. The figures in the table represent the ratio of the number of the viable colony on 42° C plate to that on 30° C plate.

A



B

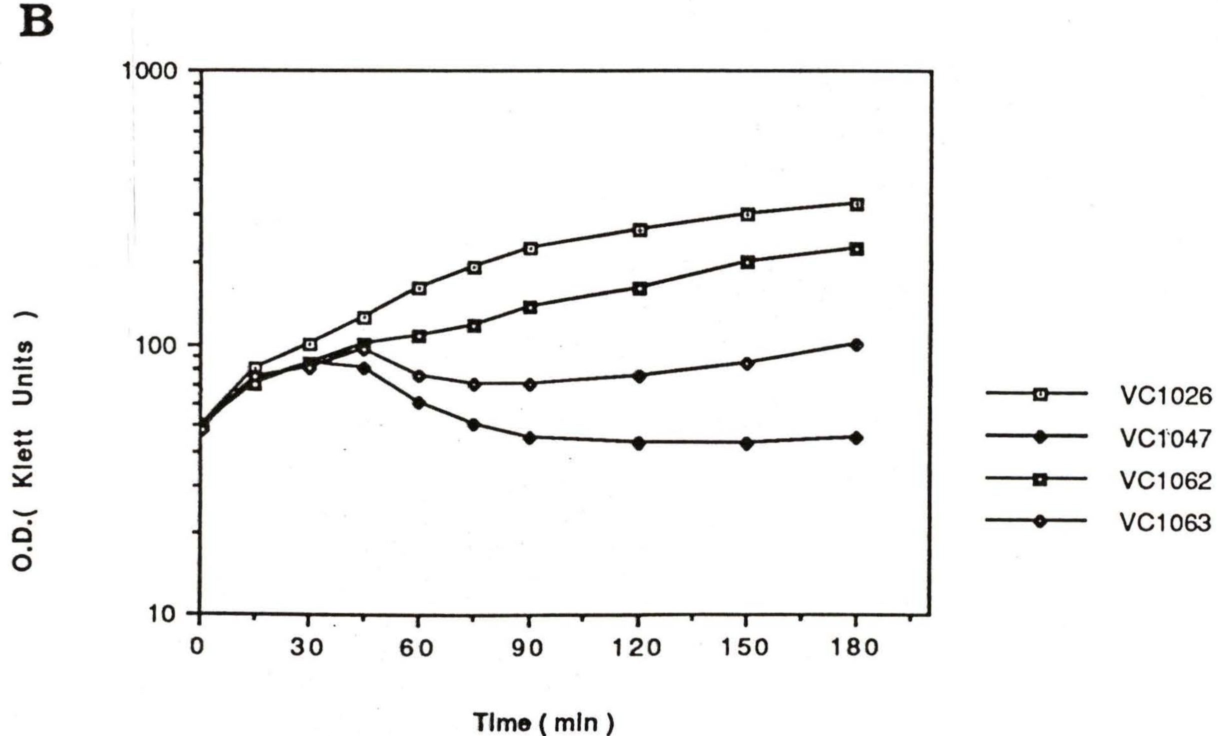


Fig. 8. Growth curves of the murE+ strain VC1026, the murE mutant strain VC1047, and the murE suppressor strains VC1062 and VC1063 in TSB medium. A: Bacterial cultures were grown at 30° C. B: Bacterial cultures were grown at 30° C to 50 klett units. The cultures were then shifted to 42° C at 0 min and incubated for another 180 min.

TSA. Furthermore, VC1063 was completely temperature-resistant on NA containing 1% NaCl but was temperature-sensitive on NA. When these results are compared with those obtained with VC1047 (murE16), it is clear that the effect of the extragenic suppressor mutation in both VC1062 and VC1063 on the restoration of growth at 42° C was dependent on medium osmolarity, i.e., the suppressors effectively restored growth when the medium had a relatively high osmolarity (TSA or NA plus 1% NaCl) but not when the medium was low in osmolarity (NA).

An unsuccessful attempt was made to locate the extragenic suppressor in VC1062 on the genetic linkage map of E. coli. The approach involved the preparation of a pool of random transposon Tn10 insertions in VC1062. The pool contained 16,000 random insertions. This should have been sufficient to include numerous insertions linked to the extragenic suppressor. These insertions were screened for linkage to the suppressor by phage P1-mediated generalized transduction into the murE mutant strain, VC1047. Tetracycline-resistant (encoded by transposon Tn10) transductants were screened for temperature resistance. A total of 50 independent transductants which were both tetracycline-resistant and temperature-resistant were re-screened. Phage P1 lysates prepared on these transductants were used to attempt the cotransduction of tetracycline resistance and temperature-resistance into VC1047 (murE16). Linkage between the Tn10 insertion and the extragenic suppressor activity could not be demonstrated during this re-screening procedure, and the original transductants were therefore apparently due to false positive results.

IV. Discussion

The isolation and characterization of extragenic suppressor mutants has proven to be a useful approach in the analysis of complex biochemical phenomena such as cell division and DNA replication (142, 145). This approach is based on the hypothesis that a mutation which inactivates one protein may be compensated for by another mutation affecting a second protein which interacts directly or indirectly with the original mutant protein. The method has been valuable in the identification of the proteins involved in a complex process. We have shown here that it is possible to obtain extragenic suppressor mutations which suppress the temperature-sensitive lysis phenotype of the murE16 allele. In one case (VC1062), the suppression appeared to be complete, and in a second case, only partial suppression was observed.

Osmoremedial mutations are mutations which can be suppressed by increasing the osmolarity of the growth medium (reviewed in reference 146). The phenomenon is apparently very common and occurs with all classes of mutations, including temperature-sensitive mutations. The mechanism has not been determined, and it is likely that several different mechanisms may be involved. Internal osmolarity is directly related to external osmolarity in bacteria. Therefore, one possible mechanism may involve the stabilization of a temperature-sensitive protein by the increased internal solute concentration in cells grown in high osmolarity media.

The murE16 mutation was at least partially osmoremedial. For example, colony formation on NA at 42° C could be partially restored by the addition of 1% NaCl to the medium. It is interesting that both of

the suppressor mutations characterized in this study functioned as suppressor of murE16 only in high osmolarity media. Thus, both apparently were osmoremedial mutations.

Attempts to map the suppressor mutations were unsuccessful. The main problem encountered was due to the fact that the suppressor activities were expressed only in high osmolarity media. Under these conditions, the murE16 mutation was also partially suppressed, and this resulted in a high background of temperature-resistant colonies. It was therefore impossible to distinguish those temperature-resistant colonies which were specifically due to the activity of a suppressor mutation. Other approaches are clearly necessary to permit genetic mapping of the suppressor loci. The proteins affected by the suppressor mutations presumably interact directly or indirectly with the murE16 gene product, and it is obviously necessary to identify the suppressor gene products in order to determine the mechanism of suppression.

REFERENCES

1. Burman, L. G, and J. T. Park (1983) Journal of Bacteriology. 155:447-453.
2. Glauner, B, and U. Schwarz (1983) In " The Target of Penicillin ". (R. Hakenbeck, J-V. Holtje and H. Labischinski. eds) pp29-34. de Gruyter. Berlin-New York.
3. Gmeiner, J., P. Essig, and H. H. Martin (1982) FEBS Letters. 138:109-111.
4. Holtje, J-V., D. Mirelman, N. Sharon, and U. Schwarz (1975) Journal of Bacteriology. 124:1067-1076.
5. Schleifer, K. H., and O. Kendler (1972) Bacteriological Review. 36:407-477.
6. Braun, V., and K. Rehn (1969) European Journal of Biochemistry. 10:426-438.
7. Braun, V., and K. Hantke (1974) Annual Review of Biochemistry. 43:89-112.
8. Primosigh, J., H. Petzer, D. Maass, and W. Weidel (1961) Biochimica et Biophysica Acta. 46:68-80.
9. Glauner, B., J-V. Holtje, U. Schwarz. (1988) Journal of Biological Chem. 263(21):10088-10095.
10. Oldmixon, E. H., S. Glauser, and M. L. Higgins (1974) Biopolymer 13:2037-2060.
11. Formanek, H., S. Formanek, and H. Wawra (1974) European Journal of Biochemistry. 46:279-294.
12. Barnickel, G., D. Naumann, H. Bradaczek, H. Labischinski. (1983) In " The Target of Penicillin ". (R. Hakenbeck. J-V. Holtje and H. Labischinski. eds.) pp61-66. de Gruyter. Berlin-New York.
13. Labischinski, H. et al. (1983) In " The Target of Penicillin ". (R. Hakenbeck. J-V. Holtje and H. Labischinski. eds.) pp49-54. de Gruyter. Berlin- New York.
14. Lapidot, A. et al. (1983) In " The Target of Penicillin ".

(R. Hakenbeck. J-V. Holtje and H. Labischinski. Eds) pp 79-84. de Gruyter. Berlin- New York.

15. Schwarz, U. et al. (1988) In " Antibiotic Inhibition of Bacterial Cell Surface Assembly and Function ". pp33-39. American Society of Microbiology. Washington DC.

16. Park, J. T. (1987) The Murein Sacculus in " Escherichia. coli and Salmonella. typhimurium; Cellular and Molecular Biology " (Frederick. C Neidhardt ed.) Am. Soc. Microbiology Washington. DC. pp23-30.

17. Hobot, J. A., E. Carlemal, W. Villiger, and E. Kellenberger (1984) Journal of Bacteriology. 160:143-152.

18. Rosenbush, J. P. (1974) Journal of Biological Chemistry. 249:8019-8029

19. Bayer, M. E. (1979) In M. Inouye (ed.), " Bacterial Outer Membranes " pp167-202. John Wiley. & Sons, Inc,. New York.

20. Greenblatt, J., R. J. Boackle, J. H. Schwab (1978) Infection and Immunity. 19:296-303.

21. Regan, D., P. Cohen, J. H. Schwab, W. J. Cromartie (1982) Proceeding Federal Amarican Socity of Experimental Biology. 41:338

22. Schleifer, K. H., R. M. Krause (1971) Journal of Biological Chemistry. 245:986-993.

23 Schleifer, K. H., H. P. Seidl (1977) In Schlessinger. D (ed) " Microbiology 1977 " pp339-343. Amarican Socity of Micro-biology. Washington. DC.

24. Holton, J. B., J. H. Schwab (1966) Journal of Immunology. 96:134-138.

25. Dziarski, R. (1979) Infection and Immunity. 26:508-514.

26. Leclere, C., E. Bourgeois, E. Chedid (1982) European Journal of Immunology. 12:249-252.

27. Stewart-Tull, D. E. (1988) Progressive Drug Research. 32:305-328.

28. Smith, S. M., R. Burrell, I. S. Snyder (1978) Infection and Immunity. 22:568-574.

29. Kotani, S., H. Takada, M. Tsujimoto et al. (1981) In friedman, H. T. W. Klein, A. Szentivanyi. (eds). Immunomodulation by Bacteria and Their Products. pp231-273. Plenum Press. New York.

30. Chetty. C., and J. H. Schwab (1984) In " Handbook of Endotoxin " pp376-408. Vol. 1. Chemistry of Endotoxin, (I. I. Rietschet ed.) Elsevier Science Publishers B. V.

31. Heymer, B., E.T. Rietschel (1977) In Schlessiner. D (ed)" Microbiology 1977 ". pp344-349. Amarican Socity of Microbiology.

32. Schwab,J. H., W. J. Cromartie (1957) Journal of Bacteriology. 74:673-679.

33. Cromartie, W. J., J. G. Craddock (1966) Science. 154:285-287.

34. Tod et al (1987) British Journal of Experimental Pathology. 68:793-802.

35. Krueger, J., J. Bacsik, and J. Garcia-Arraras. (1980) Amarican Journal of Physiology. 238:E116-E123.

36. Krueger, J., J. Pappenheimer, and M. Karnovsky (1982) Journal of Biological Chemistry. 257:1664-1669.

37. Krueger, J., J. Pappenheimer, and M. Karnovsky (1984) Journal of Biological Chemistry. 259:J12659

38. Krueger, J., R. Rosenthal, S. Martin. (1987) Brain Research. 403:249-257.

39. Krueger, J., R. Rosenthal, S. Martin. (1987) Brain Research. 403:258-265.

40. Kady, A. B., J. Krueger, (1989) Infection and Immunity. Feb:396-403.

41. Dinarello, C. A. (1984) Review of Infectious Diseases. 6:51-95.

42. Rogers, H. J., H. R. Perkins, and J. B. Ward. (1980) " Microbiol Cell Walls and Membranes " Chapman & Hall Ltd. London.

43. Tipper, D. J., and A. Wright (1979) The structure and Biosynthesis of Bacterial Cell Walls. pp291-426 In J. R. Sokatach and L. N. Orastein. (ed.) " The Bacteria ". Vol VII. Academic Press Inc. New York.

44. Higashi, Y., J. L. Strominger, and C. C. Sweeley. (1967) Journal of Biological Chemistry. 245:3697-3702.

45. Wright, A., M. Dandert, P. Fennessy and P. W. Robbins. (1967) Proceeding National Academy of Science. USA 57:1798-1803.

46. Umbreit, J. N., and J. I. Strominger (1972) Journal of Bacteriology. 112:1306-1309.

47. Neuhaus, F. C. (1972) Acc. Chemistry Research. 4:297-303.

48. Nakagawa, J., S. Tamaki, and M. Matsuhashi. (1979) Agriculture Biological Chemistry. 43:1379-1380.

49. Fumitoshi, J., K. Mitsui, S. Tamaki, and M. Matsuhashi. (1980) Biochemical and Biophysical Research Communication. 97:287-293.

50. Ishino, F., and M. Matsuhashi. (1981) Biochemical and Biophysical Research Commun. 101:905-911.

51. Ishino, F., K. Mitsui, S. Tamaki and M. Matsuhashi (1980) Biochemical and Biophysical Research Communication. 97:287-293.

52. Ishino, F., S. Tamaki, B. G. Spratt., and M. Matsuhashi. (1982) Biochemical Biophysical Research Communication. 109:689-696.

53. Van Heijenoort, Y., M. Derrien., and J. Van Heijenoort. (1978) FEBS Letters 89:141-144.

54. Ishiguro, E. E., W. Kusser. (1988) In " Antibiotic Inhibition of Bacterial Cell Surface Assembly and Function". pp189-194 Am. Soc. Microbiology. Washington D. C.

55. Holtje, J-V., and U. Schwarz. (1985) Biosynthesis and Growth of Murein Sacculus. pp77-118. In " Molecular Cytology of E. coli " Academic Press.

56. Ishiguro, E. E., and W. D. Ramey (1978) Journal of Bacteriology. 135:766-774.

57. Ishiguro, E. E. (1983) In " The Target of Penicillin " (R. Hakenbeck. J-V. Holtje and H. Labischinski. eds.) pp631-636. de Gruyter. Berlin-New York.

58. Lugtenberg, E. J. J., and Kam. A. Van Schijudel-Van. (1972) Journal of Bacteriology. 110:35-40.

59. Broome-Smith, J. K., I. Ioannidis, A. Edelman, and B. G. Spratt. (1988) Nucleic Acids Research 16:1617.

60. Spratt, B. G. (1977a) European Journal of Biochemistry. 72:341-352.

61. Spratt, B. G. (1977b) In Schlessinger, D. (ed) " Microbiology 1977 ". pp182-190. Amarican Socity of Microbiology.

62. Suzuki, H., Y. Nishimura, and Y. Hirota (1978) Proceeding National Academy of Science. USA. 75:664-668.

63. Tamaki, S., S. Nakajima, and M. Matsuhashi (1977) Proceeding National Academy of Science. USA. 74:5472-5476.

64. Spratt, B. G. (1975) Proceeding National Academy of Science. USA. 72:2999-3003.

65. Tamura, T., H. Suzuki, et al. (1980) Proceeding National Academy of Science. USA. 77:4499-4503.

66. Spratt, B. G., and A. B. Pardee (1975) Nature (London) 254:516-517.

67. Ghuysen, J-M., and G. D. Schockman (1973) In " Bacteriol Membranes and Walls " 1st ed, pp37-130, Dedder, New York.

68. Schwarz, U., A. Ryter, et al (1975) Journal of Molecular Biology. 98:749-759.

69. De Pedro, M. A., and U. Schwarz (1981) Proceeding National Academy of Science. USA. 78:5856-5860.

70. Glaser, L. (1973) Annual Review of Biochemistry. 92:91-112.

71. Wolf-Watz, H., and S. Normark (1976) Journal of Bacteriology. 128:580-586.

72. Daneo-Moore, L., and G. P. Shockman (1977) In " Cell Surface Reviews " Vol. 4. pp597-715 (G. Poste and G. L. Nicolson, eds), Elsevier North-Holland, Amsterdam.

73. Miyakawa, T. H., H. Matsuzawa, M. Matsuhashi. (1972) Journal of Bacteriology. 112:950-958.

74. Wu, H. C., and Venkateswaran (1974) Annual New York Academy of Science. 235:587-592.

75. Lutkenhaus, J. F., H. Wolf-Watz, and W. D. Donachie (1980) Journal of Bacteriology. 142:615-620.

76. Lutkenhaus, J. F., H. C. Wu. (1980) Journal of Bacteriology. 143:1281-1288.

77. Fletcher, G., C. A. Irwin, J. M. Henson, C. Fillingim, M. M. Malone, and J. R. Walker. (1978) Journal of Bacteriology. 133:91-100.

78. Dominique, Mengin-Lelreulx., C. Michaud, C. Richaud et al. (1988) Journal of Bacteriology. 170:2031-2039.

79. Lugtenberg, E. J. J., and A. van Schijindel-van Dam (1972) Journal of Bacteriology. 110:41-46.

80. Wijsman, H. J. W. (1972) Journal of Bacteriology. 110:789-790.

81. Wijsman, H. J. W. (1972) Genetic Research. 20:269-277.

82. Wijsman, H. J. W. (1972) Genetic Research. 20:65-74.

83. Lugtenberg, E. J. J., and A. van Schijindel-van Dam (1972) Journal of Bacteriology. 113:96-104.

84. Maruyama, I. N., A. Yamamoto, T. Maruyama, and Y. Hiroto. (1983) In "The Target of Penicillin " (R. Hakenbeck, J-V. Holtje and H. Labischinski eds) pp393-408. de Gruyter. Berlin-New York.

85. Walsh, Christopher. T. (1989) Journal Biological Chemistry. 264:2393-2396

86. Spratt, B. G., A. Boyd, and N. Stoker. (1980) Journal of Bacteriology. 143:569-581.

87. Tamaki, S., H. Matsuzawa, and M. Matsuhashi (1980) Journal of Bacteriology. 141:52-57.

88. Iwaya, M., R. Goldman, D. J. Tipper, B. Feingold. (1978) Journal of Bacteriology. 136:1143-1158.

89. Spratt, B. G. (1978) Nature. 274:713-715.

90. Takeda, Y., A. Nishimura, M. Nishimura, M. Yamada, S. Yasuda, H. Suzuki, and Y. Hirota (1981) Plasmid 6:86-98.

91. Suzuki, H., Y. Nishimura, and Y. Hirota. (1978) Proceeding National Academy of Science. U.S.A. 75:664-668.

92. Matsuhashi, M., Y. Tamaki, I. N. Maruyama, S. Tamaki, Y. Nishimura, H. Suzuki, U. Ogino, and Y. Hirota. (1977) Proceeding National Academy of Science. USA. 74:2976-2979.

93. Nishimura, Y., H. Suzuki, Y. Hirota, and J. T. Park. (1980) Journal of Bacteriology. 143:531-534.

94. Matsuhashi, M., S. Tamaki, S. J. Curtis, and J. L. Strominger. (1979) Journal of Bacteriology. 137:644-647.

95. Tomioka, S., T. Nikaido, T. Miyakawa, and M. Matsuhashi. (1983) Journal of Bacteriology. 156:463-465.

96. Lida, K., Y. Hirota, and U. Schwarz. (1983) Molecular and General Genetics. 189:215-221.

97. Suzuki, H., Y. Nishimura, H. Iketani, J. Campisi, A. Hirashima, M. Inouye, and Y. Hirota. (1976) Journal of Bacteriology. 127:1494-1501.

98. Nakamura, M., and M. Inouye (1979) Cell 18:1109-1117.

99. Hirota, Y., H. Suzuki, Y. Nishimura, and S. Yasuda. (1977) Proceeding National Academy of Science. USA. 74:1417-1420.

100. Inouye, S., N. Lee, M. Inouye, H. C. Wu, H. Suzuki, Y. Nishimura, H. Iketani, and Y. Hirota. (1977) Journal of Bacteriology. 132:308-313.

101. Lin, J. J. C., H. Kanazawa, J. Ozols and H. C. Wu. Proceeding National Academy of Science. USA. (1978) 75:4891-4895.

102. Rotering, H., and V. Braun. (1977) FEBS Letters. 83:41-44.

103. Wu, H. C., C. Hou, J. J. C. Lin, and D. W. Yem. (1977) Proceeding National Academy of Science. 74:1388-1392.

104. Yem, D. W., and H. C. Wu. (1977) Journal of Bacteriology. 131:759-764.

105. Yem, D. W., and H. C. Wu. (1978) Journal of Bacteriology. 133:1419-1426.

106. Nishimura, Y., H. Suzuki, Y. Hirota, and J. T. Park. (1980) Journal of Bacteriology. 143:531-534.

107. Spratt, S. K., C. L. Ginsburgh, and W. D. Nunn. (1981) Journal of Bacteriology. 146:1166-1169.

108. Iwaya, M., R. Goldman, D. J. Tipper, B. Feingold (1978) Journal of Bacteriology. 133:196-202.

109. Matsuzawa, H., K. Hagakawa, T. Sato, and K. Imahori. (1973) Journal of Bacteriology. 115:436-442.

110. Salmond, G. P. C., J. F. Lutkenhaus, and W. D. Donachie (1980) Journal of Bacteriology. 144:438-440.

111. Dai, D., and E. E. Ishiguro (1988) Journal of Bacteriology. 170:2197-2201.

112. Maruyama, I. N., A. H. Yamamoto, and Y. Hirota (1988) Journal of Bacteriology. 170:3786-3788.

113. Parquet, C., B. Flouret, D. Mengin-Lecreulx, and J. van Heijenoort. (1989) Nucleic Acid Research. Vol. 17 (13):5379.

114. Robinson, A. C., D. J. Kenan, J. Sweeney and W. D. Donachie (1986) Journal of Bacteriology. 167:809-817.

115. Broome-Smith, J., A. Edelman, and B. G. Spratt (1983) In " The Target of Penicillin " (R. Hakenbeck, J-V. Holtje and H. Labischinski eds) pp403-408. de Gruyter. Berlin-New York.

116. Nakamura, M., I. N. Maruyama, M. Spratt, J. Kato, H. Suzuki, and Y. Hirota (1983) Molecular General Genetics. 191:1-9.

117. Keck, W., B. Glauner, U. Schwarz, J. K. Broome-Smith, and B. G. Spratt. (1985) Proceeding National Academy of Science. USA. 82:1999-2003.

118. Bachmann, B. J. (1983) Microbiological Review. 47:180-230.

119. Clarke, L., and J. Carbon (1976) Cell 9:91-99.

120. Birnboim, H. C., and T. Doly (1979) Nucleic Acids Research. 7:1513-1523.

121. Maniatis, T., E. F. Fritsch and J. Sambrook (1982) In " Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory. Cold Spring Harbor. New York.

122. Hanahan, D. (1983) Journal of Molecular Biology. 166:557-580.

123. Ahmed, A., (1985) Gene. 39:305-310.

124. Sanger, F., S. Nicklen, and A. R. Conlson (1977) Proceeding National Academy of Science. 74:5403-5467.

125. Grosjean, H., and N. Fiers. (1982) Gene. 18:199-209.

126. Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay (1982) EMBO. Journal. 1:945-951.

127. Cross, R. L. (1981) Annual Review of Biochemistry. 50:681-714.

128. Matsuoka, I., T. Watanabe, and Y. Tonomura (1981) Journal of Biochemisty. 90:967-989.

129. Asoh, S., H. Matsuzawa, F. Ishino, J. L. Strominger, M. Matsuhashi. and T. Ohta (1986) European Journal of Biochemistry. 160:231-238.

130. Pai, E. G., W. Sachsenheimer, R. H. Schirmer, and G. E. Schultz (1977) Journal of Molecular Biology. 114:27-45.

131. Neidhardt, F., V. Vanghu, T-A. Phillips, and P. L. Black. (1983) Microbiological Review. 47:231-284.

132. Beall, B., and J. Lutkenhaus. (1987) Journal of Bacteriology.

169:5408-5415.

133. Taylor, W. E., D. B. Straus, A. D. Grossman, Z. F. Burton, L. A. Gross, and R. R. Burgess. (1984) Cell. 38:371-381.

134. Qing-Ming & Lutkenhaus. (1985) Journal of Molecular Biology. 184:399-412.

135. Barry, G., C. L. Squires, and C. Squires. (1979) Proceeding National Academy of Science. U. S. A. 76:4922-4926.

136. Lee, J. S., G. An, and J. D. Friesen (1981) Cell. 25:251-258.

137. Robinson, A. C., D. J. Kenan, G. F. Hatfull, N. F. Sullivan, R. Spiegelberg, and N. D. Donachie 1984. Journal of Bacteriology 160:546-555.

138. Saraste, M., A. Eberle, N. J. Gay, M. J. Runswick, J. E. Walker. (1981) Nucleic Acids Research. USA. 95:5287-5296.

139. Gay, N. J., and J. E. Walker. (1981a) Nucleic Acids Research. 9:2187-2194, (1981b) 9:3919-3926.

140. Pai, E. G., W. Sachsenheimer, R. H. Schimer, and G. E. Schultz. (1977) Journal of Molecular Biology. 114:37-45.

141. Lewin, B. In " Gene II " (B. Lewin ed.) John Wiley & Sons. New York.

142. Botsein, D. (1982) Annual Review of Genetics. 16:61-83.

143. Miller, J. H. (1972) " Experimental Molecular Genetics " Cold Spring Harbor Laboratory. Cold Spring Harbor. N. Y.

144. Way, J. C., M. A. Davis, D. Morisato, D. E. Roberts, and N. Kleckner. (1984) Gene 32:369-379.

145. Katayama, T., Y. Murakami, C. Wada, H. Ohmori, T. Yura, and T. Nagata. (1989) Journal of Bacteriology. 171:1485-1491.

146. Csonka, L. N. (1989) Microbiological Review. 53:121-147.

VITA

Surname: TAO Given Names: JING-SONG

Place of Birth: SHANGHAI P. R. CHINA Date of Birth: Jun. 3. 1959

Educational Institutions Attended:

University of Victoria	1987 to 1990
Shanghai Chinese Traditional Medical College	1978 to 1982

Degrees Awarded:

B. Med.	Shanghai Chinese Traditional Medical College	1982

Publications:

Nucleotide sequence of the murE gene of Escherichia coli
Canadian Journal of Microbiology. Vol.35 (1989) 1051-1054

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Author

JING-SONG TAO

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