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ABSTRACT

Five new genes apparently involved in the metabolism of cell wall peptidoglycan by Escherichia coli are described. One of these, designated murH, was mapped at 99 min on the E. coli linkage map. The murH1 mutant exhibited temperature-sensitive (ts) growth which was associated with a block in a late step in peptidoglycan synthesis and with peptidoglycan hydrolase-mediated lysis at the restrictive temperature. The murH locus could not be cloned in multicopy vectors but was readily cloned in a single copy phasmid vector derived from phage λ . The instability of murH in multicopy prevented its further characterization. As an alternative approach to characterizing the murH function, extragenic mutations which suppressed the murH1 ts lysis phenotype were isolated. One suppressor mutation, designated smhA1, (25 min on the genetic linkage map) restored temperature resistance in murH1 mutants but otherwise had no distinguishable phenotype. A second extragenic murH1 suppressor, smhB1 (13 min), conferred a ts lysis phenotype by itself. Interestingly, a combination of murH1 and smhB1 resulted in cosuppression of their lysis phenotypes. The suppressor activities of the smhA1 and smhB1 alleles were relatively specific in that they failed to

suppress lysis caused by either mutational (murL or murF) or antibiotic-induced blocks in peptidoglycan synthesis. Two additional ts lysis mutations, lytD1 (mapped at 13 min) and lytE1 (25 min), arose spontaneously in smhB1 and smhA1 backgrounds, respectively. The smhA1 allele suppressed the lysis phenotype of lytE1 but not of lytD1. Furthermore, the combination of smhB1 with either lytD1 or lytE1 resulted in cosuppression of their lysis phenotypes. The specificity of the suppressor activities, combined with the similarities in the phenotypes of the mutants representing this collection of loci, suggested functional relationships between the murH, smhA, smhB, lytD, and lytE loci. Four clones which complemented the lytD1 mutation were obtained by screening an E. coli gene library, but it is shown that the complementing activity did not represent the E. coli chromosomal lytD locus. It is shown instead that 2 phage λ genes, identified as cro and cI, accounted for the lytD1 complementing activities in these clones. Evidence is presented which suggests that these clones were derived from phage λ DNA which was fortuitously present as a contaminant in the vector preparation used for construction of the gene library. Since the λ Cro and CI proteins are DNA-binding proteins which bind to identical 17 base-pair recognition sequences (the λ right operator sequences), it is hypothesized that LytD encodes a DNA-binding protein with a similar specificity (i.e., which binds to a λ right operator-like sequence) which regulates, probably

negatively, the expression of a gene(s) involved in some way with peptidoglycan hydrolysis.

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LIST OF ABBREVIATIONS

Amp ^r	ampicillin-resistant
Cam ^r	chloramphenicol-resistant
CFU	colony-forming unit
CGSC	<u>E. coli</u> Genetic Stock Center, Yale University
CSH	Cold Spring Harbor Laboratory
D-Ala	D-alanine
DAP	<u>meso</u> -diaminopimelic acid
D-Glu	D-glutamic acid
DMA	Davis minimal agar
EDTA	ethylenediaminetetraacetic acid
GlcNAc	<u>N</u> -acetylglucosamine
HPLC	high performance liquid chromatography
IPTG	isopropyl β -D-thiogalatoside
Kam ^r	kanamycin-resistant
kb	kilobase pairs
kDal	kilodalton
LB	Luria broth
LPS	lipopolysaccharide
MurNAc	<u>N</u> -acetylmuramic acid
MurNAc-pentapeptide	<u>N</u> -acetylmuramyl-L-alanyl-D-glutamyl- <u>meso</u> -DAP-D-alanyl-D-alanine
MW	molecular weight
NA	nutrient agar

NB	nutrient broth
PBP	penicillin binding protein
PG	peptidoglycan
ppGpp	guanosine 5'-diphosphate 3'-diphosphate
pppGpp	guanosine 5'-triphosphate 3'-diphosphate
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
Tet ^r	tetracycline-resistant
ts	temperature-sensitive
TSA	tryptic soy agar
TSB	tryptic soy broth
Tris	tris-(hydroxymethyl)aminomethane
UDP	uridine 5'-diphosphate
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

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CHAPTER 1

INTRODUCTION

I. The Cell Wall of Escherichia coli

Like other Gram-negative bacteria, Escherichia coli possesses a cell wall composed of two layers. The external layer, or outer membrane, is approximately 7.5 nm thick and is composed of phospholipids, lipopolysaccharide (LPS), and a set of characteristic proteins (1). Underlying the outer membrane is a layer composed of the polymer known as peptidoglycan (PG).

The outer membrane is structurally an asymmetric bilayer in which the phospholipids occur exclusively in the inner leaflet of the membrane, and the outer leaflet contains the hydrophobic lipid A portion of LPS. LPS is a unique component of the outer membrane which consists of three parts: (i) the lipid A portion which anchors LPS to the outer membrane; (ii) the core polysaccharide region which is linked to lipid A; and (iii) the hydrophilic O polysaccharide region which is linked to the core (2). The outer membrane represents a selective permeability barrier. Several outer membrane proteins, known as porins, form transmembrane channels which serve as sizing filters which permit passage of certain hydrophilic substances into the cell. This structural feature restricts entry of

many toxic substances such as certain antibiotics, detergents, and PG digestive enzymes (3). PG lipoprotein is one outer membrane protein which is not directly involved in barrier function. PG lipoprotein is an abundant protein (about 7×10^5 copies per cell) involved in a crucial structural function. An N-terminal cysteine residue of the lipoprotein is substituted with a fatty acid residue which is embedded in the lipid bilayer of the outer membrane (4). About one-third of the PG lipoprotein molecules is covalently bound to the PG layer which underlies the outer membrane, and the remainder occur in the free form. Deletion mutants completely lacking PG lipoprotein are viable but their cell envelopes are apparently unstable (5). This suggests that the physical bridge formed between the outer membrane and PG, by PG lipoprotein, may be important in maintaining the structural integrity of the wall. In this regard, it should be noted that other outer membrane proteins, such as the OmpA protein and the porins OmpC and OmpF, are also involved in stabilizing the wall through noncovalent interactions with PG (6).

PG is a porous net-like macromolecule which completely encloses the underlying cytoplasmic membrane. It serves an essential function by providing the membrane with mechanical support, without which the membrane would undergo osmotically induced rupture (7). PG is usually isolated by solubilizing the membranes with boiling sodium dodecyl sulfate (SDS), various washing steps, and protease digestion to remove the

associated outer membrane proteins. The isolated PG is a semirigid macromolecule which is responsible for determining the characteristic rod-shaped morphology of E. coli (8). As already noted, the PG layer is associated with the outer membrane through covalent as well noncovalent interactions. On the other hand, there is no direct evidence for physical contacts between PG and the cytoplasmic membrane. However, ultrastructural studies suggest the occurrence of "adhesion sites" which are proposed to represent areas where the cytoplasmic membrane, outer membrane, and PG adhere to each other (9). These adhesion sites may be regions where the outer and cytoplasmic membranes are fused but, even so, it is unclear what the state of PG is in these areas. It has been proposed that these are the sites where the outer membrane components, such as LPS and outer membrane proteins, are synthesized and exported and where the newly synthesized PG chains are inserted into the existing cell wall PG. It has been further suggested that the interactions between the outer membrane, cytoplasmic membrane, and PG in the adhesion regions may be essential for maintaining cell envelope integrity and for coordinately regulating cell envelope expansion and division during the cell cycle (10).

II. E. coli Peptidoglycan Structure

1. General Structural Features

Fig. 1 illustrates the general features of E. coli PG. It should be noted, however, that this illustration is almost surely oversimplified in view of the recent observations discussed below. PG is composed of long glycan chains interlinked by short peptide substitutions (11). The glycan moiety consists of linear strands of alternating residues of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). In E. coli, each glycan chain terminates with a nonreducing 1,6-anhydro MurNAc residue. The average glycan chain length, calculated on the basis of the ratio of 1,6-anhydro MurNAc to total amount of MurNAc, is about 33 disaccharide units long, and this is equivalent to a length of approximately 33 nm (12). Each MurNAc residue is substituted with a short peptide. The amino acid sequence of this peptide is typically L-alanyl-D-glutamyl-meso-diaminopimelic acid (DAP)-D-alanyl-D-alanine, going from N-terminus to C-terminus, but shorter derivatives of this peptide also occur. Thus, PG may be viewed as a polymer composed of a basic repeating subunit represented by the disaccharide-pentapeptide shown in Fig. 2. It is also worth noting that DAP and MurNAc occur in nature only in PG, and that the occurrence of D-amino acids is unique to PG and certain other bacterial cell wall polymers such as the teichoic acids (13).

The individual peptidoglycan chains are linked to neighboring peptidoglycan chains through direct peptide bonds formed between amino group of DAP in one peptide and the

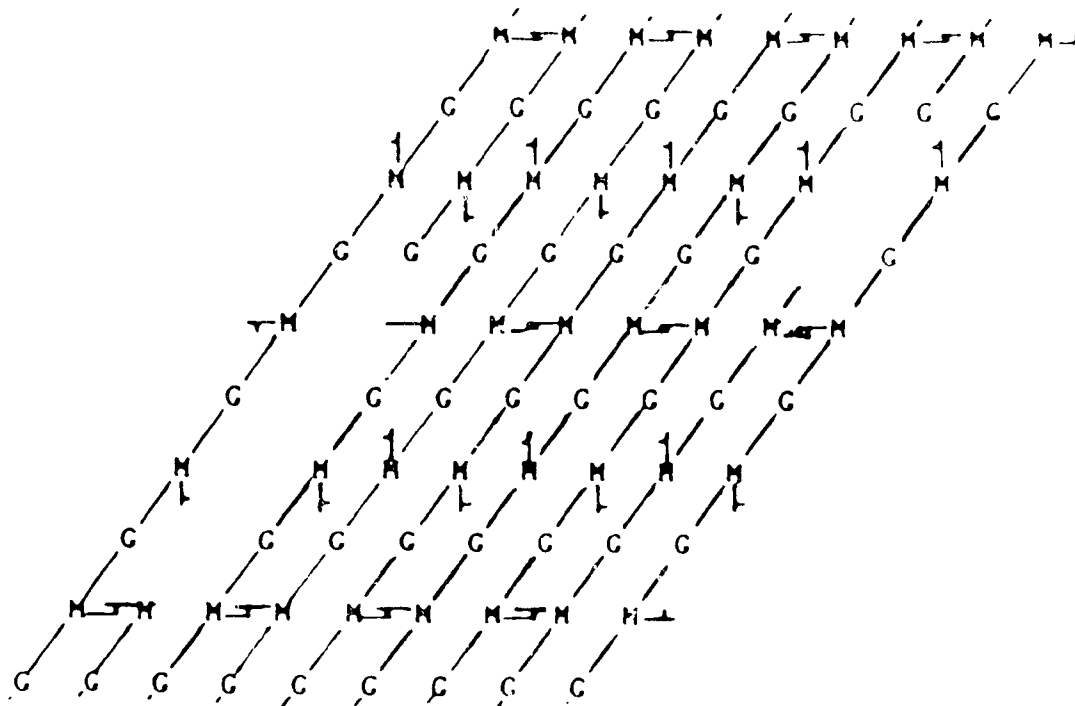


Fig. 1. General features of *E. coli* peptidoglycan. G, GlcNAc; M, MurNAc. Lines connecting G and M residues represent glycosidic bonds. \rightarrow , peptide side chain. $\text{—}\text{—}$ between two M residues represents crosslinked peptides. Model building experiments indicate that crosslinks can be formed only between every fourth peptide side chain on each side. Modified from "*Escherichia coli* and *Salmonella typhimurium*. Cellular and molecular biology", (Neidhardt, F.C., ed.), pp. 24, American Society for Microbiology, Washington, D.C.

N-acetylglucosamine N-acetylmuramic acid

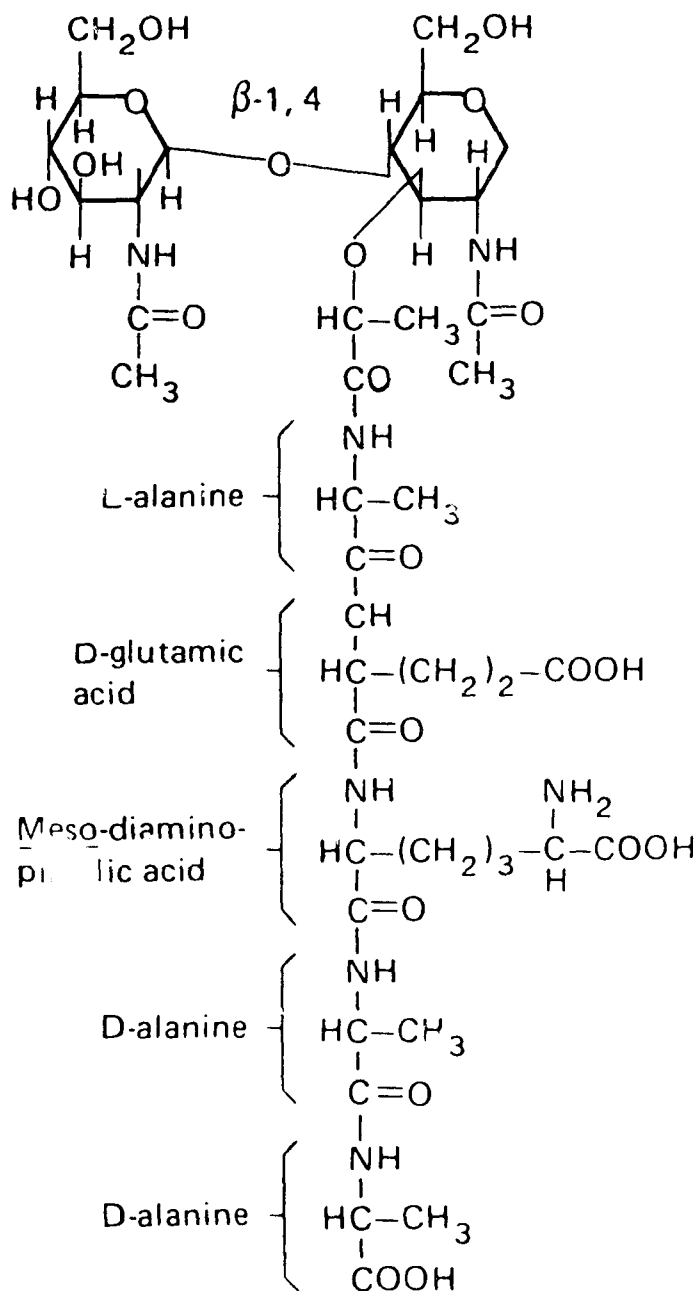


Fig. 2. Chemical structure of the disaccharide-pentapeptide unit (the so-called monomeric subunit) present in *E. coli* PG. Modified from "Developmental biology of the bacteria", (Dworkin, M., ed.), pp. 164, Menlo Park, California, U.S.A.

carboxyl group of D-alanine in an adjacent peptide. This feature gives PG the appearance of a net-like structure (Fig. 1). In E. coli, about 50% of the peptides are crosslinked (14). Furthermore, about 10% of the peptides are covalently attached to the outer membrane PG lipoprotein through a linkage formed between the carboxyl group of DAP and the amino group of the C-terminal lysine of lipoprotein (this feature is not shown in Fig. 1).

2. Recent Observations on the Chemical Composition of PG

The structure of PG, as depicted in Fig. 1, was derived primarily from the characterization and quantification of soluble fragments generated from PG by either controlled partial acid hydrolysis or enzymatic digestion. In these early studies, the soluble derivatives of PG were fractionated by paper or thin layer chromatography, and a total of eight different derivatives were separated and characterized (15). Glauner and Schwarz (16) recently used high performance liquid chromatography (HPLC) to separate soluble PG fragments generated by β -N-acetylmuramidase digestion. They identified at least eighty different PG derivatives. Their results indicated that the structure of E. coli PG was far more complex than previously realized. Their main findings are as follows. (i) They discovered peptides which terminated with glycine rather than D-alanine. (ii) A novel type of peptide cross-linkage between two DAP residues was identified. (iii)

In addition to the previously known classes of so-called monomeric subunits (disaccharide-peptides, one of which is shown in Fig. 2) and dimeric subunits (two disaccharide-peptide units joined by peptide cross-linkages), two new groups of derivatives consisting of trimeric and tetrameric disaccharide-peptide subunits were discovered. (iv) Finally, these new types of subunits carrying novel peptide substitutions were linked to each other in all possible combinations, and it is this fact that was primarily responsible for the new found complexity in E. coli PG structure.

It is undoubtedly too early to fully appreciate the significance of the findings of Glauner and Schwarz (12). Nevertheless, evidence has already started to accumulate which suggests that the novel types of PG sugar-peptides they discovered may play structural or regulatory roles in E. coli PG metabolism. While it may not be immediately possible to evaluate many of these findings, a short summary will be presented here. One significant observation is that the structure of newly synthesized PG differs from that of older or mature PG in several respects (17). (i) Newly synthesized PG is less cross-linked. (ii) Newly synthesized PG contains less covalently bound lipoprotein. (iii) The class of subunits carrying pentapeptide substitutions is found in higher amounts in newly synthesized PG. Several reports indicate that the composition of E. coli PG is influenced by growth conditions (12, 18, 19). The amount of peptides

carrying glycine or the DAP-DAP linkage were most significantly affected by changes in growth conditions. For example, the proportion of glycine-containing peptides is lower in cells grown in minimal medium without glycine as compared to cells grown in complex medium and has been found to double in amount when cells enter stationary phase (12). With respect to peptides involved in DAP-DAP cross-linkage, a marked increase was observed in stationary phase cells and in cells grown at high temperature (18). In another report, the structural composition of PG was shown to undergo changes as a consequence of amino acid deprivation, and it was suggested that this alteration in PG structure contributed, at least in part, to the mechanism of phenotypic tolerance, a phenomenon in which amino acid-deprived E. coli develops resistance to autolysis induced by inhibitors of PG synthesis such as β -lactam antibiotics (19). This matter is described in more detail in a later section.

In summary, recent structural studies indicate that E. coli PG is more complex than shown in Fig. 1. Furthermore, it appears that some of these newly recognized structural features may depend on growth conditions. An updated model based on these new data has been initiated but is far from complete. Earlier models favored a cell wall composed of a single continuous layer of PG arranged much like that shown in Fig. 1. In this layer, the polysaccharide chains are proposed to run parallel to each other in a flat plane with the peptide

side chains pointing in one direction (20). However, this simple monolayer model has been seriously challenged by the structural studies of Glauner and Schwarz (12), and any attempt at modeling must now take into consideration the possibility of a multilayered PG in E. coli.

Schwarz (21) has proposed that the cell wall PG of E. coli is composed of multilayered PG sheets. His proposal is based on the following observations: (i) Muramidase digestion of PG released fragments which were crosslinked multimers (trimers and tetramers) of the basic PG repeating subunit (Fig. 2). The existence of such structural components could not be accounted for by a monolayered PG. For example, the trimeric units would be involved in linking three separate PG strands together, and this could be possible only if the trimers were located at the ends of PG strands if the PG was arranged as a monolayer. However, about 80% of the trimers were shown to be derived from within the glycan strands (12), suggesting that at least one of the three glycan chains must lie outside the plane of the other two glycan strands as shown in Fig. 3A. (ii) Pulse-chase experiments indicated that the crosslinkages in PG had different life times, i.e., the crosslinkages formed between newly synthesized PG strands were relatively stable but those crosslinkages formed between newly inserted and preexisting PG strands were unstable (12). One possible interpretation of these results is a multilayered PG growing according to the "inside-to-outside" mechanism shown

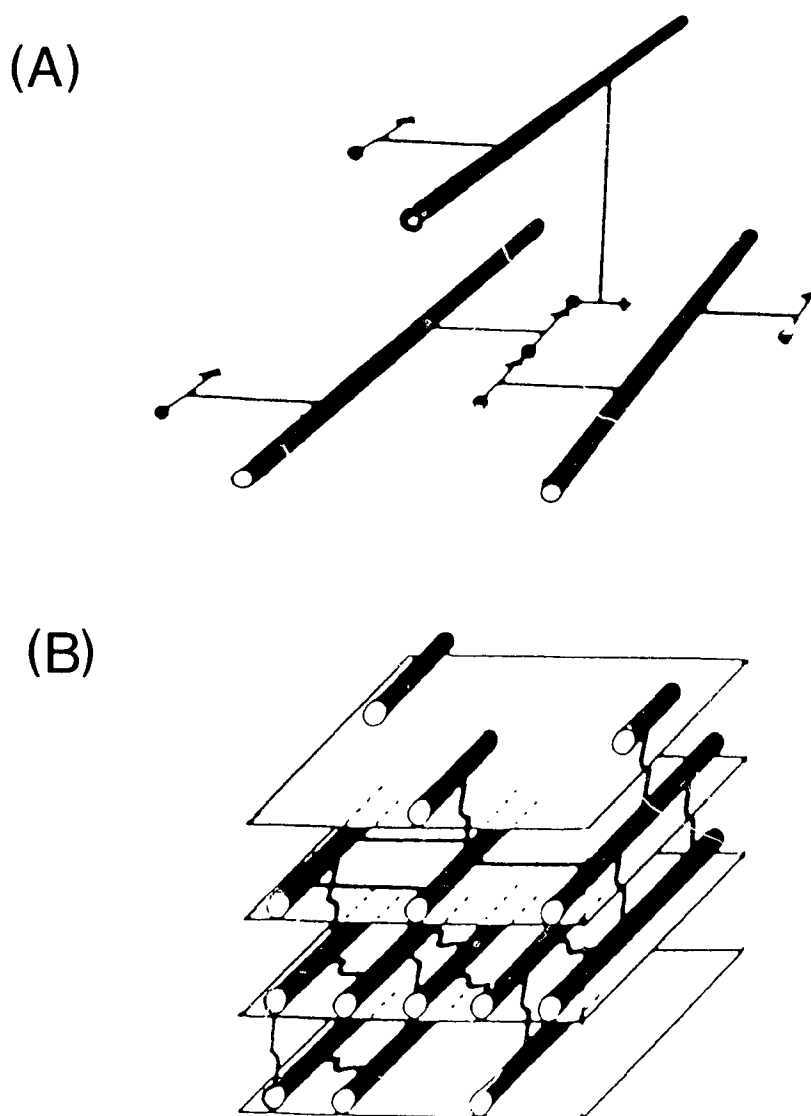


Fig. 3. Model of the multilayered PG of E. coli. (A) The structure of the trimeric subunit derived from the crosslinking of 3 individual PG strands which reside in different planes. The arrowheads indicate the donor sites and the spheres indicate the acceptor sites of the peptide side chains. The isolation of this fragment from enzymatically digested PG gave rise to the proposal that E. coli PG had to be multilayered rather monolayered as previously believed. (B) The multilayered structure of E. coli PG showing sections of 4 layers interconnected by peptide crosslinkages. The cylindrical rods represent the glycan strands, and the vertical and horizontal lines connected to them represent the crosslinked peptide side chains. Modified from ref. 21.

in Fig. 3B (21). (iii) Recent electron micrographs of E. coli specimens prepared by the new, less disruptive methods (progressive lowering of temperature in embedding and freeze-substitution) developed by Hobot and Kellenberger (22), revealed PG as a thick gel-like structure, approximately 10 nm wide, with a high water content. The estimated thickness of PG (10 nm) is equivalent to the thickness of the periplasmic space. Hobot and Kellenberger have therefore proposed that the periplasmic space is filled with a so called "periplasmic gel" which is believed to be mainly composed of PG, periplasmic proteins and polysaccharides. This gel-like PG is further proposed to be composed of several layers of highly hydrated PG. The degree of crosslinking between peptide side chains is thought to be the highest in the layer immediately adjacent to the outer membrane, and the degree of crosslinkage then decreases progressively in the underlying layers with the lowest amount found in the layer adjacent to the cytoplasmic membrane. The concept of a gel-like PG is further supported by the finding that the rate of lateral diffusion of a periplasmic protein in the periplasmic space is about 1000-fold lower than that in free aqueous environment (23). On the other hand, components other than PG must surely contribute to the viscosity, and the degree of contribution made by PG is unknown. (iv) According to the calculation by Schwarz (21), the average amount of PG found in an E. coli cell would be sufficient to form three layers. From the structural

standpoint, three layers of PG may be the minimum number necessary to guarantee the structural integrity of an E. coli cell.

3. Three Dimensional Structure of PG

The three dimensional arrangement of PG is still uncertain. Barnickel et al. (24) have recently proposed a model for PG based on computer aided calculations of the most energetically favorable conformations assumed by the glycan and peptide chains. In agreement with previous models, they suggested that the glycan chains run parallel to each other. Based on other studies (25), these glycan chains are proposed to run around the circumference of the cell cylinder, i.e., perpendicular to the long axis of the rod-shaped E. coli cell. Since the average length of a glycan strand is about 33 nm (33 disaccharide-peptide subunits), an estimated 80 strands stacked end to end would therefore be needed to cover the circumference once. Instead of forming a flat plane, Barnickel et al. (24) proposed that the sugar rings are tilted with respect to each other in such a way that each PG strand actually exists in a helical conformation. The peptides consequently cannot all protrude in the same direction from the plane formed by the glycan strands. Instead, each peptide side chain is proposed to be oriented about 90 degrees around the helical glycan relative to its neighboring peptide side chains. As a result, only one of every four peptide side

chains is positioned properly so that it can be crosslinked to a peptide in the same plane on a neighboring strand. The proposed multilayered arrangement for PG could be readily accounted for by this model. It is also possible that the glycan strands could adopt a variety of conformations based on different degrees of twisting between neighboring sugar residues. This may explain how the cell wall PG can be both rigid in some areas and flexible in other areas in order to fulfill its biological function as a stress-bearing fabric. The glycan chains and the crosslinked peptide side chains are thought to provide PG with the rigidity necessary to protect the cell from osmotic pressure, while the proposed different states of twisting between the sugar residues provide for a certain degree of flexibility to allow the cell to undergo the swelling or shrinking which have been observed to occur in response to environmental changes (26).

III. PG Biosynthesis

1. Biosynthetic Pathway of PG

Some of the main steps in PG biosynthesis in E. coli are summarized in Fig. 4. The PG biosynthetic pathway may be divided into three stages, each of which takes place in a different cellular compartment (17, 27).

In the first stage, the UDP-activated precursors, UDP-N-acetylglucosamine (UDP-GlcNAc) and UDP-N-acetylmuramyl-L-Ala-

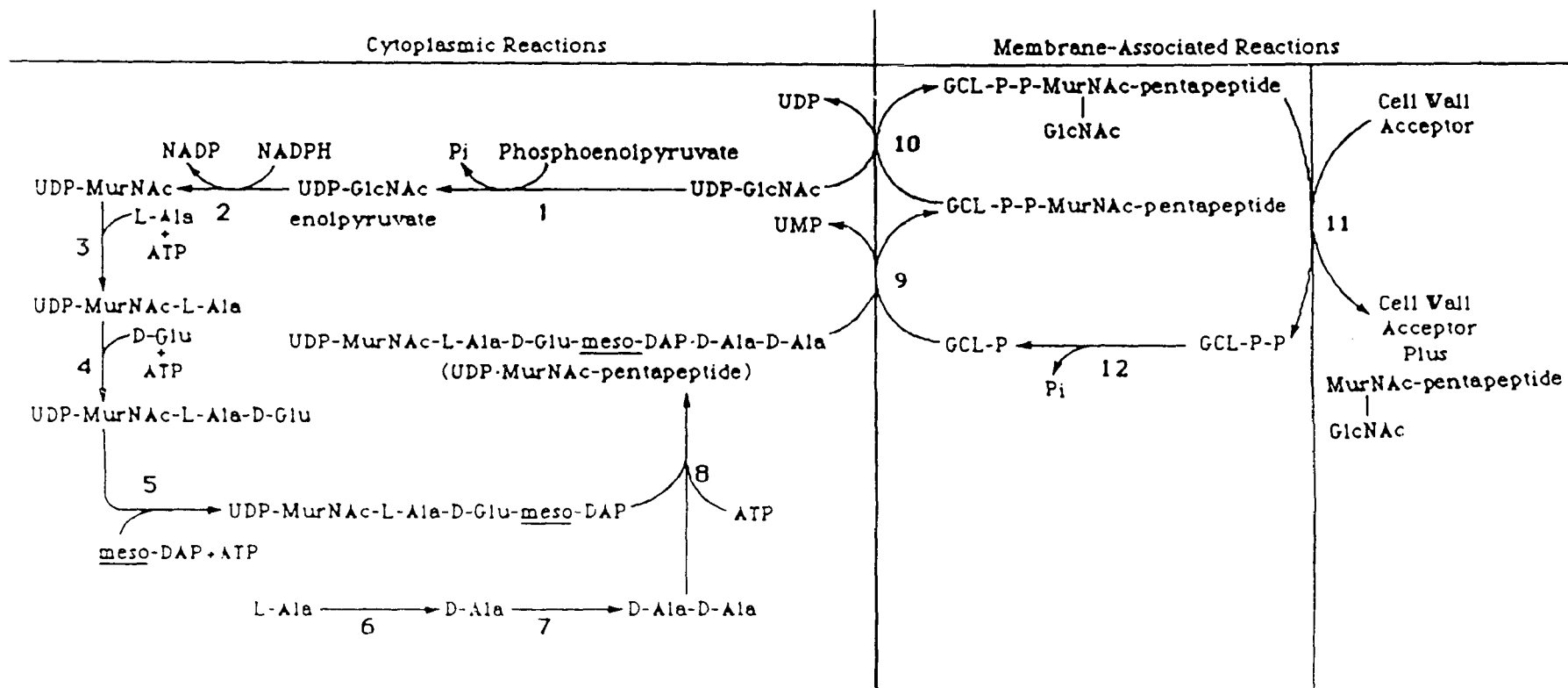


Fig. 4. The main steps in peptidoglycan biosynthesis in *E. coli*. The enzymes catalyzing the indicated reactions are: 1, phosphoenol-pyruvate:UDP-GlcNAc-pyrophosphorylase; 2, UDP-GlcNAc-enolpyruvate reductase; 3, L-alanine adding enzyme; 4, D-glutamate adding enzyme; 5, meso-DAP adding enzyme; 6, alanine racemase; 7, D-alanyl-D-alanine synthetase; 8, D-alanyl-D-alanine adding enzyme; 9, UDP-MurNAc-pentapeptide translocase; 10, UDP-GlcNAc translocase; 11, PG transglycosylase and/or transpeptidase (PBPs); 12, undecaprenol pyrophosphate pyrophosphatase. Modified, with his permission, from J.S. Tao's M. Sc. thesis, University of Victoria (1990).

D-Glu-meso-DAP-D-Ala-D-Ala (UDP-MurNac-pentapeptide) are synthesized by a set of soluble enzymes. The individual steps involved in the synthesis of UDP-GlcNac have not been studied in E. coli and are not shown in Fig. 4. UDP-GlcNac is not only the precursor for the synthesis of UDP-MurNac (reactions 1, 2) but also serves as a precursor for the synthesis of LPS, another major component of cell envelope of E. coli (28). UDP-MurNac-pentapeptide is formed by sequential addition of L-Ala, D-Glu, meso-DAP, and D-Ala-D-Ala to UDP-MurNac in a series of ATP-dependent reactions catalyzed by specific amino acid ligases (reactions 3-8).

The second stage involves the sequential translocation of P-MurNac-pentapeptide and GlcNac from the UDP-activated precursors to a membrane glycosyl carrier lipid (GCL-P) by two membrane-bound enzymes (29, 30) to yield the lipid-intermediates, GCL-P-P-MurNac-pentapeptide and GCL-P-P-MurNac-(GlcNac)-pentapeptide (reactions 9, 10). The lipid carrier has been identified as undecaprenyl phosphate, a minor membrane lipid. Undecaprenyl phosphate is also involved in the synthesis of other heteropolysaccharides such as LPS and capsular polysaccharide (31). This lipid carrier serves as means of transporting hydrophilic glycans across the cytoplasmic membrane. Since there is only limited amount of this lipid carrier in the cell, its recycling (reaction 12) turns out to be a key step in the synthesis of PG and other heteropolysaccharides. Thus, when the synthesis of any one of these

heteropolysacchrides is inhibited, its precursors are trapped on the lipid carriers, and this eventually leads to limited availability of the lipid carrier, which, in turn, interferes with the synthesis of other heteropolysaccharides (11).

The third stage involves the translocation of the disaccharide-pentapeptide units from the lipid carriers to acceptor sites in the growing PG polymer (reaction 11). This reaction is attributed to a group of cytoplasmic membrane-bound penicillin-binding proteins (PBPs), so-called because they represent the targets for the β -lactam antibiotics (32-35). The PBPs are characterized by their ability to covalently and specifically bind to penicillin. Because of their functional complexity, the roles of the PBPs in PG metabolism, including PG polymerization, will be considered separately in the following section.

2. The PBPs and Their Functions in PG Metabolism

At least 7 distinct PBPs exist in E. coli, and their genes have been mapped, cloned, and sequenced (36-41). Two approaches have been used to determine the functions of the PBPs (17). First, mutants with defects in the various PBPs have been isolated and characterized. Second, the specificity of PBP binding by different β -lactam antibiotics has been correlated with the effects of these agents on cellular morphology and viability.

The seven major PBPs are usually subdivided into two

groups according to their molecular weight because this conveniently also subdivides them functionally. The high molecular weight PBPs, designated 1A, 1B, 2, and 3, are directly involved in the final stage of PG biosynthesis, i.e., in polymerization of the PG subunits. PBPs 1A, 1B and 3 are bifunctional enzymes capable of catalyzing two distinct reactions, a transglycosylation reaction that results in the elongation of a glycan strand and a transpeptidation reaction that crosslinks adjacent peptide side chains (34, 35). As discussed further below, the transpeptidase active sites of these enzymes are the actual penicillin-binding sites, and penicillin has no effect on transglycosylation (42). Furthermore, the transglycosylase activities are specifically inhibited by another antibiotic, moenomycin (42, 43). The precise mechanism by which the PBPs insert the disaccharide-pentapeptide repeating units into a growing PG molecule is not fully understood. The crucial question of what roles the transglycosylase and transpeptidase activities play in PG polymerization has not been resolved. However, it is known that both penicillin and moenomycin inhibit the incorporation of disaccharide-pentapeptide units into PG indicating that both activities are necessary.

Since PG is the determinant of bacterial cell shape, E. coli cell wall PG may be viewed as being composed of cylinders which are closed off at their ends by hemispherical caps. The hemispherical caps represent the septal PG synthesized during

cell division. Genetic and physiological studies indicate that PBPs 1A and 1B are primarily responsible for the polymerization of cylindrical PG during cell growth elongation. Thus, β -lactam antibiotics which have a high affinity for PBPs 1A and 1B block cell elongation. Furthermore, the PBP 1A and 1B double mutant cannot elongate, and the combination of the two mutations is consequently lethal (44). On the other hand, mutants defective in either PBP 1A or 1B are viable, suggesting that PBP 1A and 1B may substitute for each other (45). Further evidence suggests the involvement of PBP 2 in the synthesis of cylindrical PG. Loss of the PBP 2 function either through mutation or by specific inhibition with the β -lactam derivative, mecillinam, results in the growth of E. coli as osmotically stable spherical cells with increased cell diameters rather than the normal rod-shaped cells (46). Thus, PBP 2 is apparently essential in the control of elongation of cylindrical PG sacculus and in maintaining the rod-shaped morphology of E. coli. Unlike the other high molecular weight PBPs, PBP 2 possesses only the transpeptidase activity (43).

Mutants defective in PBP 3 are unable to form septa, and they consequently grow as long filamentous cells (47). Furthermore, β -lactam antibiotics which have affinity for PBP 3 block cell division in E. coli. These observations indicate that PBP 3 is specifically activated at the time of cell division and is responsible for the synthesis of septal, or

hemispherical cap, PG (48).

It is noteworthy that, in addition to PBPs 1A, 1B, 2 and 3, a penicillin-insensitive transglycosylase has been identified and purified, which polymerizes uncross-linked PG (49). However, it still unclear what role, if any, this enzyme plays in PG polymerization in vivo.

The 3 low molecular weight PBPs, designated PBPs 4, 5, and 6, have been identified as DD-carboxypeptidases which hydrolyze the D-alanyl-D-alanine bond present at the N-terminus of the pentapeptide in the newly synthesized PG (50, 51). Genetic and physiological studies have so far failed to elucidate the roles of these proteins in PG metabolism. For example, mutants defective in any one of these enzymes are viable and apparently normal (52). Although these results suggest that these enzymes are not essential, it must be emphasized that mutants carrying multiple mutations affecting these PBPs have not yet been constructed. At present, it appears that the low molecular weight PBPs are involved in the maturation of nascent PG but specific details remain to be determined (53). This topic is more appropriately discussed in the following section.

Both the transpeptidase and DD-carboxypeptidase activities of the PBPs utilize the D-alanine moiety of the pentapeptide as a substrate (54). Penicillin is an analog of acyl D-alanyl-D-alanine and thus competes with this substrate to acylate the active site of the PBPs. The penicilloylated PBPs

are quite stable, and this accounts for the apparent irreversible inactivating action of β -lactam antibiotics (55). The serine residue in the conserved sequence of Ser-X-X-Lys found in all PBPs has been identified as the active site which is acylated by penicillin (38, 39, 56). Interestingly, the same amino acid sequence is found in the class A and C β -lactamases, and in these cases the serine residue has also been identified as the active site (54).

3. PG Hydrolases and Their Roles in PG Metabolism

The PG hydrolases are enzymes which hydrolyze chemical bonds in PG. They represent a diverse group in terms of mode of action. PG hydrolases occur ubiquitously in bacteria suggesting that they may have important functions (57). From the following, it can be seen that E. coli is relatively complex in this respect in that it possesses at least 10 distinct PG hydrolase activities. The activities of these enzymes are summarized in Fig. 5. Three of these enzymes, N-acetylglucosaminidase, lytic transglycosylase S, and lytic transglycosylase M, cleave interglycan linkages (58-60). One (muramyl-L-alanine amidase) cleaves a glycan-peptide bond (61). The remaining 6 cleave interpeptide bonds (45, 62-64) and note here that the low molecular weight PBPs (PBPs 4, 5, and 6), by virtue of their DD-carboxypeptidase activities, are regarded as PG hydrolases which fall into this category. In addition to the DD-carboxypeptidase activity, PBP 4 possesses

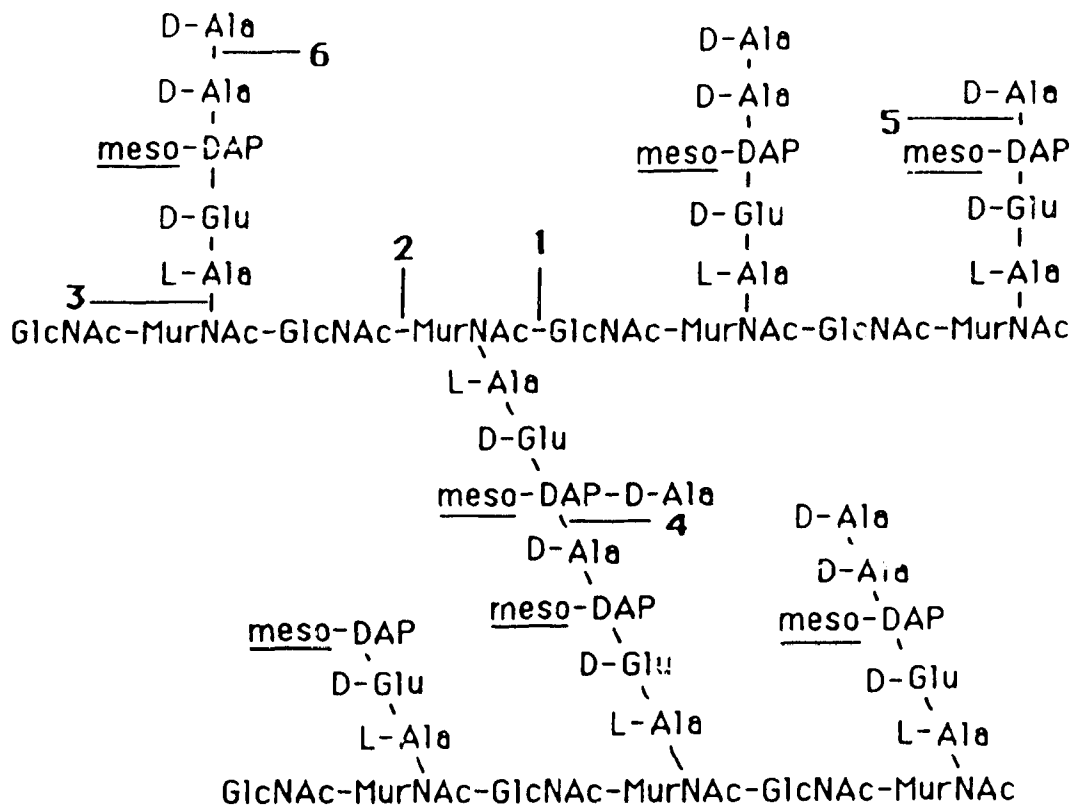


Fig. 5. Cleavage sites of the *E. coli* peptidoglycan hydrolases. 1, lytic transglycosylase S and lytic transglycosylase M. 2, N-acetylglucosaminidase. 3, muramyl-L-alanine amidase. 4, carboxypeptidase IB (PBP 4) and penicillin-insensitive endopeptidase. 5, carboxypeptidase II. 6, carboxypeptidase IA (PBP 5) and IB. Modified, with his permission, from the thesis of J.S. Tao, University of Victoria (1990).

a penicillin-sensitive DD-endopeptidase activity which hydrolyzes the intermolecular crosslinks formed between the D-alanine residue of one strand and the DAP residue of a neighboring strand (65). PBP 4 is therefore also referred to as penicillin-sensitive endopeptidase, and this is to distinguish the PBP 4 activity from that of a distinct periplasmic endopeptidase which cleaves the same bond but which is penicillin-insensitive (66). Finally, there are at least 2 distinct LD-carboxypeptidase activities which cleave the terminal D-alanine residues from tetrapeptide sidechains (53, 67).

Since the cell wall PG is a closed bag, it has been proposed that the expansion of this structure which accompanies cell growth must require hydrolysis of chemical bonds, and from this has evolved the suggestion that PG hydrolases must play an essential role in the PG biosynthesis (8). However, the exact roles of the PG hydrolases in this process are still not well understood. At the present time, two general roles appear probable. First, PG hydrolases may be involved in regulating certain aspects PG synthesis and morphogenesis (68, 69). Second, they may be involved in recycling PG building blocks in the process known as PG turnover (70). These processes are summarized below.

As indicated above, newly synthesized PG differs from old, established PG, and the changes associated with this transition ("maturation") appear to be brought about by the

action of PG hydrolases. Evidence has been presented indicating that the increase in crosslinking associated with maturation involves the pentapeptide units of the new PG serving as donors in a transpeptidation reaction apparently catalyzed by PBP 4 (53). The increased crosslinking characteristic of mature PG does not occur in PBP 4-defective mutants. Furthermore, the available evidence suggests that PBP 5 is also involved in regulating the degree of crosslinkage during PG maturation and that this activity is also involved in maintaining the normal rod-shaped morphology of PG. A strain which exhibits a 4-fold overproduction of PBP 5 synthesizes new PG which has a decreased amount of pentapeptide units, presumably due to the elevated DD-carboxypeptidase activity. Surprisingly, this was accompanied by an increased level of crosslinkage within the new PG, and a spherical cell morphology (71). The mechanistic basis for these observations has not yet been determined.

Intuitively, it would seem that PG hydrolases may be required for cell separation during the division process. In E. coli, the only evidence for this appears to be from studies on the chain-forming (i.e., cell separation-defective) mutants (envA or envC). These mutants are deficient in both muramyl-L-alanine amidase and LD-carboxypeptidase activities suggesting that these enzymes are involved in cell separation (72, 73). However, it should be noted that the envA and envC mutations are pleiotropic, and these correlations are there-

fore far from conclusive.

PG turnover is an enzymatic process resulting in the excision of fragments from the existing cell wall PG. Turnover has been demonstrated in many bacterial species, and the process seems to be associated with normal growth; i.e., it is important to stress that the solubilization of these PG fragments is not associated with cell lysis (74). In E. coli, both the glycan and the peptide portions of PG are involved, and as much as 50% of the wall PG may be excised per generation. Unlike the situation in Gram-positive bacteria, the outer membrane barrier prevents escape of most of the PG fragments generated during turnover; e.g., only about 8% of the excised peptides escapes into the culture medium (75). Most of the fragments are recycled but much of this process is not yet understood. For example, it is known that the transport of the peptide derivatives, mediated by oligopeptide permeases, is the first step in their recycling, but the metabolic fate of these peptides within the cells has not been determined (76). Based on the structure of the fragments generated, it would appear that the PG hydrolases involved are the periplasmic endopeptidase, muramyl-L-alanine amidase, LD-, and DD-carboxypeptidases (70). Since turnover is a normal event observed in growing bacteria, it is currently thought that it is part of the process of cell wall PG expansion. The phenomenon represents the most compelling proof that PG hydrolases have an essential function in bacteria.

In summary, E. coli possesses a variety of PG hydrolases. It seems likely that the assembly and morphogenesis of cell wall PG requires the activities of at least some of these enzymes working in concert with the PG biosynthetic enzymes (57, 68).

IV. Mechanism of Cell Autolysis

1. Involvement of PG Hydrolases

Bacteria generally grow in hypotonic media, and, as noted above, PG is essential for preventing the osmotically induced rupture of the cytoplasmic membrane under such conditions (7). In E. coli, cell lysis which is mediated by PG hydrolases occurs whenever any step in PG biosynthesis is inhibited in growing bacteria (77). Thus, temperature-sensitive (ts) mutants with defects in any of the PG biosynthetic enzymes exhibit a lysis phenotype at the restrictive temperature (78-87). Furthermore, if wild type bacteria are treated with an antibiotic which inhibits a step in PG synthesis, lysis results, and, in fact, lysis appears to be the basis for the bactericidal action of many of these antibiotics (88-90). In the case of penicillin-induced lysis, the activities of the lytic transglycosylase and penicillin-insensitive endopeptidase have been implicated (91-93). Since accidental cellular autolysis of growing bacteria rarely, if ever, occurs, it would appear that the PG hydrolase activities in E.

coli are tightly controlled (17, 94, 95). How this is achieved is not understood. Thus, one obviously over simplified hypothesis is that the PG hydrolases are coupled to the biosynthetic enzyme activities, and the inhibition of a step in the biosynthetic pathway results in the uncoupling of these activities and the consequent hydrolysis of PG (77, 88, 96, 97).

Nongrowing E. coli are remarkably resistant to lysis. It is well documented that they are unaffected by inhibitors of PG synthesis (98). Furthermore, bacteria suspended in buffer are stable. The stability of these cells is not due to lack of PG hydrolases because active hydrolases can be readily isolated from them (99). Furthermore, treatment of nongrowing bacteria with a variety of agents, such as trichloroacetic acid, sucrose, chelating agents, or NaCl, causes lysis of such cells (94). These findings suggest that the activities of the PG hydrolases are silenced in nongrowing bacteria. It has been proposed that this may involve an endogenous inhibitor of PG hydrolases (100) or formation of a "topological barrier" which prevents the PG hydrolases from gaining accessibility to their PG substrate (17, 101).

2. Regulation of PG Synthesis and Hydrolysis by the Stringent Response

The accumulation of guanosine 3'-diphosphate 5'-triphosphate (pppGpp) and guanosine 3'-diphosphate 5'-diphosphate

(ppGpp) occurs in relA⁺ strains of E. coli subjected to amino acid deprivation (102). These compounds apparently signal amino acid deficiency and mediate the shutdown in the syntheses of key cellular macromolecules, e.g., stable RNA species, phospholipids, and PG in a phenomenon known as the stringent response (103). The synthesis of ppGpp and pppGpp is catalyzed by an ATP:GTP 3'-pyrophosphotransferase (the product of the relA gene) which is activated during amino acid deprivation. Therefore, amino acid-deprived relA mutants do not accumulate ppGpp and pppGpp, and as a consequence, they exhibit continued macromolecular synthesis despite the inhibition of growth. It is also significant that the activation of RelA enzyme during amino acid deprivation can be blocked in relA⁺ by certain ribosome inhibitors (e.g., chloramphenicol), and this results in their phenotypic relaxation (104). The sites in the PG biosynthetic pathway which are inhibited during the stringent response have been localized to (i) an early step(s) in the synthesis of UDP-GlcNAc and UDP-MurNAc-pentapeptide and (ii) a late step in the polymerization of PG which is apparently identical to the penicillin-sensitive transpeptidation reaction (105, 107). Thus, when cell growth is inhibited by amino acid deprivation, the stringent response effectively prevents the unnecessary accumulation of UDP-linked precursors and PG in relA⁺ strains of E. coli. The accumulation of lipid-intermediates is believed to be restricted by the limited amount of undeca-

prenyl phosphate in the cell (107). The mechanism by which the stringent response inhibits PG synthesis is far from understood, but it appears that the inhibition of PG polymerization step is related to the fact that the reaction is dependent on ongoing phospholipid synthesis. Thus, it appears that the inhibition of phospholipid synthesis during amino acid starvation serves to prevent accumulation of excess membrane as well as to inhibit PG polymerization (107).

It has been shown that the stringent response results in the inhibition of penicillin-induced lysis. As noted above, nongrowing (e.g., amino acid-deprived) wild type bacteria are tolerant to penicillin. On the other hand, such bacteria can be made to undergo penicillin-induced lysis by either introducing a mutation in relA or by treating the bacteria with a RelA inhibitor such as chloramphenicol (108).

V. Purpose and Organization of this Dissertation

As discussed above, the mechanism of the control of PG hydrolase activity in E. coli is for the most part unknown. The fortuitous isolation of a ts mutant which appeared to be defective in cell wall PG metabolism prompted us to consider a genetic approach to this problem. The original objective of this work was to characterize this mutant in an attempt to identify the basis for its phenotype. Chapter 2 describes the materials and methods used in this study. The ts mutant

phenotype, as described in Chapter 3, is attributed to a mutation in a new genetic locus, designated murH. This mutation is shown to be associated with the inhibition of PG synthesis and PG hydrolase-mediated lysis at the restrictive temperature. The difficulties encountered in the cloning of the murH locus are also described. These problems forced us to take an alternative approach in the identification of the murH function. This approach, described in Chapter 4, involved the isolation of extragenic mutations which suppressed the ts lysis phenotype associated with the murH mutation. It was hoped that new clues concerning the murH function would be revealed through the characterization of such suppressors. Two suppressor loci, designated smhA and smhB, are described in Chapter 4. Also included is a characterization of two additional ts mutations in new loci designated lytE and lytD which appeared spontaneously in the smhA and smhB mutants, respectively. It is proposed that the five new loci described in this work are functionally related, and their apparent relationships are discussed. Chapter 5 describes an attempt to clone the lytD gene. It is shown that all of the positive clones isolated carried DNA derived, not from the E. coli chromosome, but from the genome of phage λ instead. Furthermore, the ts lytD phenotype is shown to be complemented by either one of 2 genes on the cloned DNA fragment. These genes are identified as the phage λ cro and cI genes which encode DNA-binding proteins involved in the

regulation of lytic infection cycle of λ . It is proposed that the lytD gene product may be a DNA-binding protein with a target specificity similar to that of the λ CI and Cro proteins. The possible implications of this proposal are discussed, particularly the possible relationship between the mutant loci described in this work and the cryptic lambdoid DNA sequences which are known to reside within the E. coli genome (109-111).

CHAPTER 2

MATERIALS AND METHODS

I. Bacteria, Bacteriophages, and Plasmids

All bacteria used in this study were derivatives of E. coli strain K-12. The bacterial strains, bacteriophages, and plasmids used are listed at the beginning of each chapter.

II. Media and Culture Conditions

Bacteria were cultured in Tryptic Soy Broth (TSB, Gibco Laboratories) or Nutrient Broth (NB, Difco Laboratories). Solidified versions of these media, designated TSA and NA, respectively, contained 1.5% agar. NaCl was added to these media at 1% where indicated. In some experiments, bacteria were grown in M9 minimal medium (112) with required nutritional supplements. Davis Minimal Agar (Difco Laboratories) containing appropriate supplements was used in genetic experiments involving auxotrophic markers. All the other media, except those mentioned above, were made according to Miller (113).

Cultures in liquid media were incubated in waterbath shakers at the indicated temperatures, and growth was monitored with a Klett-Summerson colorimeter with either a green filter (for TSB or NB) or a blue filter (for M9). When required, antibiotics were added at the following concentra-

tions: tetracycline, 20 $\mu\text{g/ml}$; ampicillin, 50 $\mu\text{g/ml}$; kanamycin, 50 $\mu\text{g/ml}$; chloramphenicol, 50 $\mu\text{g/ml}$.

III. Determination of Temperature Sensitivity

In an effort to quantify the relative degrees of temperature sensitivity exhibited by different mutant strains with respect to colony-forming capabilities, serial dilutions of cultures grown overnight in TSB at 30°C were plated in duplicate on TSA, NA, or NA containing 1% NaCl. One set of plates was incubated at 30°C and the other at 42°C. Plate counts were determined after 24 h (TSA) or 48 h (NA and NA containing 1% NaCl) of incubation. Longer periods of incubation did not change the plate counts. The efficiency of colony formation was expressed as the ratio of the plate count in terms of colony-forming units (CFU) per ml obtained at 42°C to the plate count obtained at 30°C.

To determine the effect of temperature on growth in liquid medium, bacteria were grown for 2-3 doublings in the indicated medium at 30°C to a density of about 50 Klett units (5×10^8 cells per ml). At this point, the culture was divided into two equal parts. One portion was maintained at 30°C as a control, and the other was shifted to 42°C.

IV. Solubilization of Radiolabeled PG

PG was prelabeled with [^3H]diaminopimelic acid ([^3H]DAP; 1.1 $\mu\text{Ci}/\mu\text{g}$; Amersham Corp.) as described by Ishiguro et al.

(105). Strain VC462 (murH1) was grown in M9 minimal medium at 30°C for 2 doublings to a density of 2×10^8 cells per ml. [³H]DAP was added to a concentration of 0.3 µg/ml (1.1 µCi/µg). After one doubling, labeling was terminated adding unlabeled DAP at 25 µg/ml. The labeled culture was divided into three portions. One portion was kept at 30°C, and the other two portions were shifted to 42°C. One of the cultures shifted to 42°C was simultaneously subjected to isoleucine deprivation (by adding 500 µg of L-valine per ml) and chloramphenicol (200 µg/ml, to relax the stringent control of PG synthesis) treatment to determine the effect of inhibiting protein synthesis on solubilization of radiolabeled peptidoglycan. At indicated times, 0.5-ml samples were pipetted into equal volumes of boiling sodium dodecyl sulfate (SDS) and boiled for 30 min. The SDS-insoluble fractions were collected on Millipore filters (pore size, 0.45 µm). The filters were rinsed with distilled water, dried, and counted in a Beckman LS3145T liquid scintillation counter with a toluene-based scintillation cocktail.

V. PG Synthesis

Bacteria were grown in M9 minimal medium at 30°C or 42°C. At indicated times, 0.3 ml samples of the cultures were removed and incubated for 5 min at the appropriate temperature in the presence of 1 µCi (0.2 µg) of [³H]DAP per ml. Labeling was terminated by applying duplicate 0.1 ml amounts from each

sample onto Whatman 3MM filter paper disks and immersing these in cold 10% trichloroacetic acid (TCA). The filters were then processed and counted as described by Ishiguro (114).

To identify the step in PG synthesis which was blocked by the murH1 mutation, the cellular distribution of [³H]DAP incorporated at the permissive and nonpermissive temperatures was compared. Strain VC462 was grown in M9 at 30°C to a density of 4×10^8 cells per ml. The culture was divided into three portions. One portion was maintained at 30°C without further treatment. Two portions were subjected simultaneously to isoleucine deprivation and chloramphenicol treatment; one of these was incubated at 30°C, and the other was shifted to 42°C. [³H]DAP was added to all cultures at 0.3 µg/ml (1.1 µCi/µg). After 30 min of labeling, cell samples were analyzed by paper chromatography as described by Ramey (106). Cells from 5-ml samples were collected by centrifugation and suspended in 50 µl of ice-cold distilled water. The samples were applied to a sheet of Whatman 3MM chromatography paper (57 cm long), and the chromatogram was developed in isobutyric acid-1 M NH₄OH (5:3) for 16 h. In this system, cell-bound PG remains at the origin. On the other hand, the solvent extracts the UDP-MurNAc-peptides and the lipid intermediates which migrate to R_f values of 0.2 and 0.8, respectively. When necessary, the development time was extended beyond 48 h to separate UDP-MurNAc-tripeptide from UDP-MurNAc-pentapeptide (105). The amounts of radioactivity associated with these

areas were determined.

The components of the lipid intermediate fractions were quantified after paper chromatography of mild acid hydrolysates as described in detail by Ramey et al. (106). Cultures of VC462 were labeled with [³H]DAP at 30°C and 42°C for 30 min as described above. Suspensions of labeled cells were boiled for 4 min to release soluble radiolabeled material. The washed cell suspensions were divided into two equal parts. One part, referred to as the unhydrolyzed control, was applied directly to a sheet of Whatman 3MM chromatography paper. The other part was hydrolyzed with 10% acetic acid in sealed vials at 105°C for 20 min and then applied to chromatography paper. The chromatogram was developed in isobutyric acid-1 M NH₄OH (5:3) for 16 h and analyzed (106).

VI. Genetic Techniques

To facilitate mapping, Tn₁₀, Tn₅, or Tn_{10kan} insertions linked to the locus being mapped were obtained by screening random transposon insertion pools prepared on a protrophic strain, W3110, with the λ phage derivatives, NK55 (115), λ467 (116), or λ1105 (117), respectively. The screening procedure involved the phage Pl_{vir}-mediated transduction of the random transposon insertions into different appropriate recipient strains, with the selection of transductants on TSA containing appropriate antibiotics at 30°C. Conjugation and bacteriophage Pl_{vir}-mediated generalized transduction were

performed according to the methods of Miller (113). Genetic linkages were calculated as described by Wu (118).

VII. General Recombinant DNA Techniques

Plasmid DNA was isolated by the method of Birnboim and Doly (119). Chromosomal DNA was prepared according to the procedure of Driver and Lawther (120). DNA restriction endonuclease digestions and ligations were performed as described by Maniatis (121). Transformation was according to the method of Hanahan (122). In some experiments, plasmids were introduced into bacteria by electroporation as described by Taketo (123). Agarose gel electrophoresis of DNA fragments was performed in 0.7% gels (except those indicated) following the procedure of Maniatis using the TAE buffer system (0.04 M Tris-acetate; 0.002 M EDTA) (121). Restriction endonucleases, T4 DNA ligase, and DNA molecular weight standards (λ DNA digested with HindIII) were purchased from Amersham or Boehringer Mannheim.

VIII. Cloning Methods

A genomic library of E. coli K-12 strain W3110 was constructed by ligation of DNA fragments in the 10 to 18 kb size range which were derived from a Sau3AI partial digest of chromosomal DNA into the PamHI site of the phasmid vector, λ SE6 (124). The ligation mixture was packaged in vitro using a phage λ DNA packaging kit obtained from Amersham Corp. The

packaged library was then amplified by propagating it on the P2 lysogen, strain Q359 (125). A distinctive feature of λ SE6 is that it is a phasmid (i.e., phage-plasmid hybrid) which was constructed by cloning the single-copy replicator (NR1) of plasmid pDPT427 into the phage λ 1059 genome. This DNA molecule can therefore be maintained lysogenically as an autonomous, single-copy plasmid or, upon induction, can replicate lytically under the control of the λ lytic cycle. The vector, which carries both kanamycin resistance and ampicillin resistance, has been useful for cloning genes in low-copy by complementation. It should be noted that cloning of DNA fragments into λ SE6 results in the replacement of the genes encoding ampicillin resistance as well as the λ CI repressor protein which is necessary for lysogeny (see Chapter 5). Genetic complementation assays can consequently be accomplished only in bacteria carrying a copy of the cI gene, e.g., such as λ lysogen. In this study, the genomic library was screened for clones which complemented the murH1, smhB1, lytD1, and lytE1 mutations by transducing λ lysogens carrying these mutations with the λ SE6 recombinant derivatives from the gene bank and selecting for Kan^r transductants at 42°C. Putative positive clones were then tested for Amp^s.

IX. DNA Sequencing

The strategy used for nucleotide sequencing is based on the pAA-PZ718 and pAA-PZ719 vector system first described by

Ahmed (126) and further modified by Peng and Wu (127). The DNA to be sequenced was cloned into the two vectors in opposite orientations by utilizing the polylinker sites. These vectors contain a transposon Tn9 insertion, and the fact that the excision of Tn9 promotes the deletion of neighboring DNA sequences is utilized to generate two series of overlapping deletions which extend into the DNA to be sequenced. These deletions were sized by agarose gel electrophoresis. Appropriate deletion derivatives were selected for sequencing by the dideoxynucleotide chain terminating method of Sanger (128) in the presence of [³⁵S]dATP (1000 Ci/mmol) from Amersham Corp. The sequencing was carried out with a T7 DNA polymerase sequencing kit obtained from Pharmacia.

X. Expression of Cloned DNA

The bacteriophage T7 RNA polymerase-promoter coupled system (129) was used to exclusively express a cloned gene. The DNA to be expressed was cloned in both orientations in front of the T7 promoter ϕ 10 in plasmid vectors pT7-3 and pT7-4. The resulting plasmids were then transformed into strain KX100 (130) carrying pGP1-2 which encodes T7 RNA polymerase. The transformants were grown in LB with selective drugs at 30°C to early exponential phase. A sample of 1 ml culture was centrifuged and the pellet was washed twice with M9 medium and then resuspended in 2 ml M9 medium supplemented with 0.2 ml methionine assay medium (Difco Laboratories). Cells were

further grown at 30°C for 1 h and shifted to 42°C for 20 min. Rifampin was then added to a final concentration of 200 µg/ml to inhibit selectively the host RNA polymerase. After 10 min of rifampin treatment, the cells were shifted down to 30°C for an additional 40 min. The sample was labeled with 10 µCi [³⁵S] methionine (800 Ci/mmol; Amersham Corp.) for 5 min at 30°C. The cell pellet was suspended in 120 µl of buffer (60 mM Tris-HCl pH 6.8; 1% SDS; 1% 2-mercaptoethanol; 10% glycerol; 0.01% bromophenol blue). Samples were heated to 95°C for 3 min and loaded (5-10 µl) on a SDS-polyacrylamide gel as described below.

Cell fractionation was carried out to localize the expressed gene products. The spheroplasting and lysis method of Boeke and Model (131) was used with some modifications. Cells (1 ml) with [³⁵S]methionine-labeled plasmid encoded proteins were pelleted, washed, and resuspended in 0.5 ml of buffer I which contained 20% sucrose, 100 mM Tris-HCl at pH 8.0, and 10 mM EDTA. Lysozyme (5 mg/ml) was added and the mixture was left on ice-water for 10 min. The mixture was centrifuged in a microcentrifuge for 2 min. The supernatant was saved as the periplasmic fractions. The spheroplast pellet was washed twice in buffer I and resuspended in 50 µl of buffer II (20% sucrose; 100 mM Tris-HCl pH 8.0; 10 mM MgCl₂) containing DNase (50 µg/ml). Ice-cold distilled water (22 µl) was added immediately. The spheroplasts were broken by the osmotic shock combined with six subsequent freeze (dry

ice in ethanol bath)-thaw (37°C water bath) cycles. Unbroken cells were separated by low-speed centrifugation (4,000 g, 30 min). The remainder was further centrifuged for 1 min in microcentrifuge. The supernatant represented the cytoplasmic fraction, and the pellet, which was resuspended in 200 μ l of distilled water, represented the membrane fraction. All fractions were then precipitated with 5% TCA, washed with acetone, dried, and resuspended in 50 μ l of SDS-PAGE sample buffer. After heating at 95°C for 3 min, 5-10 μ l amounts of the samples were analyzed by SDS-PAGE.

XI. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE of [³⁵S]-labeled proteins was carried out in 15% gels (0.75 mm thick) according to the protocol of Swank and Munkres (132). Protein molecular weight standards were from Sigma and Phamacia, and these included bovine albumin (66 kDal), egg albumin (45 kDal), glyceraldehyde-3-phosphate dehydrogenase (36 kDal), carbonic anhydrase (29 kDal), trypsinogen (24 kDal), trypsin inhibitor (20 kDal), α -lactalbumin (14 kDal), and fragment of sperm whale myoglobin cleavaged by CNBr (8 kDal). Samples were run at constant voltage (100 V for the stacking gel and 200 V for the separating gel). After drying, the gels were subjected to autoradiography.

XII. Southern Blot Analysis

Southern blot analyses were performed with a nonradioactive DNA labeling and detection kit obtained from Boehringer Mannheim. The DNA fragment to be used as a probe was purified by agarose gel electrophoresis. The probe was then labeled with digoxigenin (DIG)-11-dUTP. The DNA molecules to be probed were digested with the specified restriction endonucleases and subjected to electrophoresis on a 0.8% agarose gel. The separated fragments were first located by staining with ethidium bromide and then transferred to a Zeta-ProbeTM membrane (Bio-Rad) using 10 X SSC (1 X SSC is 0.15 M NaCl, 0.015 M sodium citrate), as described by Maniatis. (121). The transferred DNA fragments on the membrane were denatured, fixed, dried, and prehybridized with 10 ml hybridization solution [5 X SSC; blocking reagent, 0.5% (w/v); sodium N-lauroylsarcosinate, 0.1% (w/v); SDS, 0.02% (w/v)] for 1 h. The material on the membrane was then incubated with 10 ml of hybridization solution containing 1 µg of the DIG-11-dUTP-labeled probe. The hybridization was carried out in a sealed plastic bag for 14 h at 68°C. The membrane was washed, and DNA fragments which hybridized to the probe were detected by a color reaction. This color development was based on an immunological reaction between the hybridized DNA and anti-DIG conjugated alkaline phosphatase. The complex was detected with a color development solution consisting of 45 µl of 4-nitrobluetetrazolium chloride and 35 µl of 5-bromo-4-chloro-3-indolyl-phosphate at room temperature in the dark for 1-3

days.

XIII. Determination of Immunity to Phage λ

To determine the immunity of E. coli strains to phage λ infection, cells from fresh stationary phase cultures were collected by centrifugation and resuspended in one-half volume of sterile 10 mM MgSO_4 solution. Samples of 0.1 ml were added to 3 ml of molten λ top agar (112), and the mixtures were overlaid on LB plates. Samples of the phage λ derivatives, λ_{vir} (133) and λ_{cI857} (134), were spotted on the overlays. After an incubation period of 14 h, the plates were examined for evidence of λ -induced lysis.

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF THE murH1 MUTANT

I. Overview of Chapter Contents

The objective of the work described in this Chapter was to characterize a ts mutant of E. coli with apparent defects in PG metabolism. The mutation was assigned to a new locus which, in accordance with the current E. coli genetic terminology, was designated murH (a mnemonic for murein metabolism, locus H; "murein" is synonymous with "PG"). The phenotype associated with the murH1 mutant allele is shown to be associated with the inhibition of a late step in PG synthesis and PG hydrolase-mediated autolysis at the restrictive temperature. Attempts to clone the murH gene indicated that murH was unstable on high-copy-number vectors.

II. Bacteria, Bacteriophage and Plasmids

Table 1 lists the bacterial strains, bacteriophage and plasmids used in this study.

III. Results

1. Phenotypic Properties of the murH1 Mutant

Table 1. E. coli K-12 strains, bacteriophages and plasmids

Strain	Relevant Genotype/Description	Reference/Source
CP78	<u>thi-1</u> <u>thr-1</u> <u>leu-6</u> <u>his-65</u> <u>arg-46</u>	CGSC
JB1	<u>metB1</u> <u>uxuA1</u>	CGSC
JEF8	<u>thr-31</u> <u>carB8</u> <u>metB1</u>	CGSC
JM101	<u>thi</u> $\Delta(\text{lac-proAB})$ [F' <u>traD36</u> <u>lacI^q</u> ZAM15]	Laboratory collection
Q359	<u>hsdR</u> <u>hsdM⁺</u> P2 lysogen	(124)
VC7	<u>thi-1</u> <u>lysA23</u> <u>rpsL109</u>	Laboratory collection
VC462	VC7 <u>zaa-1::Tn5</u> <u>zaa-100::Tn10</u> <u>murH1</u>	This study
VC482	VC7 <u>zji-101::Tn10</u> <u>murH1</u>	This study
VC484	VC7 <u>murH1</u>	This study
VC486	W3104 <u>zaa-100::Tn10</u> <u>murH1</u>	This study
VC499	VC484 <u>recA</u>	This study
W3104	λ lysogen	Laboratory collection
W3110	wild type	Laboratory collection
Bacteriophage	Relevant Genotype/Description	Reference
λ SE6	Kan ^r Amp ^r	(124)

Table 1, continued

Plasmid	Relevant Genotype/Description	Reference/Source
pBR328	Amp ^r Cam ^r Tet ^r	Laboratory collection
pDD41	Kan ^r <u>murH</u> ⁺	This study
pHC79	Amp ^r Tet ^r <u>cos</u>	Laboratory collection
pLG338	Kan ^r Tet ^r	Laboratory collection
pUC18	Amp ^r	Pharmacia

The murH1 mutant was discovered fortuitously during the screening of a collection of spontaneous temperature-sensitive mutants which apparently had defects in PG metabolism. The temperature sensitivity was apparently associated with cellular autolysis. Thus, when cultures of strain VC462 (murH1) growing in either complex (data not shown) or minimal (Fig. 6A) medium at 30°C were shifted to 42°C, a decrease in culture turbidity occurred within one doubling time (based on the growth rate of the wild-type parent at 42°C). This loss in turbidity was correlated with the solubilization of PG which had been prelabeled with [³H]DAP (Fig. 6B). The lysis of strain VC462 at 42°C was prevented when growth was inhibited by amino acid deprivation, chloramphenicol treatment, or (Fig. 6A and B) by a combination of both methods. Since the temperature-dependent lysis exhibited by strain VC462 resembled the phenotypes of mutants defective in PG synthesis (78-85), the effect of temperature upshift on PG synthesis was determined. PG synthesis in VC462 was inhibited by 75% within 10 min after upshift from 30°C to 42°C (Fig. 7). This is significantly earlier than the time of onset of lysis, suggesting that lysis was a consequence of the inhibition of PG synthesis.

2. Site of Mutational Block

The step in PG synthesis blocked by the murH1 mutation was localized by using cultures of VC462 which were simulta-

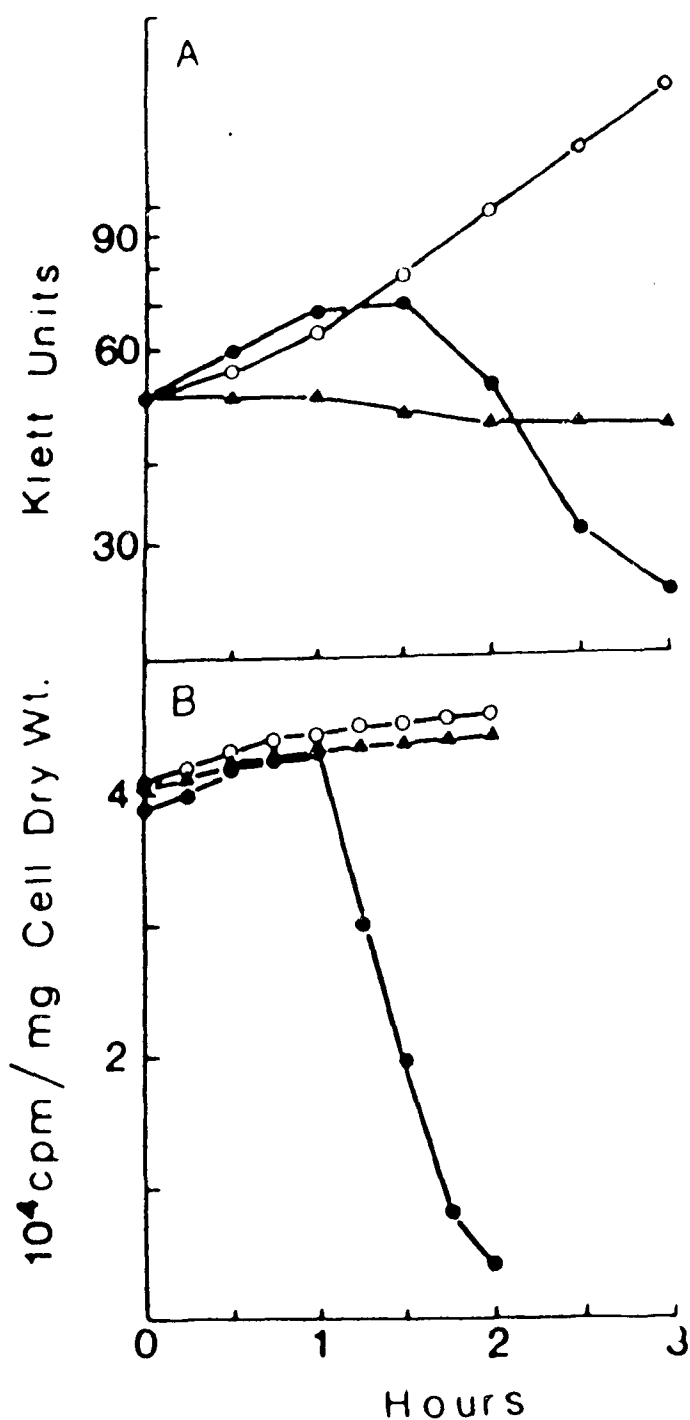


Fig. 6. Temperature-dependent autolysis of strain VC462 (*murH1*) and its prevention by inhibiting protein synthesis. Cultures were incubated at 30°C (○), 42°C (●), 42°C (▲) with 500 μ g of L-valine per ml and 200 μ g of chloramphenicol per ml. A. Lysis was determined turbidimetrically. B. Cellular peptidoglycan was pre-labeled with [3 H]DAP, and solubilization of the radiolabeled peptidoglycan was measured as an index of lysis.

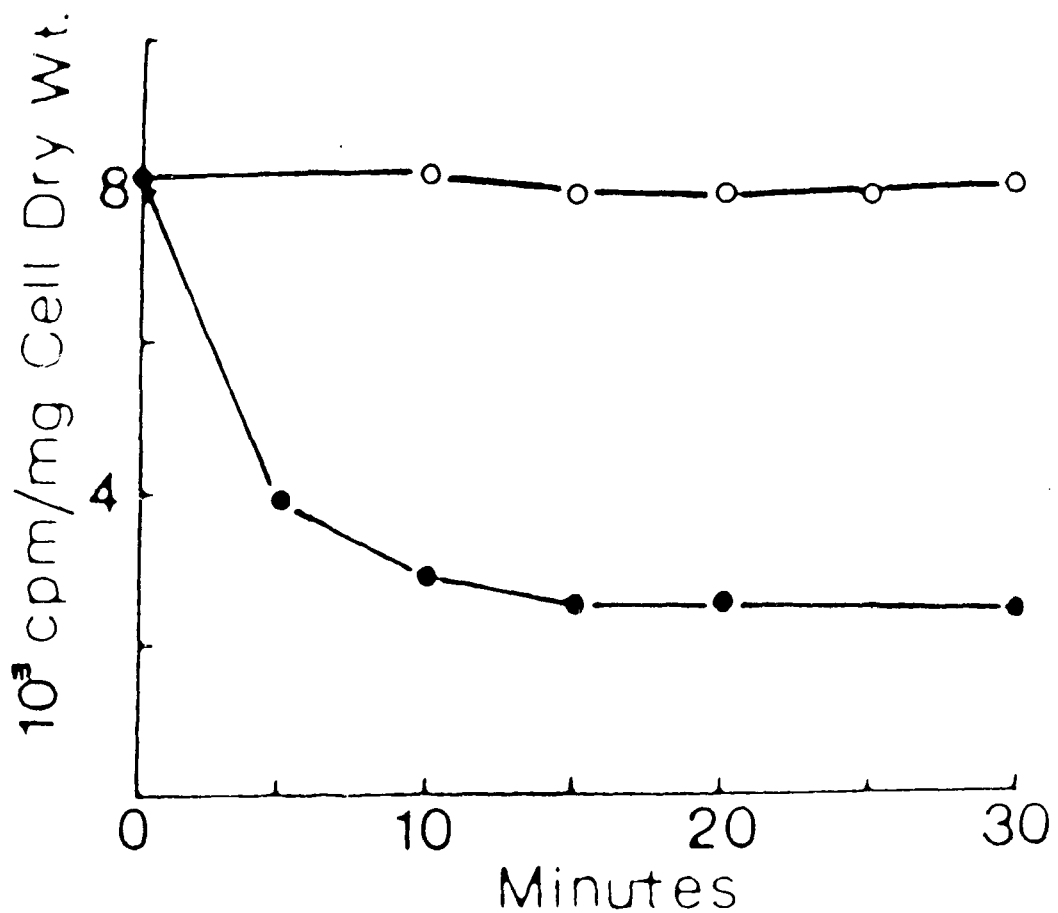


Fig. 7. Effect of growth temperature on peptidoglycan synthesis in strain VC462 (murH1). Samples from cultures incubated at 30°C (O) and 42°C (●) were pulse-labeled with ^3H DAP for 5 min and amounts incorporated into peptidoglycan fractions were determined. Average values of duplicate samples are plotted as a function of the sampling times.

neously amino acid-deprived and chloramphenicol-treated (Table 2). The main steps in PG synthesis in such nongrowing bacteria can be quantitatively monitored by following the incorporation of [³H]DAP into the various cell fractions (135, 106). Another important consideration here was that the progress of PG synthesis in strain VC462 could be followed at the nonpermissive temperature without the complication of lysis by using amino acid-deprived cells treated simultaneously with chloramphenicol, i.e., because lysis at 42°C was inhibited under these conditions (Fig. 6A and B). Nevertheless, these experiments were restricted to a 30-min period (i.e., before lysis became evident in cultures growing at 42°C) as an added precaution.

Cultures of VC462 were labeled with [³H]DAP at 30°C and 42°C for 30 min, and the amounts of radioactivity incorporated into the various cellular fractions were determined. The following results are from controls incubated at 30°C which confirm that PG synthesis in VC462 was subject to control by the stringent response (Table 2). (i) The amounts of radioactivity incorporated into the PG fractions by the untreated control culture and the chloramphenicol-treated, amino acid-deprived culture at 30°C were the same, indicating that chloramphenicol treatment relaxed PG synthesis as expected. (ii) A threefold accumulation of labeled UDP derivatives occurred in the treated culture, confirming that RelA function is necessary to prevent the overproduction of the UDP inter

Table 2. Cellular distribution of [³H]DAP incorporated by strain VC462 (murH1) at 30°C and 42°C

Treatment	Temperature	10 ³ cpm per mg cell dry weight		
		Peptidoglycan	UDP derivatives ^a	Lipid intermediates
Untreated control	30°C	43.8	3.6	1.1
Amino acid-deprived plus chloramphenicol	30°C	44.2	11.4	1.1
	42°C	25.5	16.8	1.3

^aMixture of UDP-MurNAc-tripeptide and UDP-MurNAc-pentapeptide

mediates during amino acid deprivation (105). (iii) The amounts of radioactivity in the lipid intermediate fractions were the same for both of the cultures.

To localize the step blocked in the murH1 mutant, the cellular distribution of radioactivity in amino acid-deprived cells treated with chloramphenicol at 42°C was compared with the data obtained from cells treated similarly at 30°C. The incorporation of [³H]DAP into the PG fraction was inhibited at 42°C by about 40% (Table 2). Otherwise the labeling patterns for the two sets of cells were identical. These results suggest that a late step in biosynthesis was mutationally blocked in VC462. To reinforce this conclusion, the labeled PG intermediates in the chloramphenicol treated cells were characterized further.

Over 90% of the UDP derivatives accumulated during chloramphenicol treatment at either 30°C or 42°C was accounted for as UDP-MurNAc-pentapeptide (data not shown). Similar results have been reported for wild type E. coli strains (105). This proves that the synthesis of neither UDP-MurNAc-pentapeptide nor UDP-GlcNAc (because UDP-GlcNAc is a precursor for the synthesis of UDP-MurNAc) was defective in VC462. The fact that there was no depletion of radiolabel from the lipid intermediate fraction also indicates that the synthesis of UDP derivatives was unaffected by the murH1 mutation.

The lipid intermediates in cells labeled as described in Table 2 were quantified further. Typical results are shown in

Table 3. Analysis of lipid intermediate fractions
of strain VC462 (murH1)

Sample	Growth temperature	10 ² cpm per mg cell dry weight			
		Peptido- glycan	Lipid int.	Disaccharide- pentapeptide	Un- known
un- hydrolyzed					
	30°C	492.3	12.1	-	-
	42°C	211.4	11.6	-	-
Hydrolyzed					
	30°C	487.4	-	7.6	2.2
	42°C	201.2	-	6.9	1.8

Table 3. The intracellular pools of labeled UDP derivatives and DAP were completely removed by boiling and washing the cell preparations. The PG and lipid intermediate fractions were the only detectable labeled components remaining under these conditions (the unhydrolyzed samples in Table 3). Portions of the boiled preparations were subjected to mild acid hydrolysis and then analyzed by paper chromatography. All of the labeled PG was consistently recovered after mild acid hydrolysis, and this component served as a convenient internal standard to assess the efficiency of recovery of other components. Hydrolysis of the lipid intermediate fraction was quantitative (i.e., no intact lipid intermediate was detectable following hydrolysis), but only about 60% of the radioactivity from the original starting material could be subsequently accounted for. The major product was identified as disaccharide-pentapeptide, and the only other labeled derivative was unidentifiable. We are certain, however, that the latter was not MurNAc-pentapeptide. In fact, no labeled MurNAc-pentapeptide was detected in these samples. These results suggest that the disaccharide-pentapeptide derivative was the major lipid intermediate. It is noteworthy that the lipid intermediate fractions from both 30°C- and 42°C-treated cells were identical. Therefore, although our analysis of the lipid intermediate fractions was far from ideal in terms of recovery, we feel that lipid intermediate synthesis was probably normal in VC462. If so, the defect must be in some

step involving the incorporation of disaccharide-peptide units from lipid carrier derivatives into PG.

3. Mapping of the murH Locus

To facilitate the mapping of murH, the zaa-100::Tn10, zji-100::Tn10, and zaa-1::Tn5 insertions linked to murH1 were obtained by screening random Tn10 and Tn5 insertion pools prepared on a prototrophic strain, W3110, as described in Chapter 2. The screening procedure involved the P_lvir-mediated transduction of the random Tn10 and Tn5 insertions into the murH1 mutant strain followed by the selection of temperature-resistant (i.e., murH⁺) drug-resistant transductants.

The initial mapping experiments were designed to localize the murH locus on the genetic linkage map (136). To accomplish this, the zaa-100::Tn10 insertion was transduced into a collection of Hfr strains. The location of the zaa-100::Tn10 insertion was then determined to be in the 0-min region of the linkage map through a series of conjugation experiments involving these Hfr strains and various appropriate recipient strains (data not shown). More precise mapping was accomplished by phage P1-mediated generalized transduction. Figure 8 shows the linkage map determined in this way. The murH locus was assigned to 99.2 min and appeared to represent a new locus.

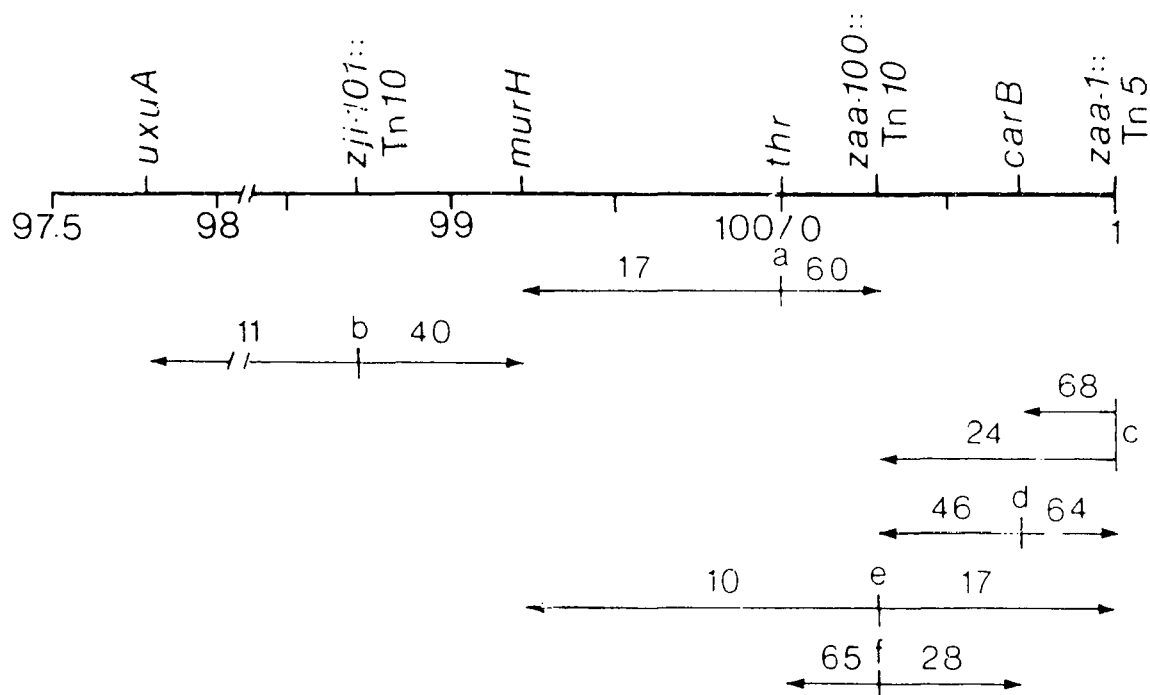


Fig. 8. Linkage map of the *murH* region. The results of the following crosses (recipient X donor) are shown with the number of recombinants scored in each case indicated parenthetically: a, CP78 X VC462 (148); b, JB1 X VC482 (183); c, JEF8 X VC462 (62); d, JEF8 X VC462 (100); e, JEF8 X VC462 (112); f, JEF8 X VC462 (112). For each set of arrows, the vertical line indicates the position of the selected marker and the arrowheads indicate the positions of the unselected markers. The numbers over the arrows represent the cotransduction frequencies (%).

4. Cloning of the murH gene

Multicopy vectors were used in initial attempts to clone the murH gene. Two separate genomic banks were prepared by ligating Sau3AI partial digests of chromosomal DNA from strain W3110 into the unique BamHI sites of pBR328 (137) and pHC79 (138). Clones carrying murH were not detected in either of these genomic banks despite repeated screening attempts. The possibility that the inability to clone murH may have been related to the high copy-number of these vectors (at least 20) was considered. Therefore, a third genomic bank was prepared by ligating a partial Sau3AI digest of strain W3110 chromosomal DNA into BamHI-restricted pLG338 (139), a plasmid with a low copy-number of 6 which encodes kanamycin resistance. Half of the ligation mixture was transformed into strain VC499 (murH1 recA). In this case, no kanamycin-resistant transformants were selected at 42°C. The other half of the ligation mixture was transformed into strain VC484 (murH1 recA⁺). A total of 4 kanamycin-resistant transformants were obtained by selection at 42°C. However, recombinant plasmids could not be recovered from these 4 transformants apparently because the plasmids had integrated or recombined into the chromosomes of these bacteria. This plasmid integration was presumably RecA-dependent because the phenomenon was not observed in strain VC499.

In view of the above results, the cloning of murH was attempted in the single copy vector designated λ SE6 which is

a derivative of phage λ (124). A partial Sau3AI digest of strain W3110 chromosomal DNA was ligated into the BamHI sites of λ SE6 as described in Chapter 2. The ligation mixture was packaged, amplified, and then transformed into the λ lysogen, strain VC486 (murH1), and kanamycin-resistant transformants were selected at 42°C. The murH gene was readily recovered from this bank, and the clone was designated pDD41.

5. Attempts to Subclone murH Gene from pDD41

As a preliminary step toward subcloning the murH gene from pDD41 to multicopy plasmid vectors, pDD41 was physically mapped with a selection of restriction endonucleases. Of the enzymes tested, only HindIII and BamHI were apparently useful for the purpose of subcloning, and each of these enzymes had two recognition sites in pDD41 (Fig. 9). There were no sites for EcoRI, SacI, SalI, XbaI, and XhoI. On the other hand, AccI, AvaI, HincII, PstI, and SphI cut pDD41 too frequently to be of use in subcloning, and the mapping of these restriction sites was not attempted.

On the basis of digestions with HindIII and BamHI, it was estimated that pDD41 contained a 13 kb fragment of E. coli chromosomal DNA (Fig. 9). An 11 kb portion of this fragment was released by BamHI digestion. HindIII digestion resulted in a 22 kb fragment which included the complete chromosomal insert and portions of the cloning vector, λ SE6. The subcloning of the BamHI and HindIII fragments into the unique BamHI

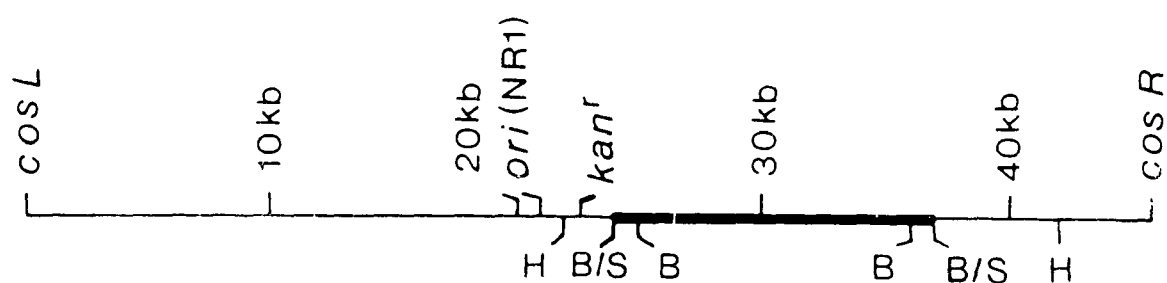


Fig. 9. Restriction map of pDD41, the λ SE6 murH^+ clone. The thin lines represent the left and right arms of the λ SE6 vector. The single-copy replication system and the kanamycin-resistance gene are indicated by ori (NR1) and Kan^r , respectively. The cohesive sites are indicated by cosL and cosR . The thick line represents the 13 kb BamHI - Sau3AI insert containing the murH^+ gene. The restriction sites are: H, HindIII ; B, BamHI ; B/S, BamHI - Sau3AI hybrid site.

and HindIII sites of pUC18 was then attempted. Both of the fragments to be subcloned were first purified by agarose gel electrophoresis and then ligated into appropriately restricted pUC18 (140). In our initial attempts, the ligation mixtures were transformed into strain VC484 (murH1), and ampicillin-resistant transformants were selected at 30°C and 42°C. No transformants were obtained at 42°C. Furthermore, all of the transformants selected at 30°C were still temperature-sensitive. An analysis of the plasmids carried by these transformants indicated that, in all cases, portions of the BamHI or HindIII inserts (presumably including the murH locus) had been deleted. These results suggested that the murH locus in multicopy was lethal. To gain further evidence for this, the attempts to subclone the BamHI and HindIII fragments were repeated in strain JM101 (i.e., a murH⁺ strain) with the selection for ampicillin-resistant, white color transformants being carried out on IPTG X-gal plates at 37°C. An analysis of plasmids purified from transformants picked at random (a total of 25 HindIII and 30 BamHI clones) again indicated that, in all cases, portions of the inserts had been deleted. Furthermore, the deletions appeared to have occurred at random sites as evidenced by the heterogeneity in the size distributions of the inserts. This is illustrated in Fig. 10 with a collection of these plasmids which have been digested with BamHI. Lane 6 represents pDD41, the source of the original 11 kb BamHI fragment carrying the putative murH locus. Lanes 1-5

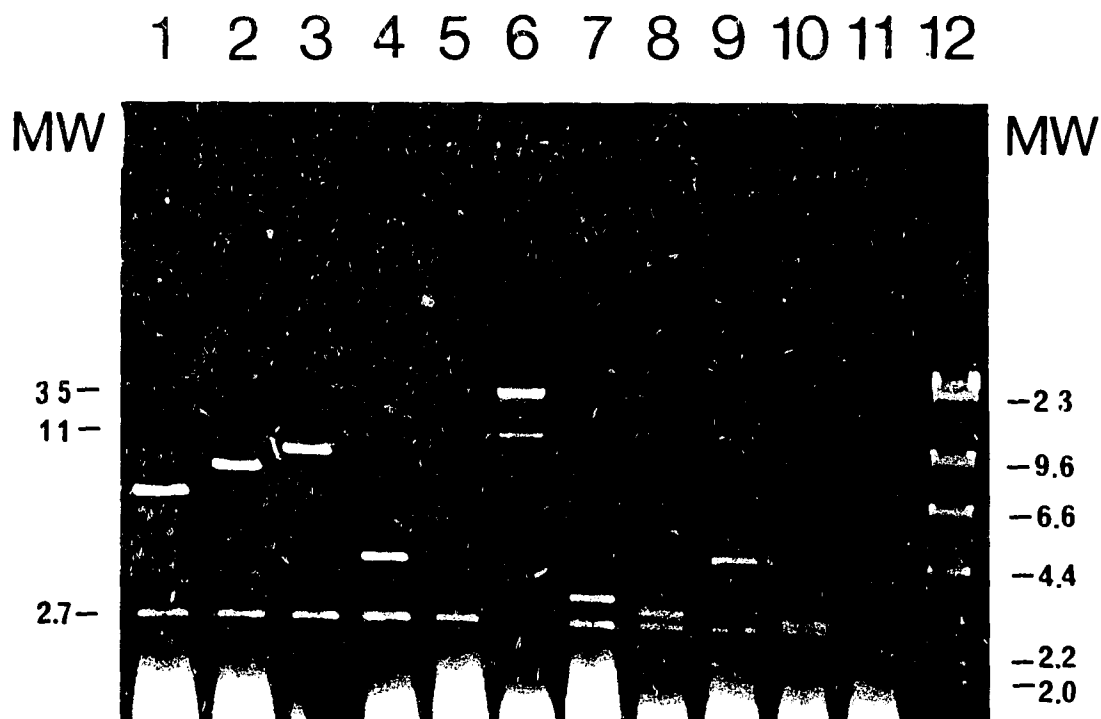


Fig. 10. Electrophoretic analysis of the size of the inserts in the recombinant plasmids obtained from the subcloning of the 11 kb BamHI fragment, derived from pDD41, into pUC18. pDD41 cleaved with BamHI is shown in lane 6; the top band represents the 33 kb λ SE6 vector plus the 2 kb portion of the insert, and the lower band is the 11 kb BamHI fragment of the insert (see Fig. 9). Recombinant plasmids from 10 independent isolates digested with BamHI are shown in lanes 1-5 and 7-11. The 2.7 kb band common to all of these plasmids is the pUC18 vector. Note the heterogeneity in the size distributions of the inserts, all of which carry deletions of varying sizes. Note that bands of less than 2 kb are not visible because of the contaminating RNA. Lane 12 contains DNA molecular weight standards as indicated by the numbers at the right (kb).

and 7-11 represent pUC18 derivatives carrying deletions within the 11 kb BamHI insert. The 2.7 kb component common to all of these derivatives represents the vector, pUC18. The deletion derivatives of the insert range from 9 to less than 2 kb. In one last attempt, the 11 kb BamHI fragment was subcloned into the low copy-number vector pLG338, and transformed into JM101. Again, in all 25 random selected clones, portions of the 11 kb BamHI fragment had been deleted (data not shown).

IV. Discussion

Cellular autolysis occurs whenever PG synthesis is blocked in growing bacteria. The basis for this is unclear. However, as mentioned in Chapter 1, PG synthesis may involve the coordinated activities of the biosynthetic enzymes and the PG hydrolases. One view is that the hydrolase activities become dissociated when a biosynthetic step is blocked, and lysis results. Thus, strain VC462 resembled other previously described mutants with defects in PG synthesis in terms of the lysis phenotype it exhibited at the nonpermissive temperature. This defect was established in VC462 by demonstrating the inhibition of [³H]DAP incorporation into PG at 42°C.

To analyze the function of the murH locus, the incorporation of [³H]DAP was traced in VC462 cultures which were simultaneously amino acid-deprived and chloramphenicol-treated. As expected, the normal tight coupling between the

soluble and the particulate enzyme systems was disrupted under these conditions, and UDP-MurNAC-pentapeptide accumulated at the permissive temperature of 30°C. The fact that UDP-MurNAC-pentapeptide also accumulated at the nonpermissive temperature of 42°C clearly indicates that all of the soluble enzyme activities were normal. As already noted, this includes the synthesis of UDP-GlcNAC because this derivative is a precursor of UDP-MurNAC.

Further identification of the biosynthetic step blocked by the mutation in murH1 has posed some difficulties. These are related mainly to the low levels of lipid intermediates normally present in E. coli and to the incomplete recoveries experienced during fractionation of these intermediates. These technical problems will not necessarily be easy to overcome. The maximum level to which the lipid intermediates can accumulate under conditions of relaxed control is fixed by the level of undecaprenyl phosphate present, and this appears to be low. Our results suggest that the disaccharide-pentapeptide derivative was the major product synthesized by VC462, and the amounts observed at the permissive and nonpermissive temperatures were identical. We therefore tentatively conclude that the synthesis of lipid intermediates was not affected by the mutation in VC462. The same conclusion was drawn from results (data not shown) obtained with the conventional in vitro assay method using particulate cell envelope preparations. It is also worth noting that the only known

mutation affecting lipid intermediate synthesis has been assigned to the mrB locus at 90 min and is thus unlinked to murH.

These results suggest that the murH1 allele most likely conferred a defect in some aspect of the terminal step in PG synthesis. However, the work presented here does not allow us to draw any conclusions concerning the precise action of murH1 on PG metabolism. Nevertheless, the work described in subsequent chapters suggests that the murH1 mutation may not be directly eliciting an inhibitory effect on PG synthesis; i.e., murH1 may be a pleiotropic mutation which may cause, among other things, an alteration in a membrane-associated function which indirectly upsets PG synthesis. There are clear indications that the final steps in PG synthesis are complex and not completely understood (17). It is possible that these reactions are influenced, in general, by other membrane functions.

The difficulties encountered in cloning murH were apparently due to the toxicity of either murH or a closely linked marker when present in multiple copies. It is noteworthy that this toxicity extended to a relatively low copy-number of 6 as demonstrated with pLG338. Our results suggest that the toxic marker lies within the 11 kb BamHI fragment derived from the chromosomal DNA inserted in pDD41. Our results also suggest that the murH1 allele was apparently recessive and could be complemented by pDD41.

Other examples of E. coli genes which are toxic in multicopy have been reported. These include the following: (i) the closely linked pbpA and dacA genes which encode penicillin-binding proteins 2 and 5, respectively (141); (ii) the ompA which encodes a major outer membrane structural protein (142); (iii) the dnaA gene which encodes a key protein involved in the initiation of chromosomal DNA replication as well as in the regulation of gene expression (143); (iv) the polA gene which encodes DNA polymerase I (144, 145); and (V) the lpp which encodes lipoprotein (146). It is worth noting that these examples are gene products which are either found in the cell envelope or are involved in regulatory functions but whether the murH gene product falls into any of these categories remains to be determined.

The murH⁺ locus was detectable when it was subcloned in the relatively low copy-number vector, pLG338, provided that recA⁺ murH1 hosts were used. However, the recombinant plasmids could not be recovered from the transformants, presumably because the plasmids had integrated into the chromosome through a process of homology-dependent recombination. Thus, the multicopy toxicity was alleviated by integration of the plasmid into the chromosome and curing of the autonomous copies of the plasmid. These were apparently rare events as evidenced by the low frequency of occurrence of such transformants. When vectors with significantly higher copy-numbers were used (e.g., pBR328, pHC79, or pUC18),

temperature-resistant transformants of this type were never encountered. Whether this may be related to the higher copy-number of the vector is not known. But it seems reasonable that the higher copy-number could result in a relatively higher level of toxicity, and this may preclude the selection of plasmid integration derivatives perhaps by decreasing the probability of plasmid curing. The apparent high-copy toxicity of the murH region was also exhibited in murH⁺ strains as illustrated by the attempts to subclone murH in pUC18 in strain JM101. All of the transformants carried plasmids with deletions within the original chromosomal DNA insert containing the putative murH locus. In view of the low frequency at which these clones appeared, it would appear that we had selected rare spontaneous deletions. The phenomena of plasmid-chromosome recombination and spontaneous deletions have been encountered before as apparent means of reducing the copy-number of toxic genes (e.g., see ompA in ref. 142)

CHAPTER 4

EXTRAGENIC SUPPRESSORS OF murH1: FOUR NEW GENES (smhA, smhB,
lytD, and lytE) APPARENTLY FUNCTIONALLY RELATED TO murH

I. Overview of Chapter Contents

Because it was not possible to characterize the cloned murH gene, an alternative approach was taken which involved the isolation of extragenic mutations which suppress murH1. Suppressor mutations are mutations occurring either within or outside an original mutant gene which are able to correct the phenotypic defect of the original mutant (147, 148). Such second-site mutations have often been a useful genetic approach for identifying the functional interactions of a specific gene product with products of other genes and for obtaining additional mutations in previously unidentified genes whose products are functionally related to that of the original mutant gene. Two extragenic suppressors of murH1 are characterized in this study which apparently represented new loci and were designated smhA and smhB (mnemonics for sup-pressor of murH, loci A and B). Two additional mutations, also representing new loci, arose spontaneously in the smhA and smhB mutants. These loci were designated lytE and lytD to highlight the ts lysis phenotypes associated with them. The

results presented below are divided into 2 parts. The first part deals with the smhB and lytD mutants, and the second part deals with the smhA and lytE mutants. Finally, the possibility that these loci are functionally related is discussed.

II. Bacterial Strains

The E. coli strains used in this chapter are listed in Table 4 (for the studies on smhB1 and lytD1) and Table 8 (for the studies on smhA1 and lytE1). The sets of isogenic strains carrying various combinations of wild type and mutant alleles of the murH, smhA, smhB, lytD, and lytE loci, which are described below, were constructed in order to assess the phenotypic characteristics associated with these loci. The genotypes of these isogenic strains were confirmed by backcrossing the various markers into strain VC7 by phage Pl_{vir}-mediated transduction using appropriate linked transposon insertions as means of selection.

III. Results: the smhB and lytD Mutants

1. Isolation of the smhB1 Mutant

Since the murH locus was apparently essential for growth, we considered it possible that suppressors of the murH1 (ts) allele may occur in other essential genes. In order to be detectable, such extragenic suppressor mutations would

Table 4. E. coli K-12 strains

Strain	Relevant Genotype/Description	Reference/Source
CSH57B	<u>leu</u> <u>purE</u> <u>trp</u> <u>his</u> <u>met</u> <u>ilv</u> <u>argG</u> <u>thi</u> <u>ara</u> <u>lac</u> <u>xyl</u> <u>mtl</u> <u>gai</u> <u>rpsL</u>	CSH
CV2-4	<u>tonA22</u> <u>phoA8</u> <u>ush-1</u> <u>ompF627</u> <u>fadL701</u> <u>glpD3</u> <u>glpR2</u> <u>pit-10</u> <u>spoT1</u>	CGSC
JF701	<u>proC24</u> <u>aroA357</u> <u>ompC264</u> <u>his-53</u> <u>purE41</u> <u>met-65</u> <u>ilv-277</u> <u>lacY29</u> <u>xyl-14</u> <u>rpsL97</u> <u>cycA1</u> <u>cycB2?</u> <u>tsx-63</u>	CGSC
TKL11	<u>thr-1</u> <u>leuB6</u> <u>murE1</u> <u>codA1</u> <u>pyrF101</u> <u>his-108</u> <u>thyA6</u> <u>argG66</u> <u>ilvA634</u> <u>thi-1</u> <u>deoC1</u> <u>lacY1</u> <u>tonA21</u> <u>tsx-95</u> <u>supE44</u>	CGSC
TKL46	<u>thr-1</u> <u>leuB6</u> <u>murF2</u> <u>codA1</u> <u>pyrF101</u> <u>his-108</u> <u>thyA6</u> <u>argG66</u> <u>ilvA634</u> <u>thi-1</u> <u>deoC1</u> <u>lacY1</u> <u>tonA21</u> <u>tsx-95</u> <u>supE44</u>	CGSC
UT181	<u>cysS818</u> <u>zbc104::Tn10</u> <u>metA</u> <u>rpoB</u> <u>argG</u> <u>thi</u> <u>nalA</u>	L. Isaacson ^a
VC7	<u>thi-1</u> <u>lysA23</u> <u>rpsL209</u>	Laboratory Collection
VC460	VC7 <u>zaa-1::Tn5</u> <u>murH1</u>	This study
VC461	VC7 <u>zaa-1::Tn5</u>	This study
VC494	VC460 <u>smhB1</u>	This study
VC4007	VC494 <u>zbc-103::Tn10</u>	This study
VC4007a	VC4007 <u>lytD1</u>	This study
VC4014	VC7 <u>zbc-103::Tn10</u> <u>lytD1</u>	This study
VC4023	VC7 <u>zbc-103::Tn10</u>	This study
VC4024	VC7 <u>zbc-103::Tn10</u> <u>lytD1</u> <u>smhB1</u>	This study
VC4073	VC7 <u>lytD1</u>	This study
VC4076	VC460 <u>zbc-103::Tn10</u> <u>smhB1</u>	This study

Table 4, continued

VC4077	VC7 <u>zbc-103::Tn10</u> <u>smhB1</u>	This study
VC4110	JF701 <u>purE⁺ zbc-103::Tn10</u>	This study
VC4111	JF701 <u>zbc-103::Tn10</u>	This study
VC4117	VC7 <u>lytD1 zbc-103::Tn10</u>	This study
VC4132	VC460 <u>zbc-103::Tn10</u>	This study
VC4133	VC461 <u>zbc-103::Tn10</u> <u>smhB1</u>	This study
VC4134	VC461 <u>zbc-103::Tn10</u>	This study
VC4174	JF701 <u>zbc-104::Tn10</u>	This study
VC4189	TKL11 <u>zbc-103::Tn10</u> <u>smhB1</u>	This study
VC4190	TKL46 <u>zbc-103::Tn10</u> <u>smhB1</u>	This study

^aDepartment of Microbiology, Biomedical Center, Uppsala, Sweden. The Tn10 allele number was not provided, and the number shown is one that we assigned in this laboratory.

obviously have to be of the conditional variety, e.g., with a *ts* phenotype. We designed the following procedure for the isolation of such mutants (Fig.11). Strain VC460 (murH1) was plated on TSA and incubated at 42°C. Spontaneous temperature-resistant derivatives appeared at a frequency of 3×10^{-6} within 2 days of incubation, and further incubation did not result in the appearance of more colonies. As shown below, the majority of these temperature-resistant derivatives were pseudorevertants carrying extragenic suppressor mutations in the smhA locus, or at least closely linked to it. These temperature-resistant colonies were scored, and the plates were then incubated for an additional 24 hours at 30°C. The temperature downshift resulted in the further appearance of a small number of colonies. This procedure was designed to select derivatives carrying suppressors which prevented expression of the lethal lysis phenotype associated with the murH1 allele at 42°C but which could not restore colony-forming capability because the suppressors themselves conferred a *ts* phenotype. We reasoned that it may be possible to rescue such double mutants by a temperature downshift. One isolate obtained by this method (designated strain VC494) was characterized, and as confirmed below, much of our rationale was substantiated. The presence of the murH1 *ts* allele in strain VC494 was confirmed by cotransducing the locus into strain VC7 with direct selection for the linked zaa-1::Tn5 marker. This preliminary experiment also indicated that the

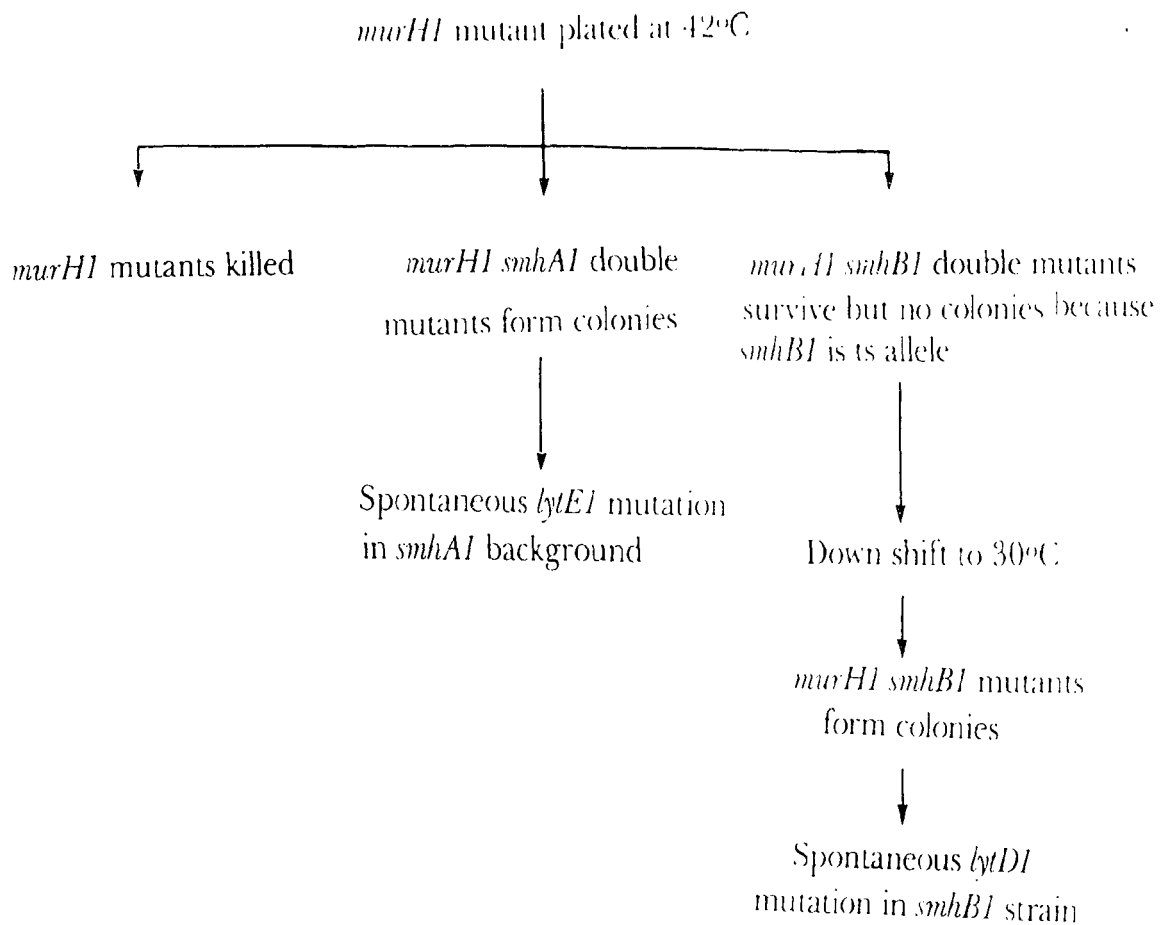


Fig. 11. Procedure used for the isolation of two murH1 suppressor mutations (smhA1 and smhB1), and two additional temperature-sensitive lysis mutations (lytE1 and lytD1). For details, see text.

suppressor mutation in VC494, designated smhB1, was not linked to murH locus.

2. Isolation of the lytD Mutant and Genetics of smhB and lytD

To facilitate the manipulation of smhB1, the zbc-103::Tn-10 insertion closely linked to smhB1 (98% cotransduction) was obtained by screening random Tn10 insertion pools prepared on the smhB1 mutant strain, VC494, with the phage NK55 as described in Chapter 2. The screening procedure involved the transduction of the random insertions into the murH1 strain, VC460. The Tn10 insertion was localized to the 10 to 20 min region of the linkage map in interrupted mating experiments involving a collection of Hfr strains carrying the Tn10 insertion and appropriate F⁻ strains. Subsequent linkage mapping by phage Plyir-mediated transduction placed both the Tn10 insertion and the smhB1 locus at 12.5 min on the E. coli linkage map (136) as summarized in Fig. 12. The insertion was consequently designated zbc-103::Tn10. It should be noted that the order of zbc-103::Tn10 and smhB relative to the purE locus is uncertain.

The lytD1 mutation apparently arose spontaneously in strain VC4007. The relevant genotype of VC4007 was murH1 smhB1 zbc-103::Tn10. The derivative carrying the lytD1 mutation was designated VC4007a (Table 4) to distinguish it from its parent strain, VC4007. The lytD1 allele was accidentally discovered during the construction of new smhB1

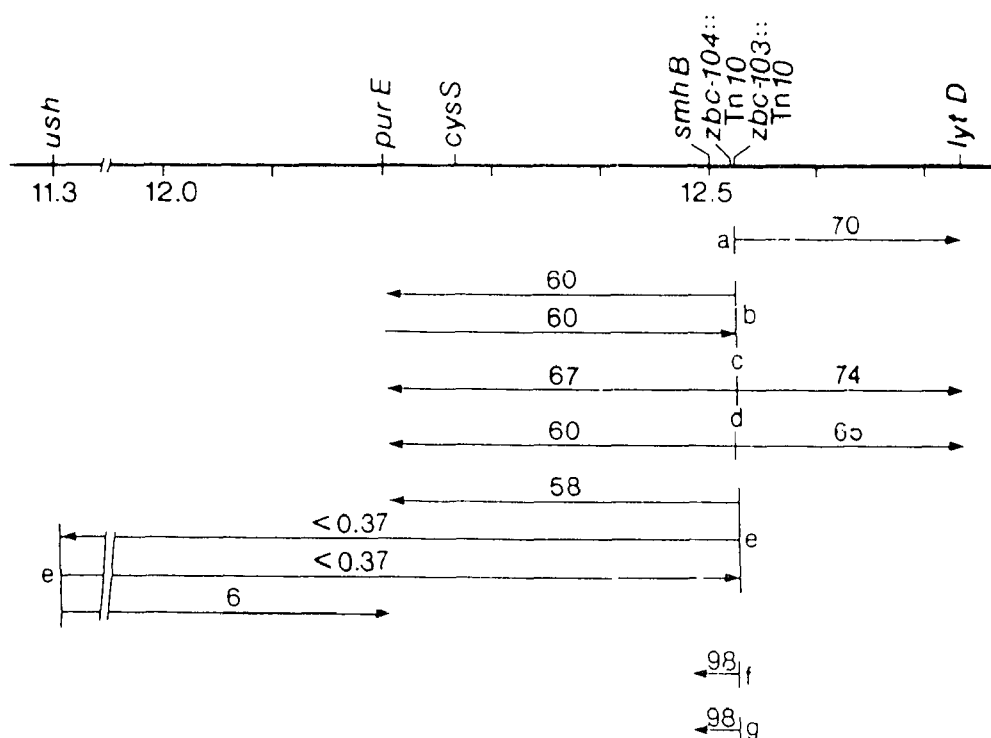


Fig. 12. Linkage map of the *smhB* and *lytD* loci region. The results of the following crosses (recipient X donor) are shown, with the number of recombinants scored in each case indicated parenthetically: a, VC4073 X VC4110 (100); b, JF701 X VC4110 (100); c, VC4073 X VC4111 (90); d, VC4073 X VC4174 (92); e, CV2-4 X VC4111 (270); f, VC7 X VC4133 (150); g, VC460 X VC4133 (200). For each set of arrows, the vertical line indicates the position of the selected marker and the arrowheads indicate the positions of the unselected markers. The numbers represent the cotransduction frequencies (%).

derivatives using what turned out to be VC4007a as the donor. The zbc-103::Tn10 insertion was used as a convenient, cotransducible selective marker for the ts smhB1 allele in such constructions. The lytD1 mutation was also a ts allele which was linked to zbc-103::Tn10. However, the phenotype conferred by lytD1 was distinguishable from smhB1 (see below), and this resulted in its discovery. The lytD locus was mapped at 12.7 min, about 0.2 min from smhB. The location of lytD relative to the zbc-103::Tn10 and zbc-104::Tn10 insertions and the purE locus was established by the series of three-factor crosses summarized in Table 5. As discussed below, there were technical problems in performing large scale screenings for the smhB1 and lytD1 markers, and we therefore do not present data for crosses between these two markers. Nevertheless, we placed both Tn10 insertions between smhB and lytD because in crosses where the Tn10 insertions were in the donor strains and served as the selected marker, the donor lytD and smhB alleles could be readily segregated, and recombinants inheriting both lytD and smhB donor alleles were comparatively rare (data not shown).

3. Temperature Sensitivity of the smhB1 and lytD1 Mutants

Two sets of isogenic strains carrying combinations of the wild type and mutant alleles of the murH, lytD and smhB loci were constructed to assess the characteristics of smhB1 and its effects on murH1 and lytD1. The genotypes were confirmed

Table 5. Three-factor cross analyses for zbc-103::Tn10,
zbc-104::Tn10, purE, and lytD1

Donor	Recipient	Selected marker ^a	Unselected markers	No. of transductants
VC4111 (<u>Tn10</u> <u>purE</u>)	VC4073 (<u>lytD1</u>)	<u>Tn10</u>	<u>lytD</u> ⁺ <u>purE</u>	43
			<u>lytD</u> ⁺ <u>purE</u> ⁺	25
			<u>lytD1</u> <u>purE</u>	18
			<u>lytD1</u> <u>purE</u> ⁺	4
VC4174 (<u>Tn10</u> <u>purE</u>)	VC4073 (<u>lytD1</u>)	<u>Tn10</u>	<u>lytD</u> ⁺ <u>purE</u>	35
			<u>lytD</u> ⁺ <u>purE</u> ⁺	25
			<u>lytD1</u> <u>purE</u>	19
			<u>lytD1</u> <u>purE</u> ⁺	13
VC4117 (<u>Tn10</u> <u>lytD1</u>)	CSH57B (<u>purE</u>)	<u>Tn10</u>	<u>lytD</u> ⁺ <u>purE</u>	14
			<u>lytD</u> ⁺ <u>purE</u> ⁺	31
			<u>lytD1</u> <u>purE</u>	31
			<u>lytD1</u> <u>purE</u> ⁺	18

^aStrains VC4111 and VC4117 carry zbc-103::Tn10 and strain VC4174 carries zbc-104::Tn10.

by backcrossing the murH, lytD, and smhB loci from these strains into strain VC7 using appropriate linked transposon insertions as the means of selection. Table 6 compares the colony-forming capabilities of the strains on 3 different media at 30°C and 42°C. Strains VC4132 (murH1) and VC4014 (lytD1) were ts on all 3 media, in contrast to their wild type counterparts, strains VC4134 and VC4023. On the other hand, the smhB1 mutants, VC4133 and VC4077 were ts on NA but not on TSA. The ts colony-forming capabilities of both VC4133 and VC4077 on NA were suppressed by the addition of 1% NaCl to the medium, suggesting that their abilities to form colonies on TSA at 42°C were due to the relatively high salt concentration in this medium. Strain VC4076 (murH1 smhB1) had a temperature-resistant phenotype on TSA indicating that the smhB1 allele was indeed a suppressor of the murH1 mutation. Furthermore, the suppressor activity of smhB1 was apparently salt-dependent because VC4076 was ts on NA, but not on NA containing 1% NaCl. The smhB1 allele also suppressed the ts phenotype associated with lytD1 in strain VC4024 in a salt-dependent fashion.

The effects of the smhB1 mutation on the survival of strains carrying either the murH1 or the lytD1 mutations at 42°C were determined. Serial dilutions of stationary phase cultures of the various strains were plated on NA (6 plates per dilution); it is important to note that survival was measured under conditions where the smhB1 allele was not being

Table 6. Colony formation by isogenic lytD1 smhB1 and murH1 smhB1 strains as a function of growth medium and temperature

Strain and Relevant Genotype	CFU per ml at 42°C/CFU per ml at 30°C		
	TSA	NA	NA + 1%NaCl
VC4076 (<u>murH1 smhB1</u>)	0.7	2.3×10^{-7}	0.8
VC4132 (<u>murH1 smhB⁺</u>)	3.1×10^{-6}	3.4×10^{-7}	5.2×10^{-7}
VC4133 (<u>murH⁺ smhB1</u>)	0.9	1.4×10^{-7}	0.9
VC4134 (<u>murH⁺ smhB⁺</u>)	1.0	0.8	0.8
VC4024 (<u>lytD1 smhB1</u>)	0.8	1.5×10^{-7}	0.9
VC4014 (<u>lytD1 smhB⁺</u>)	5.0×10^{-6}	1.1×10^{-8}	2.3×10^{-5}
VC4077 (<u>lytD⁺ smhB1</u>)	0.9	2.1×10^{-7}	0.9
VC4023 (<u>lytD⁺ smhB⁺</u>)	1.1	0.7	0.8

salt-suppressed. Two plates of each dilution were incubated at 30°C, and the remainder were placed at 42°C. After 48 h, the colonies on all plates were counted, and duplicate plates of each dilution were transferred from 42°C to 30°C. After 72 h, the colonies on all plates were recounted. No differences were seen in the 48- and 72-h counts of the sets of plates incubated at 30°C and at 42°C, and the data from the 72-h plates are summarized in Table 7. As shown above (Table 6), all of the strains used in these experiments plated poorly at 42°C, and the colonies which did appear were apparently those of spontaneous temperature-resistant derivatives. The colonies which subsequently appeared upon downshift to 30°C presumably represented those of ts cells which survived the 48-h incubation period at 42°C, and the magnitude of the increases in the plate counts after the temperature downshift therefore reflected the relative survival capabilities of the strains. The temperature downshift did not change the plate counts of the smhB1 mutant strains, VC4133 and VC4077 (Table 7), meaning that they survived poorly during incubation at 42°C. In contrast, the temperature downshift resulted in increases in the plate counts for all of the other strains. Thus, the plate counts increased 13-fold for VC4132 (murH1), 2.4 X 10⁴-fold for VC4076 (murH1 smhB1), 300-fold for VC4014 (lytD1), and 1 X 10⁵-fold for VC4024 (lytD1 smhB1). These results indicate that the murH1 and the lytD1 mutants exhibited some survival capacity at 42°C, but the survival of

Table 7. Comparative survival of murH1 smhB1 and lytD1 smhB1 strains at 42°C on NA

Strain	Relative colony formation	
	42°C ^a	42°C to 30°C Downshift ^b
VC4076 (<u>murH1 smhB1</u>)	3.2 x 10 ⁻⁷	7.6 x 10 ⁻³
VC4132 (<u>murH1 smhB</u> ⁺)	2.4 x 10 ⁻⁷	3.2 x 10 ⁻⁶
VC4133 (<u>murH</u> ⁺ <u>smhB1</u>)	8.0 x 10 ⁻⁷	9.5 x 10 ⁻⁷
VC4024 (<u>lytD1 smhB1</u>)	1.1 x 10 ⁻⁷	1.5 x 10 ⁻²
VC4014 (<u>lytD1 smhB</u> ⁺)	1.7 x 10 ⁻⁸	6.2 x 10 ⁻⁶
VC4077 (<u>lytD</u> ⁺ <u>smhB1</u>)	2.2 x 10 ⁻⁷	3.7 x 10 ⁻⁷

^aCFU per ml at 42°C divided by CFU per ml at 30°C. These determinations are based on plate counts after 72 h of incubation.

^bCFU per ml after 42°C to 30°C downshift divided by CFU at 30°C. These determinations are based on plate counts after 72 h of incubation.

these mutants was markedly enhanced by the introduction of the smhB1 allele.

4. Temperature-Dependent Lysis Phenotypes and Lysis Suppressor Activities of the smhB Mutant

Fig. 13 shows the growth of strain VC4133 (smhB1) in NB and NB containing 1% NaCl at 30°C and 42°C. VC4133 lysed in NB at 42°C, and lysis was, therefore, apparently responsible for the inability of VC4133 to form colonies on NA at 42°C (Table 6). The temperature-dependent lysis of VC4133 in NB was suppressed by the addition of 1% NaCl, suggesting that the ability of VC4133 to form colonies on NA containing 1% NaCl (Table 6) was at least partly attributable to a salt-dependent inhibition of the smhB1-associated lysis phenotype. However, growth was still severely inhibited in NB with 1% NaCl, at least over the short duration of this experiment (150 min). It is possible that solidification of the medium also played a role in enhancing colony formation on NA with 1% NaCl.

Fig. 14 shows the effects of smhB1 on the lysis phenotype conferred by murH1. Strain VC4132 (murH1) lysed in NB at 42°C, and this lysis was not prevented by the addition of 1% NaCl. Strain VC4076 (murH1 smhB1) did not lyse in either medium, although growth was severely inhibited. Thus, the individual lysis phenotypes of murH1 and smhB1 were suppressed when the two alleles were combined.

The phenotypes of the lytD smhB isogenic strains are

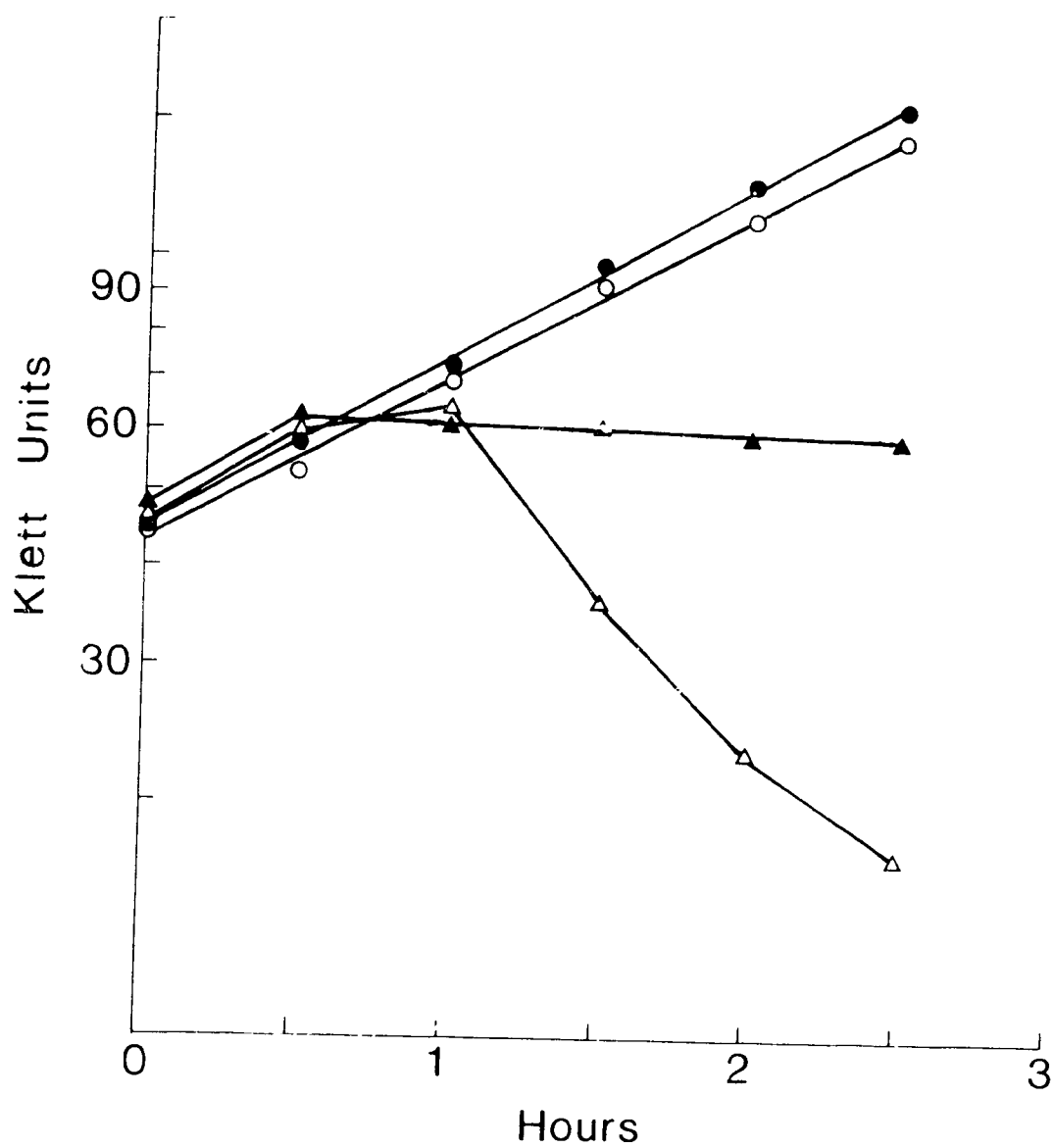


Fig. 13. Temperature-dependent autolysis of strain VC4133 (smhB1). Growth of VC4133 at 30°C (O, ●) and 42°C (Δ, ▲) in NB (open symbols) and NB containing 1% NaCl (closed symbols).

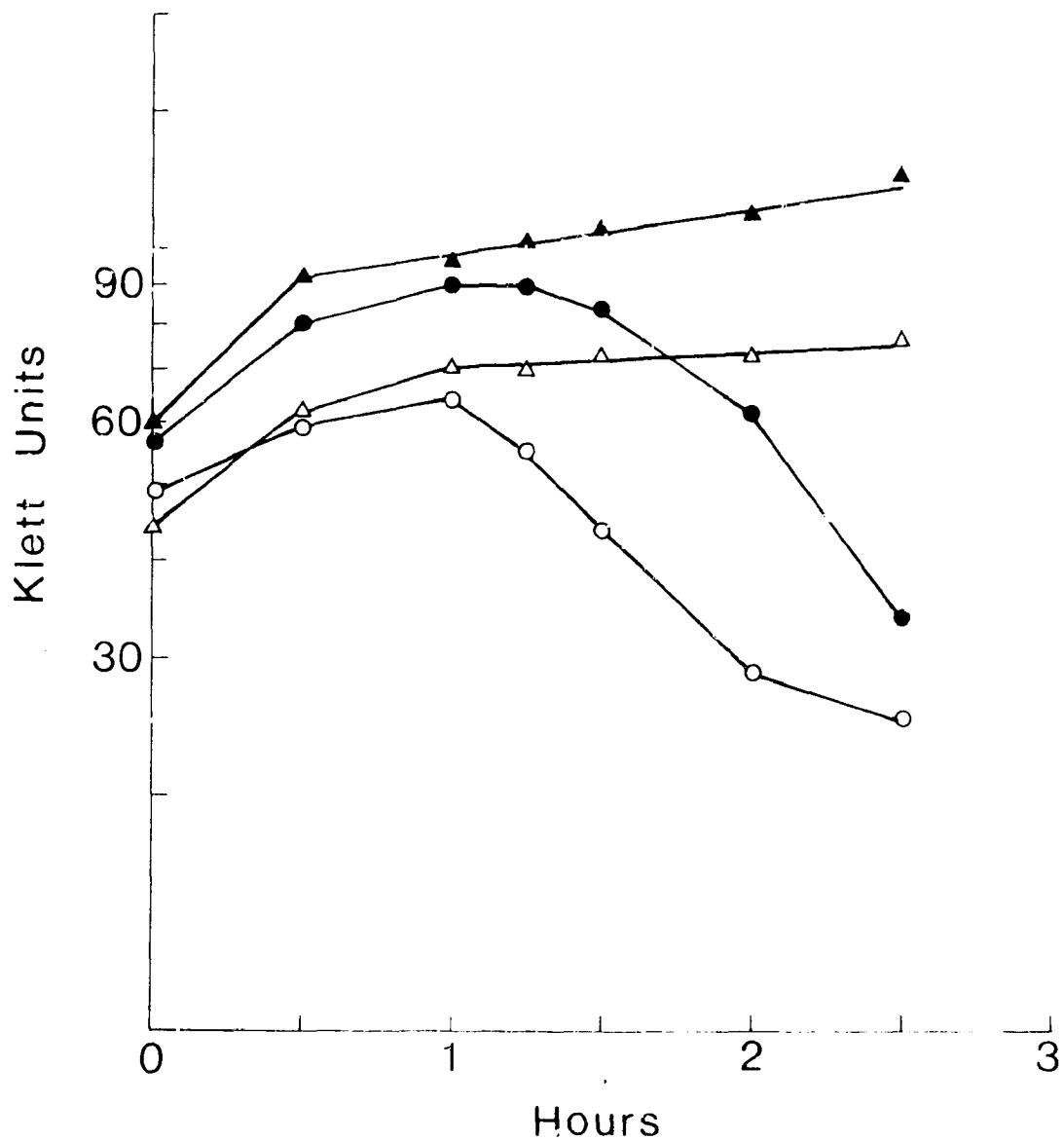


Fig. 14. Suppression of murH1 by smhB1. Growth of strains VC4132 (smhB murH1) and VC4076 (smhB1 murH1) at 42°C in NB (open symbols) and NB containing 1% NaCl (closed symbols). VC4132, ○, ●; VC4076, △, ▲.

shown in Fig. 15. Strain VC4077 (lytD⁺ smhB1) (Fig.15A), like VC4133 (Fig. 13), lysed in NB at 42°C, and its lysis was suppressed by the addition of 1% NaCl. Fig. 15B shows that the lytD1 mutant, VC4014, also lysed at 42°C in NB. However, in contrast to the lysis of the smhB1 mutant, the lytD1-induced lysis of VC4014 was not suppressed by the addition of 1% NaCl. In addition, the smhB1 lytD1 double mutant, strain VC4024, was similar to the murH1 smhB1 strain, VC4076 (Fig. 14), in that it did not lyse in either NB or NB containing 1% NaCl although growth was markedly inhibited 30 min after the temperature upshift. Thus, the lytD1 and the smhB1 alleles, by themselves, exhibited temperature-dependent lysis phenotypes, but these lysis phenotypes were suppressed when the two mutant alleles were combined.

Further experiments indicated that the smhB1 allele did not suppress the lysis phenotypes conferred by the murE1 (79) and murF2 (78) mutations in strains VC4189 and VC4190, respectively, and also did not prevent the ampicillin-induced lysis of VC4076 (data not shown).

5. Other smhB-Like Mutants

Three additional independent isolates of smhB-like suppressor mutants were characterized. They were all identical with respect to phenotypic properties, and the mutant loci were mapped at 12.5 min at a position indistinguishable from that of the smhB locus.

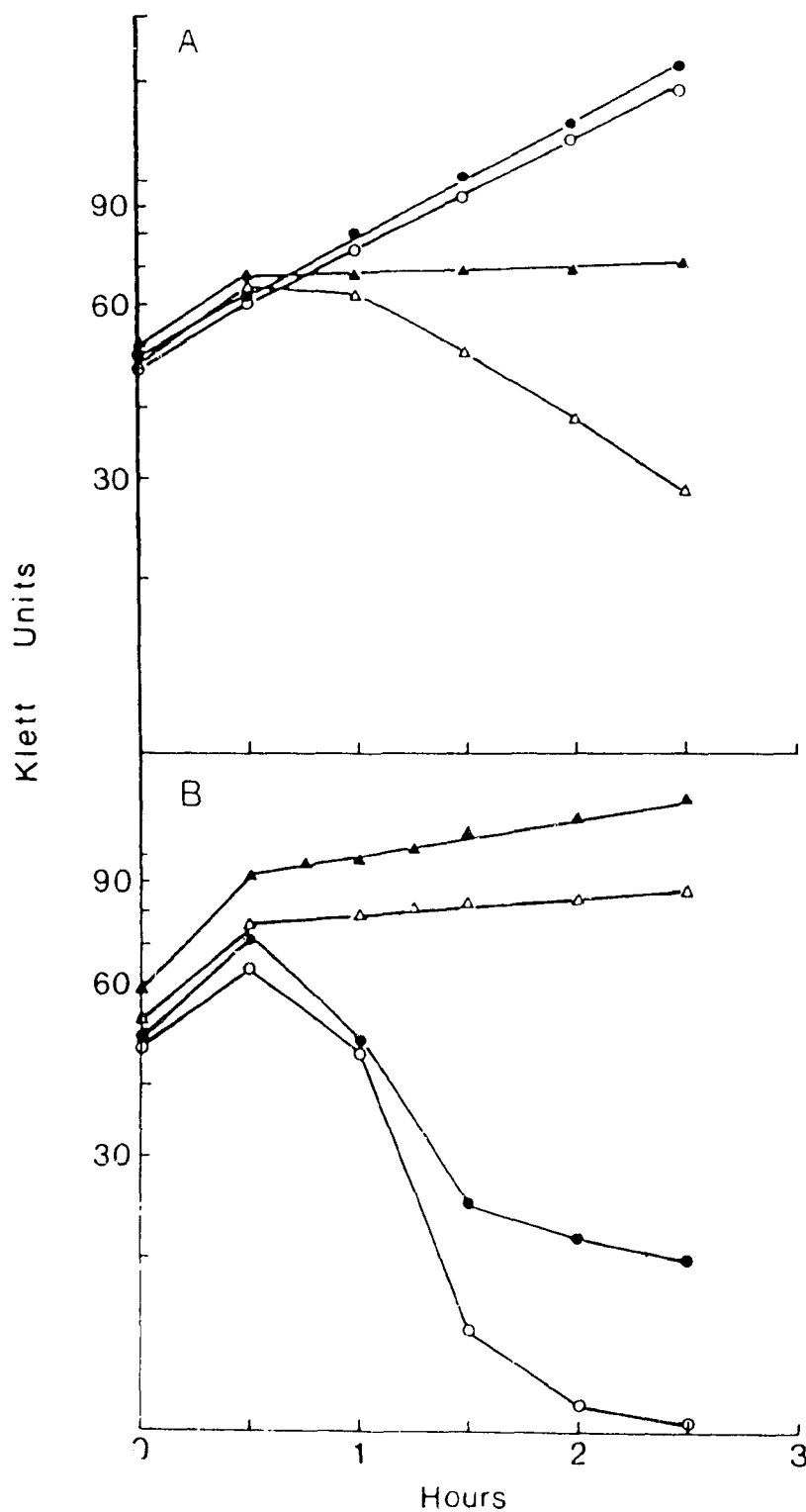


Fig. 15. Suppression of *lytD1* by *smhB1*. A. Growth of strain VC4077 (*smhB1 lytD+*) in NB at 30° (○) and 42°C (△) and in NB plus 1% NaCl at 30° (●) and 42°C (▲). B. Growth of strain VC4014 (*smhB+ lytD1*) in NB (○) and NB plus 1% NaCl (●) and strain VC4024 (*smhB1 lytD1*) in NB (△) and NB plus 1% NaCl (▲) at 42°C.

IV. Results: the smhA and lytE Mutants

1. Isolation of smhA Mutants

As already noted, spontaneous temperature-resistant derivatives of the murH1 ts mutant, strain VC460, occurred at a frequency of 3×10^{-6} . Ten such derivatives were chosen at random in a preliminary experiment designed to determine the basis for their temperature resistance. To achieve this, the zaa-1::Tn5 insertion in these derivatives was transduced into strain VC7, and the kanamycin-resistant transductants were screened for temperature sensitivity and lysis phenotypes at 42°C. All ten VC460 derivatives were shown by this method to still possess the original murH1 ts allele, indicating that the majority, and possibly all, of the observed spontaneous temperature-resistant derivatives carried extragenic suppressor mutations. The mutant strain designated VC493 was chosen from among the original ten isolates for more detailed characterization. The suppressor mutation in VC493 was apparently a new genetic locus and was designated smhA1.

2. Isolation of the lytE Mutant and Genetics of smhA and lytE

The zce-102::Tn10 which was closely linked to smhA1 was obtained by screening random Tn10 insertion pools prepared on the smhA1 mutant strain, VC493, with the phage derivative, NK55. The random insertions were transduced into the murH1 strain, VC460, with phage Plvir. The zce-1::Tn10kan inser-

Table 8. E. coli K-12 strains

Strain	Relevant Genotype/Description	Reference/Source
JRG33	<u>thi-1 his-4 proA2 purB15 lip-9 mtl-1</u> <u>xyl-5 galK2 lacY1 rpsL35 supE44?</u>	CGSC
Sφ1263	<u>araD139 Δlac U169 thi ΔpyrC rpsL</u>	R.A.Kelln ^a
TKL11	<u>thr-1 leuB6 murE1 codA1 pyrF101 his-108</u> <u>thyA6 argG66 ilvA634 thi-1 deoC1</u> <u>lacY1 tonA21 tsx-95 supE44</u>	CGSC
TKL46	<u>thr-1 leuB6 murF2 codA1 pyrF101 his-108</u> <u>thyA6 argG66 ilvA634 thi-1 deoC1</u> <u>lacY1 tonA21 tsx-95 supE44</u>	CGSC
VC7	<u>thi-1 lysA23 rpsL109</u>	Laboratory Collection
VC460	VC7 <u>zaa-1::Tn5 murH1</u>	This study
VC461	VC7 <u>zaa-1::Tn5</u>	This study
VC484	VC7 <u>murH1</u>	This study
VC493	VC460 <u>smhA1</u>	This study
VC4023	VC7 <u>zbc-103::Tn10</u>	This study
VC4041	VC493 <u>zce-102::Tn10</u>	This study
VC4045	VC4041 <u>lytE1</u>	This study
VC4077	VC7 <u>zbc-103::Tn10 smhB1</u>	This study
VC4084	VC7 <u>zce-102::Tn10 lytE1</u>	This study
VC4112	VC7 <u>zce-102::Tn10 smhA1 lytD1</u>	This study
VC4113	VC7 <u>zce-102::Tn10 lytD1</u>	This study
VC4119	VC7 <u>zce-102::Tn10 lytE1 smhA1</u>	This study
VC4120	VC7 <u>zce-102::Tn10 smhA1</u>	This study
VC4122	VC7 <u>zce-102::Tn10</u>	This study

Table 8, continued

VC4127	VC460 <u>zce-102</u> ::Tn10 <u>smhA1</u>	This study
VC4128	VC460 <u>zce-102</u> ::Tn10	This study
VC4135	VC461 <u>zce-102</u> ::Tn10 <u>smhA1</u>	This study
VC4136	VC461 <u>zce-102</u> ::Tn10	This study
VC4146	VC7 <u>zbc-103</u> ::Tn10 <u>lytE1</u> <u>smhB1</u>	This study
VC4147	VC7 <u>zbc-103</u> ::Tn10 <u>lytE1</u>	This study
VC4170	VC7 <u>zce-102</u> ::Tn10 <u>lytE2</u>	This study
VC4173	VC7 <u>zce-102</u> ::Tn10 <u>lytE3</u>	This study
VC4195	VC4120 <u>zce-1</u> ::Tn10 <u>kan</u>	This study
VC4200	TKL11 <u>zce-102</u> ::Tn10 <u>smhA1</u>	This study
VC4201	TKL46 <u>zce-102</u> ::Tn10 <u>smhA1</u>	This study
VC4205	VC4077 <u>zce-1</u> ::Tn10 <u>kan</u> <u>smhA1</u>	This study

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tions which was linked to both smhA1 and zce-102::Tn10 was isolated from a random Tn10kan insertion pool prepared on strain VC4120 (smhA1 zce-102::Tn10) with phage λ 1105. The random Tn10kan insertions were transduced into the murH1 strain, VC484, according to procedures described in Chapter 2. The zce-102::Tn10 insertion was transduced into a collection of Hfr strains, and its map location was determined to be in the 23- to 28-min region of the E. coli genetic linkage map (136) in interrupted mating experiments involving these Hfr strains and appropriate F^- strains (data not shown). Subsequent mapping by phage P1vir-mediated generalized transduction yielded the linkage map shown in Fig. 16. The smhA1 mutation was mapped at 24.5 min. The order of zce-102::Tn10, smhA, and zce-1::Tn10kan was based on the results of the three-factor crosses summarized in Table 9.

The lytE1 mutation apparently arose spontaneously in strain VC4041, and the derivative carrying this mutation was subsequently designated VC4045. The lytE1 allele was discovered as a ts mutation which was closely linked to zce-102::Tn10 during attempts to construct new smhA1 derivatives using VC4045 as a donor. We placed lytE at 25 min on the linkage map (Fig. 16), and its position reflects the fact that smhA and lytE were readily segregated in crosses involving zce-102::Tn10.

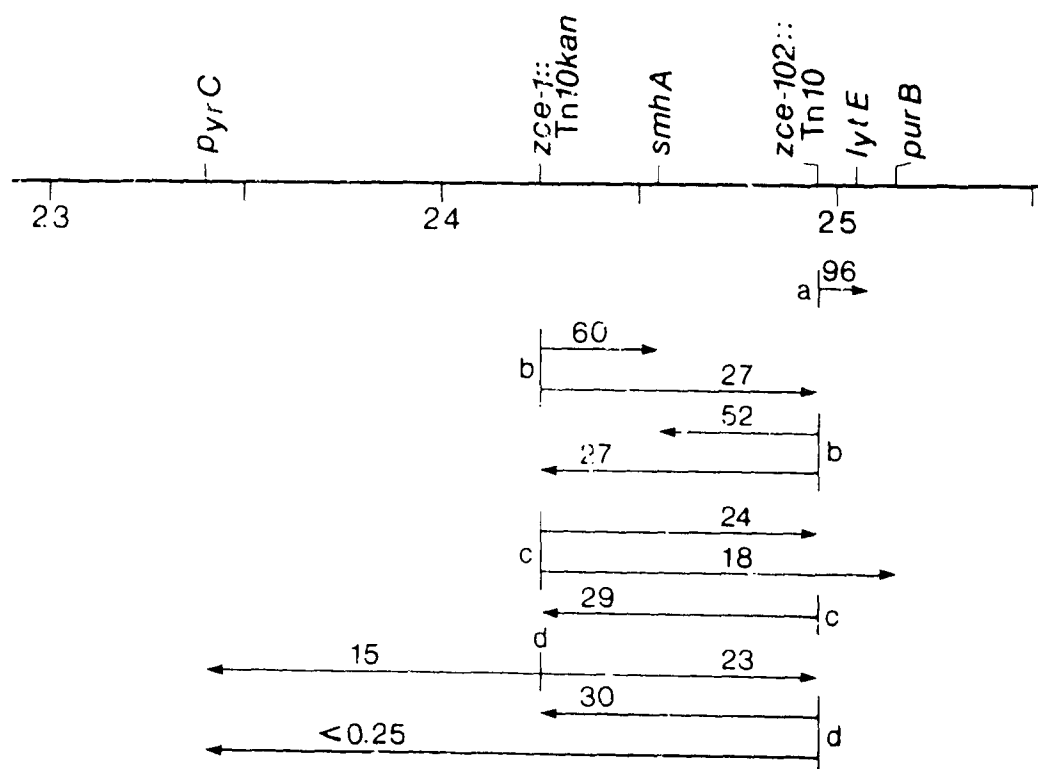


Fig. 16. Linkage map of the *smhA* and *lytE* region. The results of the following crosses (recipient X donor) are shown, with the number of recombinants scored in each case indicated parenthetically: a, VC7 X VC4084 (200); b, VC484 X VC4195 (94); c, JRG33 X VC4195 (94); d, S ϕ 1263 X VC4195 (188). For each set of arrows, the vertical line indicates the position of the selected marker and the arrowheads indicate the positions of the unselected markers. The numbers represent the cotransduction frequencies (%).

Table 9. Three-factor cross analysis for zce-102::Tn10,
zce-1::Tn10kan, and smhA

Donor	Recipient	Selected marker	Unselected marker	Number
(A)				
VC4195	VC484	Kan ^r	<u>smhA1</u> Tet ^r	24
(Kan ^r <u>smhA1</u> Tet ^r)	(<u>murH1</u>)		<u>smhA</u> ⁺ Tet ^r	2
			<u>smhA1</u> Tet ^s	32
			<u>smhA</u> ⁺ Tet ^s	36
		Tet ^r	Kan ^r <u>smhA1</u>	22
			Kan ^r <u>smhA</u> ⁺	1
			Kan ^s <u>smhA1</u>	26
			Kan ^s <u>smhA</u> ⁺	45
(B)				
VC4195	JRG33	Kan ^r	Tet ^r <u>purB</u> ⁺	13
(Kan ^r <u>smhA1</u> Tet ^r)	(<u>purB</u>)		Tet ^r <u>purB</u>	9
			Tet ^s <u>purB</u> ⁺	3
			Tet ^s <u>purB</u>	69

The zce-102::Tn10 and zce-1::Tn10kan markers are indicated by the phenotypic designations, Tet^r and Kan^r, respectively. Tet^s and Kan^s refer to sensitivity to tetracycline and kanamycin, respectively.

3. Phenotypic Properties of the smhA1 and lytE1 Alleles

The smhA1 allele did not have a recognizable phenotype of its own, and smhA1 mutants such as strains VC4135 (e.g., Table 10) and VC4120 (e.g., Tables 10 and 11) grew as well as wild type strains on various media at temperatures ranging from 30°C to 42°C. Sets of isogenic strains carrying various combinations of the murH and smhA or the lytD and smhA alleles were constructed in order to assess the effects of smhA1 on the ts growth phenotype associated with the murH1 and lytD1 mutations. A comparison between strains VC4127 and VC4128 in experiment A of Table 10 confirms that smhA1 suppressed the ts phenotype of murH1 on both low (NA) and high osmolarity (NA containing 1% NaCl) media. On the other hand, the lytD1 ts phenotype was not suppressed by smhA1 as demonstrated with strains VC4112 and VC4113 in experiment B of Table 10. Fig. 17A shows that strain VC4128 (murH1) lysed about 60 min after a temperature upshift from 30°C to 42°C in NB. On the other hand, strain VC4127 (smhA1 murH1) grew as well as strain VC4135 (smhA1) after the temperature upshift indicating that the smhA1 allele suppressed the lysis phenotype associated with murH1. Fig. 17B shows that smhA1 did not suppress the lysis phenotype of the lytD1 mutation in NB, and identical results were obtained in NB containing 1% NaCl (data not shown). These results collectively demonstrate a correlation between the suppression of temperature sensitivity (Table 10) and the suppression of lysis (Fig. 17) by smhA1.

Table 10. Colony formation by isogenic murH1 smhA1 (A) and lytD1 smhA1 (B) strains as a function of growth medium and temperature

Strain and Relevant Genotype	CFU per ml at 42°C/CFU per ml at 30°C	
	NA	NA + 1%NaCl
(A)		
VC4127 (<u>murH1 smhA1</u>)	0.4	0.8
VC4128 (<u>murH1 smhA</u> ⁺)	1.4 x 10 ⁻⁷	1.2 x 10 ⁻⁷
VC4135 (<u>murH</u> ⁺ <u>smhA1</u>)	0.4	1.0
VC4136 (<u>murH</u> ⁺ <u>smhA</u> ⁺)	0.9	0.9
(B)		
VC4112 (<u>lytD1 smhA1</u>)	3.2 x 10 ⁻⁷	2.0 x 10 ⁻⁶
VC4113 (<u>lytD1 smhA</u> ⁺)	2.0 x 10 ⁻⁷	1.5 x 10 ⁻⁶
VC4120 (<u>lytD</u> ⁺ <u>smhA1</u>)	0.4	1.0
VC4122 (<u>lytD</u> ⁺ <u>smhA</u> ⁺)	0.9	0.9

Table 11. Colony formation by isogenic lytE1 smhA1 (A) and lytE1 smhB1 (B) strains as a function of growth medium and temperature

Strain and Relevant Genotype	CFU per ml at 42°C/CFU per ml at 30°C		
	TSA	NA	NA + 1%NaCl
(A)			
VC4119 (<u>lytE1 smhA1</u>)	1.0	0.4	0.9
VC4064 (<u>lytE1 smhA</u> ⁺)	2.0 x 10 ⁻⁶	1.2 x 10 ⁻⁸	6.4 x 10 ⁻⁶
VC4120 (<u>lytE</u> ⁺ <u>smhA1</u>)	0.9	0.4	1.0
VC4122 (<u>lytE</u> ⁺ <u>smhA</u> ⁺)	1.0	0.8	0.9
(B)			
VC4146 (<u>lytE1 smhB1</u>)	0.9	1.3 x 10 ⁻⁷	0.9
VC4147 (<u>lytE1 smhB</u> ⁺)	2.2 x 10 ⁻⁶	1.1 x 10 ⁻⁸	3.1 x 10 ⁻⁶
VC4077 (<u>lytE</u> ⁺ <u>smhB1</u>)	0.9	2.1 x 10 ⁻⁷	0.9
VC4023 (<u>lytE</u> ⁺ <u>smhB</u> ⁺)	1.1	0.7	0.8

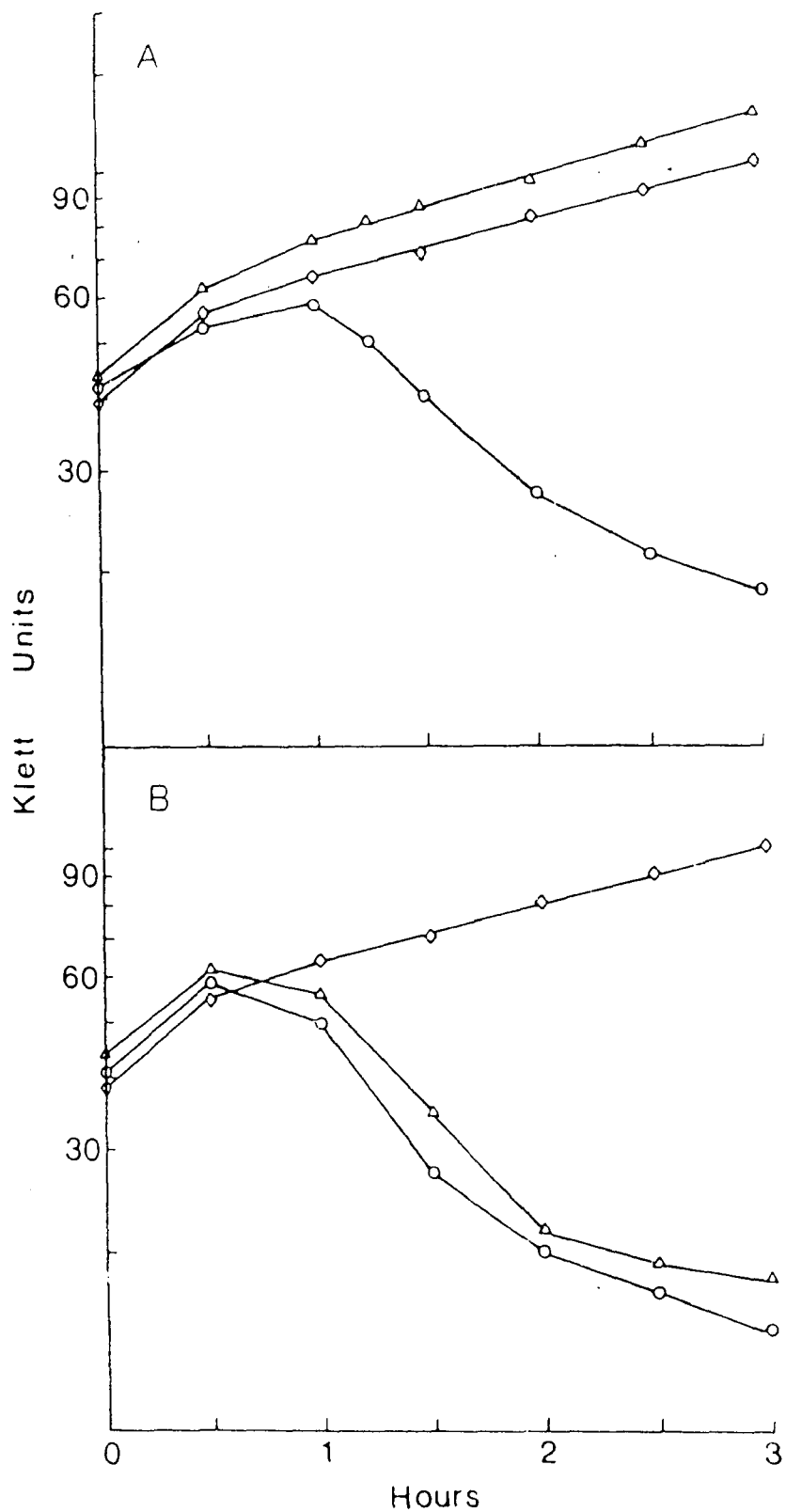


Fig. 17. Suppression murH1 by smhA1. A. Growth of strains VC4127 (smhA1 murH1), Δ , VC4128 (murH1), \circ , and VC4135 (smhA1), \diamond , at 42°C in NB. B. Growth of strains VC4112 (smhA1 lytD1), Δ , VC4113 (lytD1), \circ , and VC4135 (smhA1), \diamond , at 42°C in NB.

Table 11 shows that the ts phenotypes associated with the lytE1 mutation in strains VC4084 and VC4147 were exhibited on both high and low osmolarity media. The 2 strains belong to sets of isogenic strains constructed to test the lysis suppression activities of the smhA1 and smhB1 alleles on lytE1. The results for strain VC4119 (experiment A, Table 11) indicate that smhA1 suppressed the ts phenotype of lytE1 on both high and low osmolarity media. Furthermore, smhB1 also suppressed lytE1 ts in strain VC4146, but only on high osmolarity media (experiment B, Table 11). It should be noted that, as shown above, the smhB1 allele by itself (e.g., strain VC4077 in experiment B of Table 11) conferred ts growth on low but not high osmolarity media.

Fig. 18 demonstrates the lysis phenotype of strain VC4084 (lytE1 smhA⁺) and the smhA1 lysis suppressor activity in strain VC4119 (lytE1 smhA1) in NB at 42°C. Lysis induced by lytE1 also occurred in NB containing 1% NaCl, and this, too, was suppressed by smhA1 (data not shown). The wild type strain in Fig. 18, VC4122, characteristically exhibited biphasic growth in NB with a sharp break in the growth rate occurring at a density of about 100 Klett units. Furthermore, a comparison of strains VC4120 (lytE⁺ smhA1) and VC4122 confirms that the smhA1 allele did not have a demonstrable phenotype of its own.

Fig. 19A illustrates the phenotype of smhB1 in strain VC4077. The smhB1 allele was associated with lysis at 42°C in

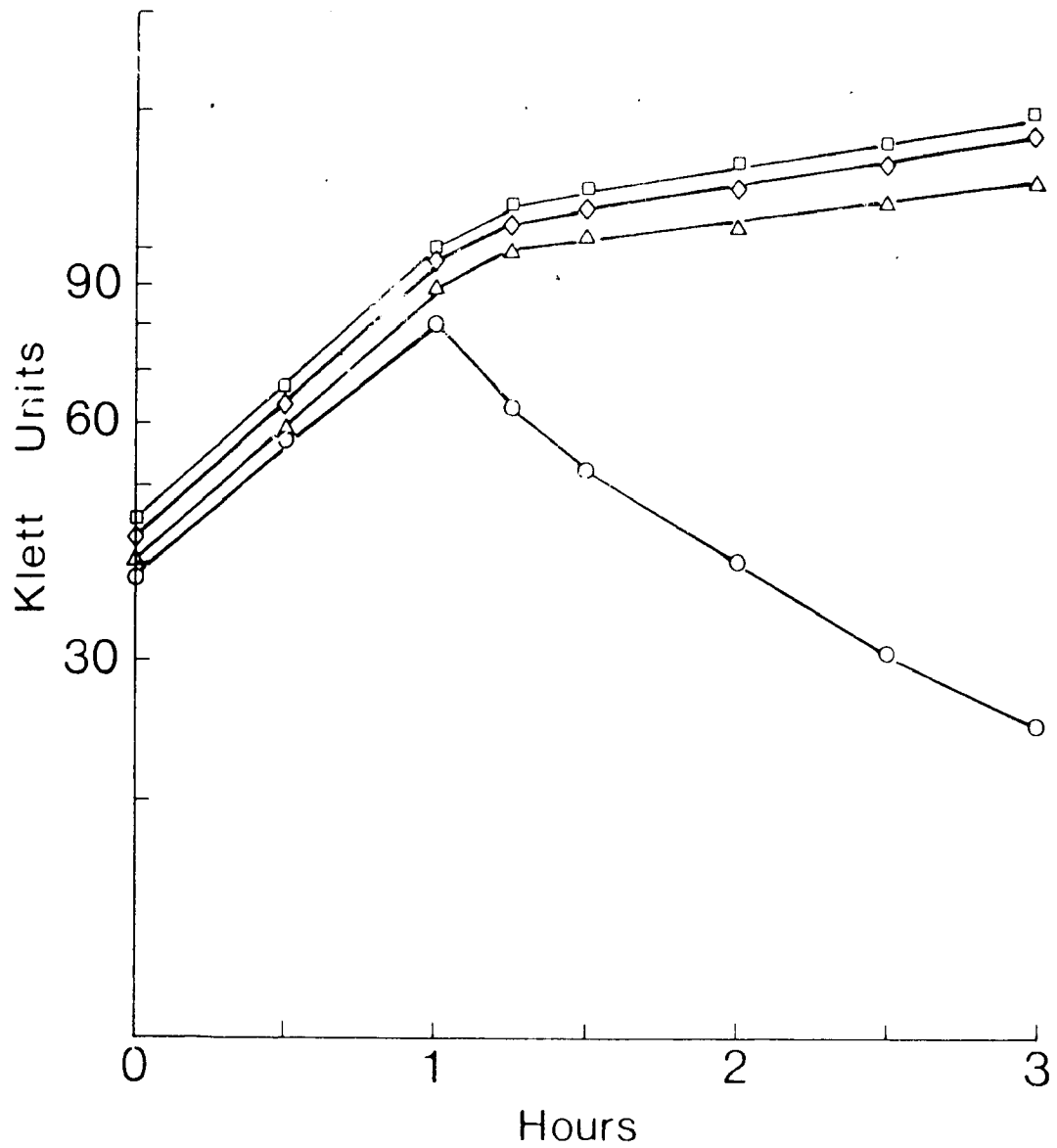


Fig. 18. Suppression of lytE1 by smhA1. Growth of strains VC4119 (lytE1 smhA1), Δ , VC4084 (lytE1), O, VC4120 (smhA1), \diamond , and VC4122 (wild type), \square , at 42°C in NB.

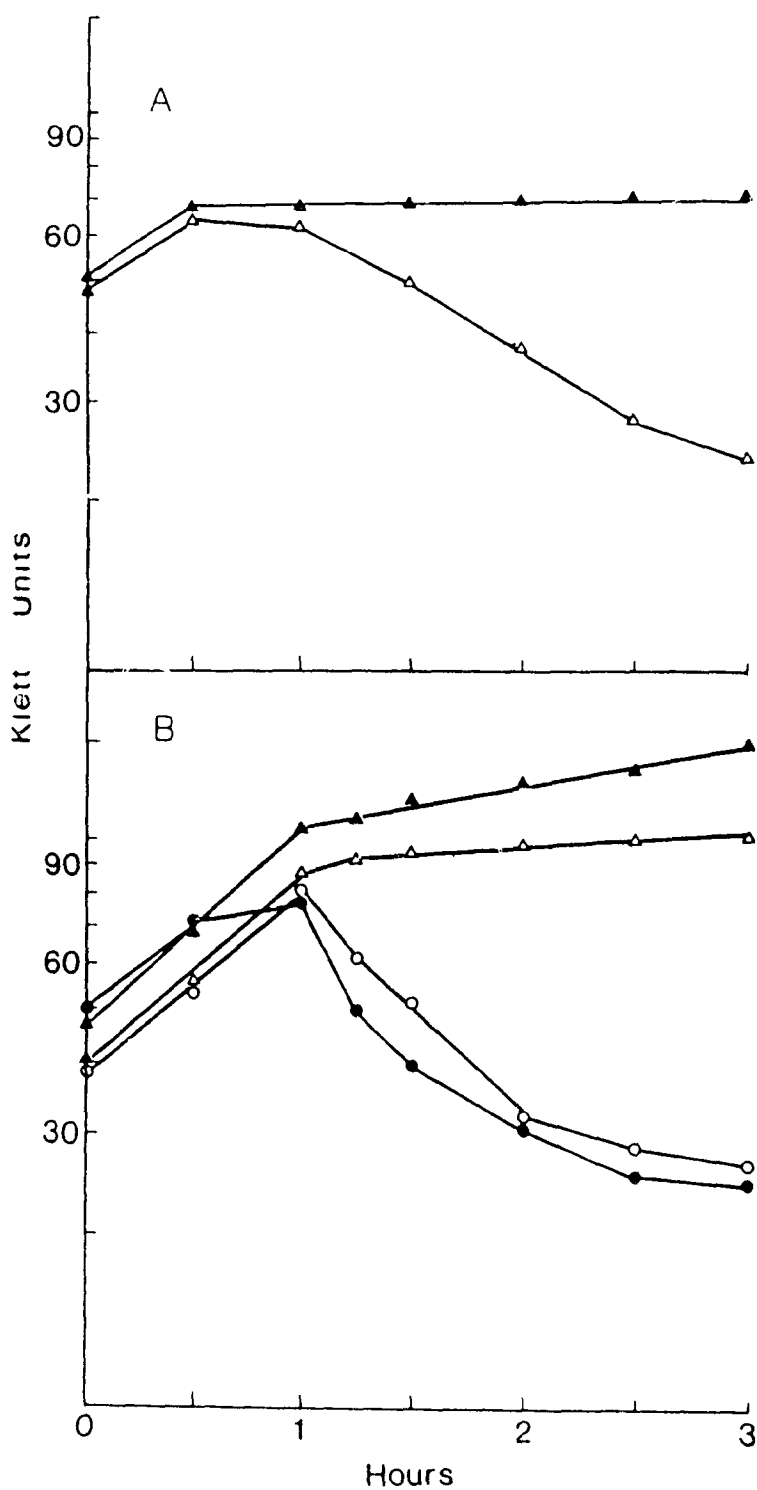


Fig. 19. Suppression of *lytE1* by *smhB1*. A. Growth of strain VC4077 (*smhB1*) at 42°C in NB (△) and NB containing 1% NaCl (▲). B. Growth of strains VC4146 (*lytE1 smhB1*) and VC4147 (*lytE1*) at 42°C in NB (open symbols) and NB containing 1% NaCl (closed symbols). VC4146 (△, ▲); VC4147 (○, ●).

NB. Furthermore, this lysis did not occur in NB containing 1% NaCl although the growth rate was severely inhibited. Fig. 19B shows that the lytE1 smhB⁺ strain, VC4147, lysed at 42°C in both NB and NB containing 1% NaCl whereas the lytE1 smhB1 strain, VC4146, did not.

Additional experiments (data not shown) indicated that (i) smhA1 did not suppress lysis caused by mutations in either the murE (strain VC4200) or the murF (strain VC4201) genes; (ii) the suppressor activity of smhA1 did not protect strain VC4127 from ampicillin-induced lysis; and (iii) smhA1 did not suppress the ts growth and lysis phenotype of the smhB1 mutation in strain VC4205.

4. Nature of the Relationship Between smhA and lytE

Because of the similarities in the phenotypes of murH1 and lytE1, we considered the possibility that the spontaneous occurrence of the lytE1 mutation in strain VC4041 was not merely coincidental. The following demonstrates that lytE-type mutations do indeed occur spontaneously in smhA1 genetic backgrounds. The smhA1 zce-102::Tn10 strains VC4127 (also murH1) and VC4135 were subjected to 4 serial subcultures as described in Methods. Phage Plvir was propagated on each of the subcultures, and these were used to transduce the zce-102::Tn10 marker (which is linked to the lytE and smhA loci) of the 2 strains into strain VC7 with the selection being carried out 30°C. In each experiment, 100 tetracycline-

resistant transductants were screened for temperature-sensitivity. No ts Tn10 cotransductants were detected with phage prepared on the first 3 serial subcultures of either strain. However, the fourth subculture of both strains yielded 2 ts tn10 transductants. One ts transductant from each strain was studied further. The ts mutations in these strains, VC4170 (derived from VC4127) and VC4173 (derived from VC4135), were shown to be 90% and 98% linked to zce-102::Tn10, respectively. Furthermore, both mutations were phenotypically indistinguishable from lytE1. We have designated these mutations lytE2 and lytE3, but it must be emphasized that these are preliminary assignments since the complementation studies to demonstrate their allelic relationships have not yet been performed.

5. Preliminary characterization of Additional smhA-Like Suppressor Mutants

As shown above, extragenic suppression of murH1 was apparently a common occurrence, and it was of interest to estimate the number of loci involved in this phenomenon. To this end, a genetic analysis of additional spontaneous temperature-resistant derivatives of strain VC4128 (zaa-1::Tn5 murH1 zce-103::Tn10) was conducted to determine whether extragenic suppression of murH1 may involve more than one locus. Six independent temperature-resistant isolates were analyzed in a two-step process. In the first step, all six were shown to carry the murH1 allele by cotransduction of the

marker into VC7 using the linked zaa-1::Tn5 insertion as the means of selection, confirming that the temperature resistance of these derivatives was due to extragenic suppression. The second step was designed to determine whether the suppressor may be located in the vicinity of the smhA locus. The zce-103::Tn10 insertions from the temperature-resistant derivatives were transduced into VC460 (murH1), and Tet_r transductants were selected at 30°C. The tetracycline-resistant transductants were then screened for temperature resistance, i.e., for cotransduction of a suppressor of the murH1 mutation. All six derivatives of VC4128 were shown in this way to carry suppressors of murH1 which were linked to zce-103::Tn10, and the degree of linkage in each case was about 50% cotransduction. Since this is similar to the linkage between zce-103::Tn10 and smhA, it is possible that these new suppressors were alleles of smhA but we have been unable to demonstrate this because complementation analyses so far have not been feasible.

V. Discussion

Two phenotypic classes of extragenic mutations which suppressed murH1 were isolated. One class, as represented by smhA1, was isolated directly from the spontaneous temperature-resistant derivatives of the murH1 mutant (Fig. 11). The high frequency occurrence of spontaneous temperature-resistant

derivatives of the murH1 mutant was attributed primarily to extragenic suppressor mutations, and we found no evidence for reversion of murH1 in this limited survey. The genetic analysis of six independently isolated suppressor mutants indicated that all mapped in the 25-min region of the linkage map. Thus, although the lack of complementation data does not allow us to distinguish the actual number of loci involved, it appears that extragenic suppression of murH1 occurs primarily in a single locus or perhaps in no more than a few closely linked loci. The second class of murH1 suppressor, represented by smhB1, conferred a temperature-sensitive phenotype of its own. The mutations in other isolates carrying this phenotype were mapped close to the smhB locus, but, as in the case of the smhA-type isolates, it is not known whether these mutations are allelic because complementation studies were not possible. Nevertheless, the results suggest that the genetics of murH1 suppression is relatively simple in that only two regions of the linkage are involved, and each of these areas may contain only a single suppressor locus. Interestingly, both the smhA1 and smhB1 mutants spontaneously acquired additional mutations, designated lytE1 and lytD1, respectively, which conferred ts lysis phenotypes.

The smhB1 mutant was isolated by following a strategy designed to select ts extragenic suppressors of murH1 (Fig. 11). It is notable that the ts colony-forming phenotype associated with the smhB1 allele, e.g., in strain VC4133, was

attributed to temperature-dependent lysis of the bacteria on NA and in NB, respectively. Both characteristics were suppressed by the addition of 1% NaCl, and smhB1 may therefore be classified as a ts osmoremedial mutation (149). The basis for the osmoremedial property of smhB1 has not been determined. Osmotically fragile cell envelope mutants which are osmoremedial have been previously isolated (79, 81, 86, 150, 151), and it is possible that the increased osmolarity serves to provide cell membrane support in view of the lysis phenotype associated with smhB1. On the other hand, it has been noted that ts mutations are very often osmoremedial, including those which have nothing to do with cell envelope function (149). Therefore, other osmoremedial mechanisms are possible and should be considered. For example, the high concentration of solute may serve to stabilize a mutant protein. Whatever the cause, it is important to note in the following discussion that the lysis phenotypes of the murH1, lytD1 and lytE1 alleles, unlike smhB1, were not osmoremedial characteristics.

As already noted, the lytD1 ts mutation apparently arose spontaneously in the murH1 smhB1 strain, VC4007. The lytD and smhB loci were located close to each other, but the 2 loci were not directly linked as determined by linkage mapping and by the placement of transposon Tn₁₀ insertions between the 2 loci. These Tn₁₀ insertions played an instrumental role in the discovery of the lytD locus during construction of

derivatives carrying smhB1. In these constructions, the lytD1 derivatives were distinguished from the smhB1 derivatives as a class of tetracycline-resistant transductants with ts properties which were not osmoremedial. It should be noted that although the lytD1 and smhB1 alleles could be differentiated on the basis of the inability of lytD1 mutants to grow on high osmolarity media such as TSA at 42°C, the method in practice was inconvenient for large scale genetic screening because the ts characteristic of lytD1 was leaky. Genetic screening by replication, which inherently results in the transfer of relatively large inocula, always resulted in the appearance of residual growth by lytD1 mutants, and our judgement consequently had to be based on the amount of growth observed. However, we were able to confirm our results, although not on a large scale, by picking transductants and individually testing for colony formation by the determination of plating efficiencies on TSA at 42°C as described in Table 6 and for lysis in NB plus 1% NaCl at 42°C as described in Figure 15.

Isogenic strains carrying combinations of wild type and mutant alleles of the murH, lytD and smhB loci were constructed in order to assess the interactions between these loci. Studies on the reconstructed murH1 smhB1 strain, VC4076, revealed an apparent discrepancy. In our isolation procedure (Fig. 11), the original murH1 smhB1 mutant isolate (VC494) failed to develop a colony on TSA at 42°C, and this

property, in fact, distinguished the murH1 smhB1 mutant from the murH1 smhA-type mutant which exhibited a completely temperature-resistant phenotype. Therefore, the ability of strain VC4076 to form colonies on TSA at 42°C was unexpected. Moreover, VC4076 had virtually identical plating efficiencies on TSA at 42°C and 30°C (Table 6) although its growth rate at the higher temperature was markedly reduced. The plating efficiencies of the original murH1 smhB1 mutant isolate (VC494) and the reconstructed murH1 smhB1 mutant (VC4076) on TSA were subsequently determined, and a comparison indicated that both strains behaved identically. Thus, it would appear that certain unidentified factors prevented the original murH1 smhB1 isolate, VC494, from forming colonies on TSA at 42°C during its initial isolation. On the other hand, it is noteworthy that the combination of murH1 and smhB1 resulted in enhanced survival at 42°C (Table 7), and this is clearly a manifestation of the smhB1 suppressor activity.

Since the murH1, lytD1, lytE1, and the smhB1 alleles by themselves conferred ts lysis phenotypes, the phenotypes of the double mutants, strains VC4076 (murH1 smhB1), VC4024 (lytD1 smhB1), and VC4146 (lytE1 smhB1) are interesting in that none of them exhibited lysis at 42°C (Figs. 14, 15B and 19B). The lysis suppression observed in both high and low osmolarity media when smhB1 was combined with murH1, lytD1 or lytE1 suggests a mechanism in which the individual alleles interact either directly or indirectly with each other to

ectively suppress their lysis phenotypes. The smhB1 allele also had the same effects on the ts growth phenotypes of murH1, lytD1 and lytE1. The ts colony formation on high osmolarity media such as TSA or NA plus 1% NaCl associated with either murH1, lytD1 or lytE1 was suppressed by smhB1. However, it was curious that the combination of smhB1 with murH1, lytD1 or lytE1 did not result in the suppression of temperature sensitivity on NA in view of the suppression of ts growth under these conditions (Tables 6 and 11). The similar behavior exhibited by the murH1 smhB1, lytD1 smhB1, and lytE1 smhB1 mutants further strengthens the resemblance between the alleles murH1, lytD1 and lytE1. In an effort to explain this, we hypothesize that the smhB1 gene product may be involved in two separable suppressor activities at the restrictive temperature: (i) lysis suppression, and (ii) restoration of colony-forming ability. Our results suggest that lysis suppression by smhB1 (activity i) is not dependent on medium osmolarity, whereas restoration of colony formation (activity ii) is. This proposal is further supported by the demonstration that smhB1 combined with murH1 (strain VC4076) or with lytD1 (strain VC4024) resulted in enhanced survival at 42°C on NA but did not fully support colony formation under these conditions (Table 7).

The spontaneous occurrence of the lytE1 mutation in the smhA1 mutant strain, VC4041, was probably not a fortuitous event because we were subsequently able to demonstrate the

spontaneous occurrence of lytE-like mutations during serial passage of smhA1 mutants. The serial passage experiment also indicated that the presence of the murH1 mutation was not essential for the appearance of the lytE-like mutations in the smhA1 genetic background since identical results were obtained with strains VC4127 and VC4135. On the basis of these results, we suspect that lytE may be a suppressor of the smhA1 mutation, but, if so, this effect is not obvious because the smhA1 mutant already behaves like a wild type strain and does not appear to be handicapped. On the other hand, the suppression of the ts lysis phenotype of lytE1 by smhA1 was readily demonstrable, suggesting that it is advantageous for the lytE1 mutant to carry the smhA1 allele. In this connection, it is interesting that the lytD1 ts lysis mutation apparently also occurred spontaneously in the smhB1 suppressor mutant, but in this case we were unable to demonstrate that spontaneous lytD-like mutations occurred during subculture of the smhB1 mutant. Nevertheless, There are reasons to suspect that a mutually beneficial relationship, similar to that between smhA1 and lytE1, exists between smhB1 and lytD1. For example, the smhB1 and the lytD1 mutants survived poorly when incubated at the restrictive temperature on a low osmolarity medium whereas survival was markedly enhanced when the two mutant alleles were combined (Table 7).

The lysis suppression activities of smhB1 and smhA1 appeared to be relatively specific in that the suppression

activity of smhB1 was expressed only with murH1, lytD1, or lytE1, while smhA1 suppressed only the ts phenotypes induced by either the murH1 or the lytE1 mutations. For example, neither smhB1 nor smhA1 prevented lysis caused by imposing either mutational (murE or murF) or antibiotic-induced (e.g., ampicillin) blocks in peptidoglycan synthesis. This apparent specificity suggests that the murH, lytD, lytE, smhB and smhA loci may be functionally related. We should also note that no suppressor activity was observed between murH1 and lytD1, between murH1 and lytE1, between lytD1 and smhA1, and between smhB1 and smhA1 (data not shown).

The mechanistic bases for the induction of lysis by murH1, lytD1, lytE1 and smhB1 are uncertain, but their lysis-inducing mechanisms may be similar if these loci are functionally related. In this respect, it was demonstrated in Chapter 3 that a late step in peptidoglycan synthesis was inhibited in the murH1 mutant at the restrictive temperature, and it was proposed that the lysis observed, which was peptidoglycan hydrolase-mediated, was a direct consequence of this blockage in peptidoglycan synthesis. However, this proposal now seems unlikely in view of the failure of smhB1 or smhA1 to suppress the lysis induced by well characterized mutational blocks (murE and murF) in peptidoglycan synthesis. Two other possibilities are therefore being considered. First, these mutations may be pleiotropic, and they may, in some as yet undetermined way, simultaneously inhibit peptido-

glycan synthesis and induce peptidoglycan hydrolase-mediated lysis; i.e., these mutations directly interfere with peptidoglycan hydrolase control. Alternatively, the murH1-, lytD1-, lytE1-, and smhB1-induced lysis processes may follow a pathway which is different from that of the lysis processes caused by murE-, murF-, or antibiotic-induced blocks in peptidoglycan synthesis. However, it must be stressed that the basis for lysis and lysis suppression by these mutations has not yet been determined. The elucidation of this problem will require the functional identification of the murH, lytD, lytE, smhA and smhB gene products.

In summary, the results presented here collectively suggest that the murH, lytD, lytE, smhA, and smhB loci are functionally related as shown in Fig. 20. One possible interpretation of the reciprocal lysis suppression data is that the smhB1 allele interacts, directly or indirectly, with all of the other alleles except smhA1. Furthermore, the smhA1 allele may interact in some way with murH1 and lytE1 but apparently not with either smhB1 or lytD1. For example, the data demonstrating the cosuppression of 2 ts mutant alleles may be indicative of a direct protein-protein interaction between the 2 mutant gene products.

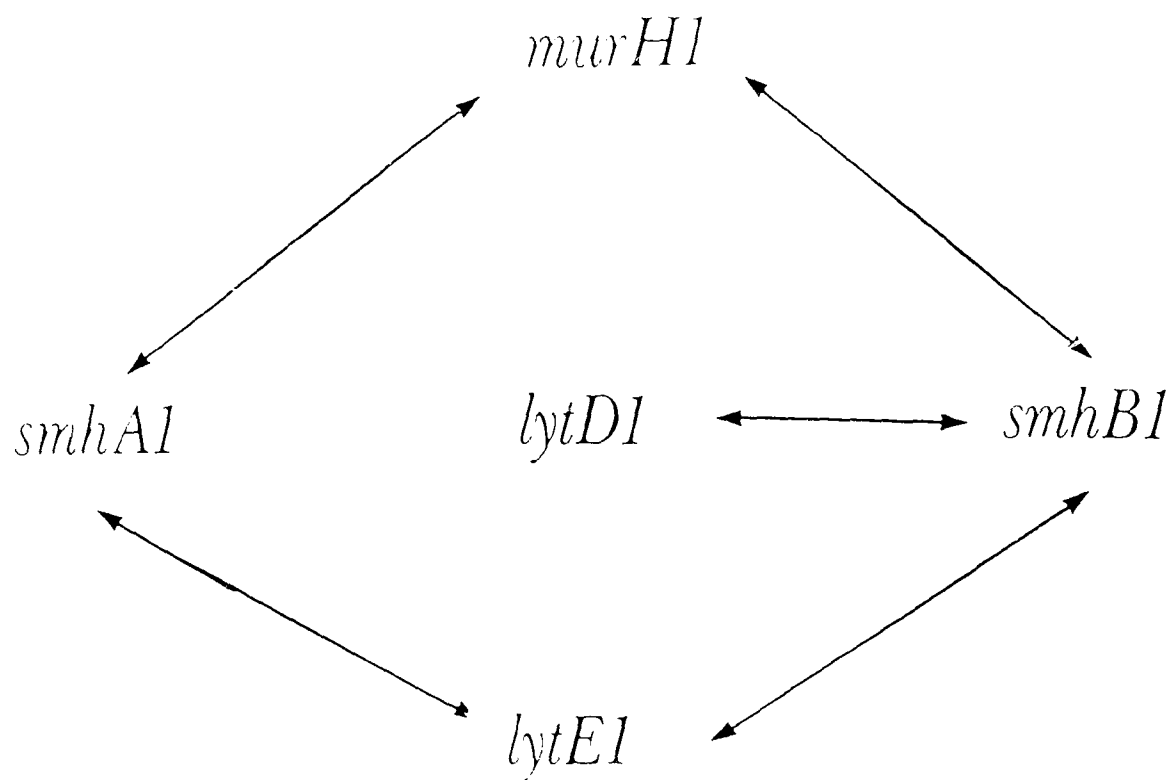


Fig. 20. Proposed relationships between the murH1, lytD1, lytE1, smhA1, and smhB1 alleles based on suppression studies. The relationships shown may involve either direct or indirect interactions, but this has not been directly demonstrated. The smhA1 allele interacts with the murH1 and lytE1 alleles but not with the lytD1 and smhB1 alleles. The smhB1 allele interacts with all of the other alleles except smhA1. No interaction was detected between murH1 and lytD1, murH1 and lytE1, or lytD1 and lytE1.

CHAPTER 5

SUPPRESSION OF THE lytD1 MUTATION BY THE PHAGE λ
cro AND cI GENES

I. Overview of Chapter Contents

This chapter describes an attempt to clone the lytD gene by screening an E. coli gene bank for clones which complemented the lytD1 allele. Such positive clones are shown to carry DNA derived from the genome of phage λ and not from the E. coli chromosome. Furthermore, 2 phage λ genes, identified as λ cro and cI, are shown to exhibit lytD1 complementing activity.

Bacteriophage λ is a well known temperate phage of E. coli. Upon infection of its host, λ may enter a lytic pathway which results in the replicative production of progeny phage and lytic death of the host. Alternatively, λ may enter a lysogenic pathway by integrating its genome into a specific site in the host chromosome. During lysogeny, the phage genes involved in the lytic pathway are silenced. These two different pathways are genetically determined by two alternate programs regulated primarily by two λ genes designated cI and cro which encode the CI (also known as λ repressor) and Cro proteins, respectively (152). CI and Cro have been extensively studied and are among the best characterized DNA-binding

proteins known (153). These proteins play antagonizing roles in determining the life style of phage λ . To initiate the lytic pathway, the cro gene is expressed, and the Cro protein subsequently represses the transcription of cI gene. To initiate the lysogenic pathway, the synthesis of the repressor protein, CI, must be established and maintained in order to prevent the expression of the cro gene (154). In addition, CI represses the transcription of the genes involved in the lytic pathway. Fig. 21 diagrammatically represents the regulatory region of the phage λ genome. The CI repressor binds to three 17-base pair sites in the so-called right operator region designated O_R1, O_R2, and O_R3. Cro also binds to the same three sites (155). In fact, CI and Cro apparently contact many of the same functional groups in binding to these sites, but there are no obvious similarities in the amino acid sequences of the two proteins (156, 157). The nucleotide sequences of these sites are similar but not identical (158), and CI and Cro bind to each of these sites with different affinities and orders. At increasing concentrations, CI binds to O_R1 and O_R2 simultaneously, but O_R3 is bound only at much higher concentrations (159). In comparison, Cro binds initially to O_R3, and then at higher concentrations, to O_R2 and O_R1 (160). As shown in Fig. 21, the three right operator sites are involved in regulating transcription of the divergent cI and cro genes from their promoters designated P_{RM} and P_R, respectively. Two aspects of these DNA-protein interactions are important (refer

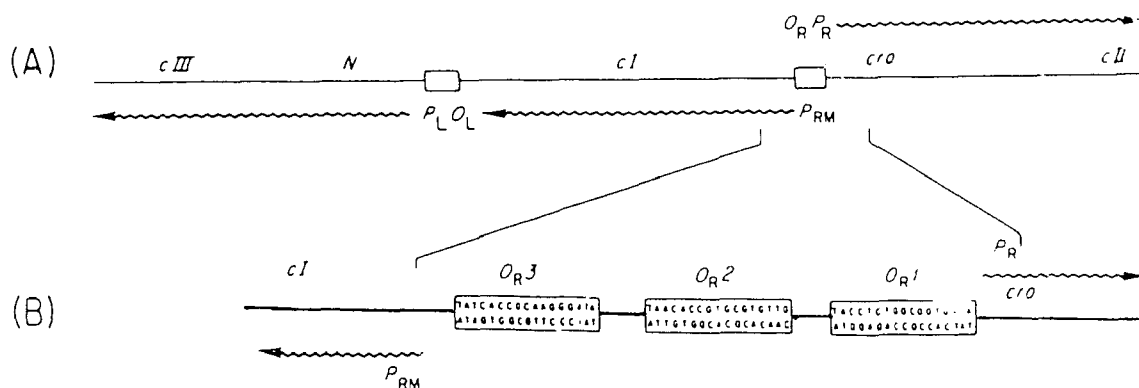


Fig. 21. A portion of the phage λ genome showing the regulatory elements which control the lytic and lysogenic cycles. (A) The arrows on the wavy lines indicate the direction of transcription of various genes. O_L and O_R are the left and right operator regions, and P_L , P_R , P_{RM} are their associated promoter regions. These promoters are subject to repression by either the C_I repressor or the Cro protein as described in the text. (B) Expanded diagram of the λO_R region. O_{R1} , O_{R2} and O_{R3} are C_I and Cro binding sites, each of which consists of 17 bp. The binding of RNA polymerase to either the P_R or the P_{RM} sites will be prevented by repressor proteins occupying the operator sites because of the functional overlaps in the repressor and operator sites. Modified from ref. 154.

to Fig. 21). First, Cro and CI have reciprocally antagonistic effects on gene expression. At moderate concentrations, Cro binds to O_R3 and thereby blocks transcription of cI . Alternatively, at moderate concentrations, CI binds to O_R1 and thereby blocks transcription of cro . In both cases, the protein-DNA interactions physically exclude the binding of RNA polymerase to the promoter sequences (161). The second important aspect is the autoregulatory consequence of these protein-DNA interactions in regulating expression of cI and cro . CI is a positive regulator of its own synthesis in that CI binding to O_R2 stimulates transcription of cI (162). Moreover, the binding of CI to O_R3 blocks cI transcription but this would occur only at very high CI concentrations (163). Furthermore, Cro negatively regulates its own synthesis; at high concentration, the binding of Cro to O_R1 prevents transcription of cro (154). In summary, CI blocks the λ lytic cycle by preventing the expression of genes involved in lytic growth, including cro , which are expressed from P_R . In addition, CI also binds to the left operator sequence, O_L , to prevent expression lytic cycle genes expressed from P_L (164). In contrast, Cro induces the lytic cycle by preventing the expression of cI (165). The fate of phage λ to grow in either one of the two alternate modes thus depends on the competition between CI and Cro for the three binding sites in the right operator region of the λ genome (154).

II. Bacteria, Bacteriophages and Plasmids

The E. coli strains, bacteriophages and plasmids used in this chapter are listed in Table 12.

III. Results

1. Attempt to Clone the lytD Gene

An E. coli gene bank was prepared by ligating a partial Sau3AI digest of chromosomal DNA into the unique BamHI site of the phasmid vector, λ SE6, as described in Chapter 2. This bank was screened for clones which complemented the smhB1, lytD1, and lytE1 mutations. No smhB and lytE clones were found in three attempts using 3 independently prepared gene banks. On the other hand, lytD clones were readily isolated. Fig. 22 shows the BamHI digests of recombinant phasmids purified from 4 positive lytD clones. The 33 kb fragment common to all of the phasmids represents the λ SE6 vector. Furthermore, all of the phasmids carried a 7 kb insert. Two of the phasmids (lanes 3 and 4) contained 2 additional inserts of 5.5 (lane 3) and 12 kb (lane 4). These results suggested that the lytD complementing activity may reside on the 7 kb fragment common to all of the phasmids.

2. lytD1 Complementation by the Phage λ cro Gene

The lytD1 complementing activity was subcloned in the

Table 12. E. coli K-12 strains, bacteriophages and plasmids

Strain	Relevant Genotype/Description	Reference/source
AA102F'lac	<u>thi</u> <u>pro</u> <u>endA</u> <u>recA</u> $\Delta(\text{gal-chl-pql-att})$ [F' <u>traD36</u> <u>proAB</u> <u>lacI^q</u> <u>ZAM15</u>]	GBT ^a
CSH66	<u>thi</u> $\Delta(\text{lac})$ (λ <u>cI857S7</u>)	CSH
DH5 α	<u>thi</u> <u>hsdR17</u> <u>recA1</u> <u>relA1</u> <u>endA1</u> <u>gyrA96</u> ϕ 80 <u>dIac</u> <u>ZAM15</u>	BRL ^b
JM101	<u>thi</u> $\Delta(\text{lac-proAB})$ [F' <u>traD36</u> <u>proAB</u> <u>lacI^q</u> <u>ZAM15</u>]	Laboratory collection
JM109	<u>thi</u> <u>endA1</u> <u>recA1</u> <u>relA1</u> <u>gyrA96</u> <u>hsdR17</u> $\Delta(\text{lac-proAB})$ [F' <u>traD36</u> <u>proAB</u> <u>lacI^q</u> <u>ZAM15</u>]	Laboratory collection
KX100	<u>hsdR514</u> <u>lacY1</u> <u>galK2</u> <u>galT22</u> <u>metB1</u> <u>trpR55</u> <u>recA56</u> <u>srl::Tn10</u>	(130)
Q359	<u>hsdR</u> <u>hsdM⁺</u> P2 lysogen	(125)
VC7	<u>thi-1</u> <u>lysA23</u> <u>rpsL109</u>	Laboratory collection
VC4014	VC7 <u>zbc-103::Tn10</u> <u>lytD1</u>	This study
VC4040	VC4014 λ lysogen	This study
VC4211	pDD65 in VC4014	This study
VC4214	pUC19 in VC4014	This study
W3104	W3110 λ lysogen	Laboratory collection
W3110	wild type	Laboratory collection

Table 12, continued

Bacteriophage Relevant Genotype/Description			Reference/Source
λ SE6	Kan ^r Amp ^r		(124)
λ cI857	temperature-sensitive cI		(134)
λ vir	O _R O _L mutation		(133)

Plasmid	Relevant Genotype/Description	Reference/Source
pAA-PZ718	Amp ^r Cam ^r IS1-Tn9 <u>ori</u> (M13)	GBT ^a
pAA-PZ719	Amp ^r Cam ^r IS1-Tn9 <u>ori</u> (M13)	GBT ^a
pDD51	Kan ^r <u>cro</u> ⁺ on 7 kb <u>Bam</u> HI fragment in λ SE6	This study
pDD52	Amp ^r <u>cro</u> ⁺ on 7 kb <u>Bam</u> HI fragment in pUC19	This study
pDD53	Amp ^r <u>cro</u> ⁺ on 4.3 kb <u>Bam</u> HI- <u>Hind</u> III fragment in pUC19	This study
pDD54	Amp ^r Cam ^r <u>cro</u> ⁺ on 2.5 kb <u>Sma</u> I- <u>Hind</u> III fragment in pAA-PZ718	This study
pDD55	Amp ^r Cam ^r <u>cro</u> ⁺ on 2.5 kb <u>Sma</u> I- <u>Hind</u> III fragment in pAA-PZ719	This study
pDD56	Amp ^r <u>cro</u> ⁺ deletion derivative of pDD54	This study
pDD57	Amp ^r <u>cro</u> ⁺ deletion derivative of pDD54	This study
pDD58	Amp ^r <u>cro</u> ⁻ deletion derivative of pDD54	This study
pDD59	Amp ^r <u>cro</u> ⁻ deletion derivative of pDD55	This study
pDD60	Amp ^r <u>cro</u> ⁻ deletion derivative of pDD55	This study

Table 12, continued

pDD61	Amp ^r <u>cro</u> ⁺ deletion derivative of pDD55	This study
pDD63	Amp ^r <u>cro</u> ⁺ on 1.1 kb <u>HindIII</u> - <u>PstI</u> fragment in pT7-3	This study
pDD64	Amp ^r <u>cro</u> ⁺ on 1.1 kb <u>HindIII</u> - <u>PstI</u> fragment in pT7-4	This study
pDD65	Amp ^r <u>cI</u> ⁺ on 2.4 kb <u>BglIII</u> fragment in pUC19	This study
pDD70	Kan ^r (<u>cro</u> ⁺) on 12.5 (7+5.5) kb <u>BamHI</u> fragment in λSE6	This study
pDD71	Kan ^r (<u>cro</u> ⁺) on 19 (7+12) kb <u>BamHI</u> fragment in λSE6	This study
pDD72	Kan ^r (<u>cro</u> ⁺) on 7 kb <u>BamHI</u> fragment in λSE6	This study
pDD73	Kan ^r (<u>cro</u> ⁻) on 18 kb <u>BamHI</u> fragment in λSE6	This study
pGP1-2	Kan ^r T7 RNA polymerase	(129)
pT7-3	Amp ^r T7 promoter φ10	(129)
pT7-4	Amp ^r T7 promoter φ10	(129)
pUC19	Amp ^r	Pharmacia

^aGold Biotechnology Inc. St. Louis, MO. U.S.A.

^bBethesda Research Laboratories, Gaithersburg, MD. U.S.A.

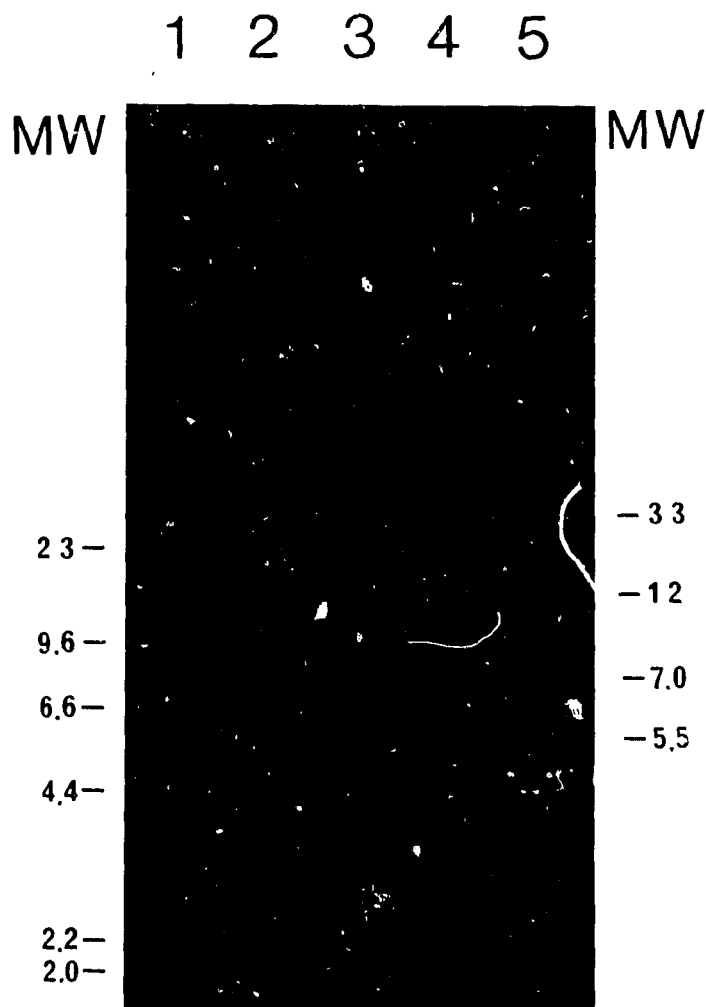


Fig. 22. Electrophoretic analysis of 4 *lytD1* complementing recombinant plasmids independently isolated from an *E. coli* gene bank. The plasmids (lane 2, pDD51; lane 3, pDD70; lane 4, pDD71; lane 5, pDD72) were digested with *Bam*HI and subjected to electrophoresis in a 0.8% agarose gel. Lane 1 is the DNA molecular standard marked in kb on the left. The numbers on the right side indicate the estimated sizes (kb) of the fragments generated by the *Bam*HI digestion of the plasmids.

following way. The 7 kb BamHI fragment from pDD51 (lane 2; Fig. 22) was purified by agarose gel electrophoresis and ligated into the BamHI site of pUC19. This derivative, pDD52, complemented lytD1. Fig. 23 shows a restriction map of the 7 kb BamHI fragment and summarizes further subcloning strategy. The lytD1 complementing activity was localized further on a 4.3 kb HindIII-BamHI fragment in a derivative designated pDD53. A 2.5 kb HindIII-SalI fragment derived from pDD53 was subsequently shown to complement lytD1 when cloned in either orientation in either pAA-pZ718 (pDD54) or pAA-pZ719 (pDD55). The complementation activity was localized further with sets of nested deletions derived from pDD54 (pDD56, pDD57, and pDD58) and from pDD55 (pDD59, pDD60, and pDD61). These deletion derivatives were used to determine the nucleotide sequence of the lytD1 complementing gene according to the strategy outlined at the bottom of Fig. 23. The lytD1 complementation activity, as defined by the deletion analysis in Fig. 23, coincided with a single open reading frame specifying a small protein consisting of only 66 amino acid residues (Fig. 24). The 1.1 kb fragment carrying the lytD1 complementing activity on the deletion derivative, pDD61 (Fig. 23), was obtained by utilizing the HindIII site at the 5'-end of the open reading frame and a unique PstI site (derived from the vector and therefore not shown in Fig. 23) at the 3'-end. This fragment was subcloned in both orientations in front of the phage T7 promoter ϕ 10 in the expression vectors, pT7-3 and

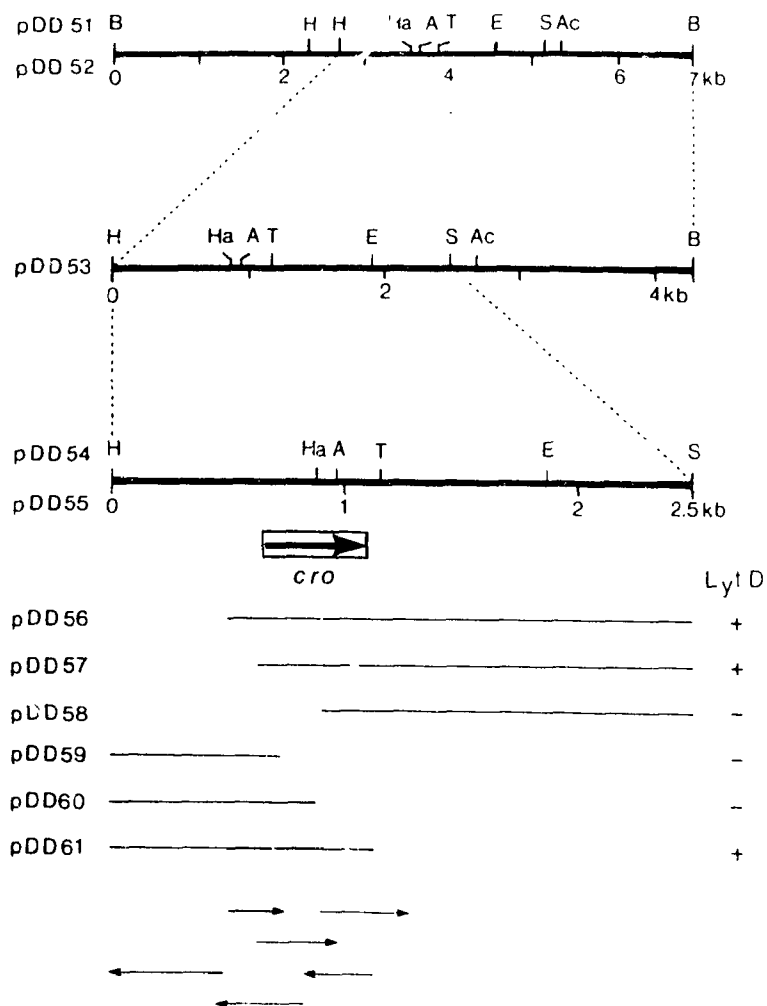


Fig. 23. Restriction map of the 7 kb *Bam*HI fragment carrying the λ *cro* gene and the strategies for subcloning and DNA sequencing of the λ *cro* gene. Locations of restriction sites used for plasmid construction are shown at the top. Abbreviations for restriction enzyme cleavage sites: A, *Ava*I; B, *Bam*HI; E, *Eco*RI; Ha, *Hae*I; H, *Hind*III; S, *Sma*I; T, *Taq*I. Thick horizontal lines denote the fragments cloned into the corresponding vectors (see Table 12). The boxed thick arrow represents the location and transcriptional direction of the open reading frame of the *cro* gene as determined from the DNA sequencing data and the expression experiments (see text). Plasmids pDD56 through pDD61 are deletion derivatives of pDD54 and pDD55, which were used to define the *lytD* complementing activity (results are shown on the right) and to sequence the gene according to the strategy indicated by the arrows at the bottom.

pT7-4 to yield plasmids pDD63 and pDD64, respectively. As expected, both plasmids complemented lytD1, further confirming that the 1.1 kb fragment contained the complete lytD1 complementing gene including its promoter. The open reading frame was expressed from the T7 promoter ϕ 10 as described in Chapter 2. Fig. 25 (lane 1) confirms that the open reading frame on the 1.1 kb fragment in pDD64 specified a small polypeptide estimated to have a molecular weight of 7,400 by SDS polyacrylamide gel electrophoresis. When the open reading frame was cloned in the opposite orientation in pDD63, no such product was observed (lane 2). Fig. 25 (lanes 5-8) also shows that the 7.4 kDal protein was localized exclusively in the cytoplasmic fraction. This protein is undoubtedly the phage λ Cro protein because the nucleotide sequence shown in Fig. 24 was identified as that of the phage λ cro gene through a search of the EMBL DNA database.

The lytD1 complementation data summarized in Fig. 23 is quantitatively expressed as plating efficiencies in Table 13. The data confirm that lytD1 complementation required the complete cro open reading frame. Deletions extending into either the 5'- or 3'-end of the cro gene resulted in loss of lytD1 complementation as measured by colony formation at 42°C. Complementation was correlated with the ability of cro to suppress the lysis phenotype associated with lytD1 at 42°C. This is illustrated with 2 examples in Fig. 26. Strain VC4014 was lysis-tolerant when it carried the complete cro gene in

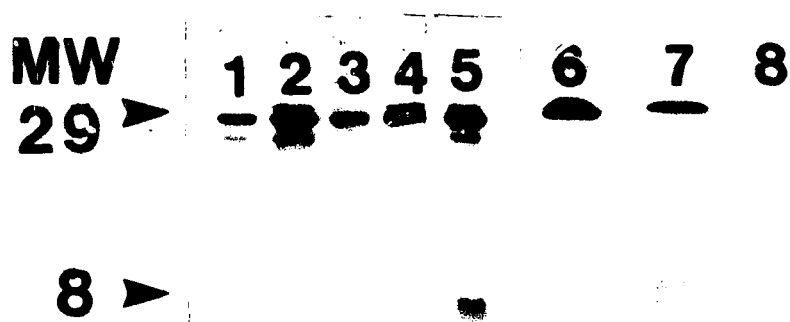


Fig. 25. Expression (lanes 1-4) and localization (lanes 5-8) of the *lytD1* complementing protein as directed in a phage RNA polymerase and promoter system. Autoradiogram showing [³⁵S]methionine-labeled, plasmid encoded proteins synthesized in strain KX100 (pGP1-2) that had been transformed with the following plasmids: pDD64 (lane 1); pDD63 (lane 2); pT7-4 (lane 3); pT7-3 (lane 4). For protein localization, KX100 carrying pGP1-2 and pDD64 was labeled with [³⁵S]methionine and then fractionated into periplasmic (lane 6), cytoplasmic (lane 7), and cell envelope (lane 8) fractions by spheroplast formation and lysis. Lane 5 is the unfractionated cell control. Positions of relevant protein molecular weight standards (carbonic anhydrase, 29 kDal; sperm whale myoglobin I, 8 kDal) are indicated on the left.

Table 13. lytD1 complementation activity of recombinant plasmids in strain VC4014

Plasmid	CFU per ml at 42°C/CFU per ml at 30°C
none	8.6 X 10 ⁻⁶
pDD54	1.0
pDD55	1.0
pDD56	0.9
pDD57	1.0
pDD58	7.2 X 10 ⁻⁶
pDD59	4.4 X 10 ⁻⁶
pDD60	6.0 X 10 ⁻⁶
pDD61	1.0

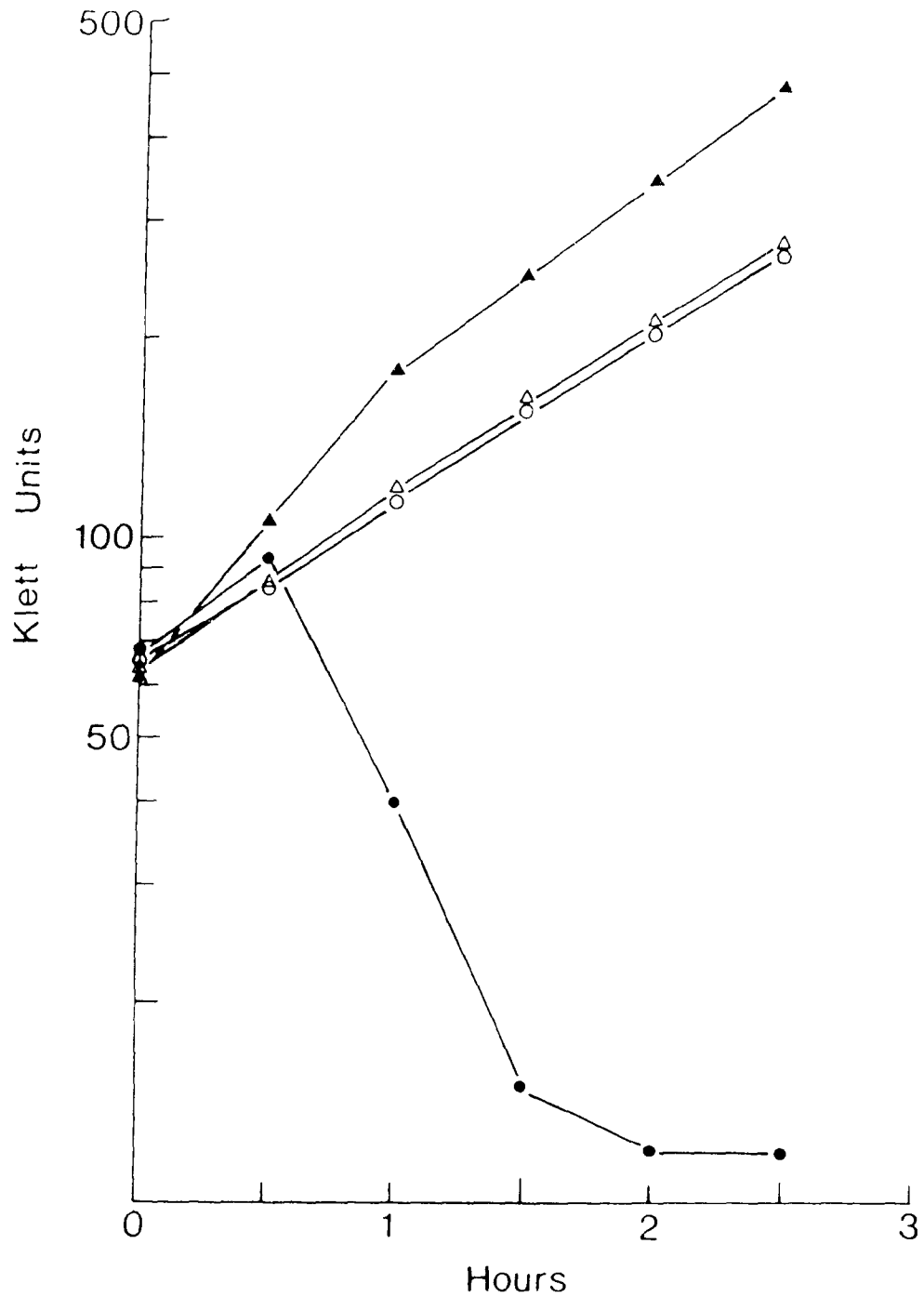


Fig. 26. Suppression of *lytD1*-mediated lysis by the λ *cro* gene. Growth of strain VC4014 (*lytD1*) carrying pDD60 (*cro*) at 30°C (O) and at 42°C (●) in TSB. Growth of strain VC4014 carrying pDD61 (*cro*⁺) at 30°C (Δ) and at 42°C (▲) in TSB.

pDD61 but not when it carried the deletion derivative, pDD60.

The 1.1 kb fragment derived from pDD61 which carries the cro gene was used as a probe in a Southern blot analysis of 2 of the clones described above as well as of the phage λ and E. coli genomes in Fig. 27. This experiment was designed primarily to determine the origin of the cloned cro gene in the gene library; i.e., we wished to verify that it could not have come from the nonlysogenic strain, W3110 (the source of the DNA for the gene library). As expected, the probe hybridized to the 2.5 kb SmaI-HindIII fragment of pDD54 (lane b) as well as to the 4.3 kb BamHI-HindIII fragment of pDD53 (lane c). The probe also hybridized to the 7 kb BamHI (lane d), and 6.6 kb HindIII (lane e) fragments derived from the λ genome, and this is in agreement with the known physical map of the λ genome. It is noteworthy that the probe hybridized to both the 33 kb and the 7 kb BamHI fragments of pDD51 (lane f); i.e., pDD51 contains 2 copies of cro, one on the vector (33 kb) and one on the insert (7 kb) (The apparent quantitative differences in the degree of hybridization are attributed to the incomplete transfer of the 33 kb fragment from the gel to the Zeta-probe membrane). Finally, the BamHI-digested chromosomal DNAs of strains W3104 (λ lysogen) and W3110 (nonlysogenic) were compared. A 7 kb fragment which hybridized with the probe was undoubtedly derived from the λ prophage of W3104 (lane g). This component was not observed in W3110 (lane h). Interestingly, both strains exhibited at

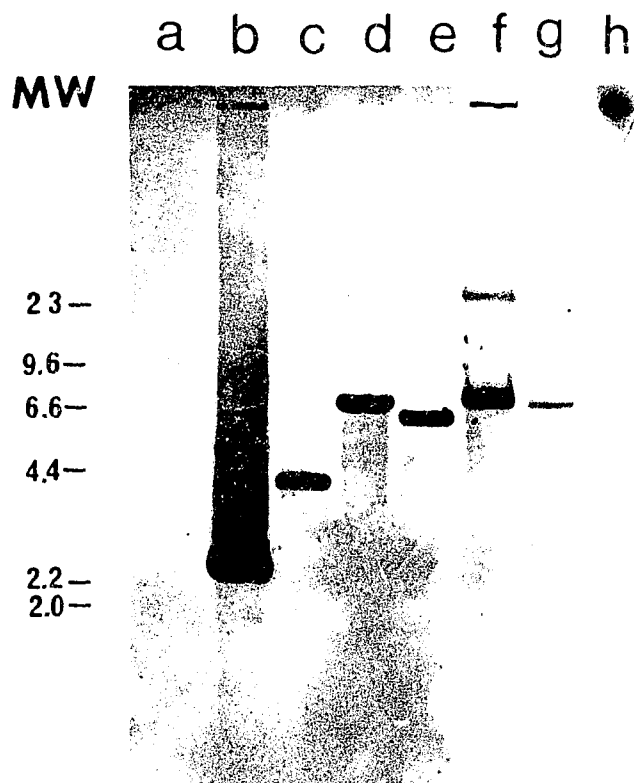


Fig. 27. Southern blot analysis of *E. coli* and λ phage genomes probed with a 1.1 kb PstI-HindIII fragment, carrying the λ *cro* gene, derived from pDD61. (a) no DNA control; (b) SmaI-HindIII-digested pDD54; (c) BamHI-HindIII-digested pDD53; (d) BamHI-digested λ DNA; (e) HindIII-digested λ DNA; (f) BamHI-digested pDD51; (g) BamHI-digested W3104 (λ) chromosomal DNA; (h) BamHI-digested W3110 chromosomal DNA. DNA standard markers are shown on the left (kb).

least 6 common fragments (31, 26, 13, 6.8, 5.5, and 5 kb) which weakly hybridized with the probe, suggesting that E. coli K-12 possesses several genes related to cro. Collectively, these results confirm that the 7 kb BamHI fragment carrying the cro gene most likely originated from a λ genome and could not have come from W3110. As discussed below, we propose that the λ SE6 vector preparation was contaminated with DNA derived from the λ helper phage.

The 1.1 kb cro probe was also used for a Southern blot analysis comparing BamHI digests of all of the 4 lytD1 complementing λ SE6 derivatives described in Fig. 22. A noncomplementing clone, pDD73, was included as a control. Fig. 28 shows that, in all cases, the probe hybridized with the 33 kb fragment representing the λ SE6 vector as expected. The noncomplementing clone (lane b) did not exhibit any other hybridizing fragments. All of the lytD positive clones exhibited a 7 kb fragment carrying cro. The 5.5 kb and 12 kb fragments in pDD70 (lane e) and pDD71 (lane d), respectively, did not hybridize with the cro probe. These results suggest that the lytD complementing activity of all 4 recombinant phasmid clones was attributable to the same 7 kb BamHI fragment containing the cro gene of phage λ .

3. Demonstration of the cI Gene in pDD51

A re-examination of the restriction map indicated that the original 7 kb BamHI fragment in phasmid pDD51 represented

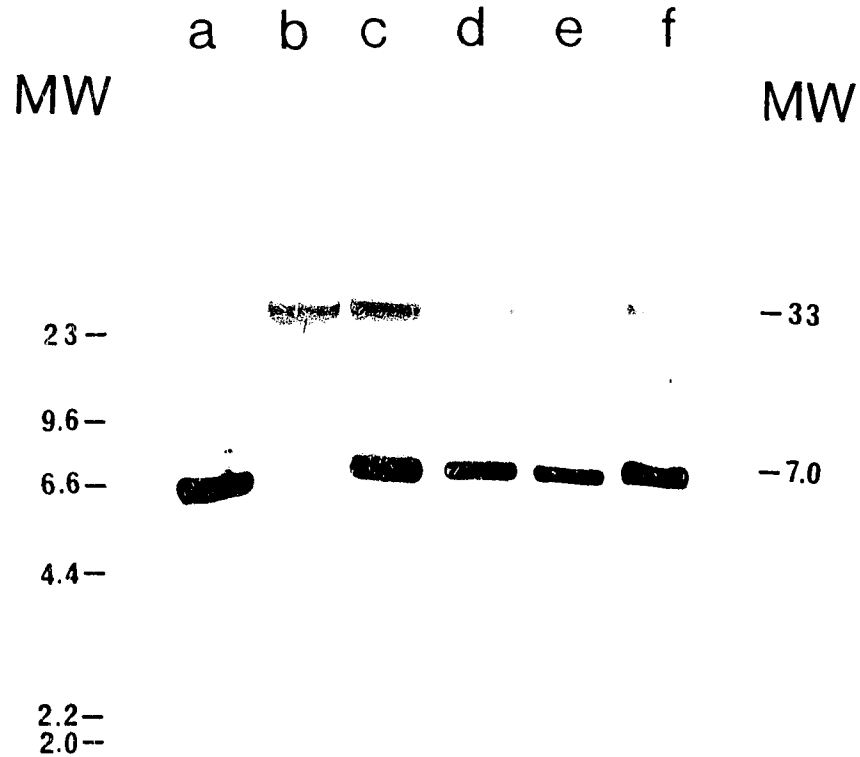


Fig. 28. Southern blot analysis of the 4 *lytD1* complementing clones probed with a 1.1 kb *Pst*I-*Hind*III fragment, carrying *cro* gene, derived from pDD61. (a) *Hind*III-digested λ DNA; (b) *Bam*HI-digested pDD73; (c) *Bam*HI-digested pDD72; (d) *Bam*HI-digested pDD71; (e) *Bam*HI-digested pDD70; (f) *Bam*HI-digested pDD51. DNA molecular standard markers were shown on the left (kb).

the segment from the N gene to the ren gene of the phage λ genome (166) and, therefore, included the cI gene as well as the cro gene. As noted above, the recombinant λ SE6 vector used for cloning the lytD1 complementing activity, does not contain a copy of cI and must be maintained in the presence of a copy of cI in trans in order to repress the λ lytic cycle genes carried on the vector, e.g., in λ lysogenic hosts. The presence of a functional wild type cI gene on the 7 kb BamHI insert in pDD51 was consequently easily verified by demonstrating that the phasmid pDD51 was stably maintained in the absence of a cI gene provided in trans, i.e., in nonlysogenic hosts. The presence of cI on the 7 kb BamHI fragment was important to establish in view of the fact that CI is a repressor of cro expression. Therefore, the presence of a functional CI was further assessed by testing the immunity of strains carrying the 7 kb BamHI fragment in single copy (pDD51) or in multicopy (pDD52) to infection by either phage λ vir or phage λ cI857. λ vir carries mutations in both the right operator and the left operator of the λ genome which reduce the binding affinity of CI; λ vir is consequently virulent and unable to lysogenize (133). λ cI857 possesses a temperature-sensitive CI repressor which is inactivated at high temperature (e.g., 42°C); i.e., the lytic cycle is induced at the restrictive temperature (134). Table 14 confirms the λ sensitivity of strains DH5 α and VC4014 (lytD1) (the λ sensitivity of VC4014 could not be tested at 42°C

Table 14. λ immunity assay of strains DH5 α (lytD⁺), and VC4014 (lytD1) carrying different plasmids

Strain	Plasmid	<u>λvir</u>		<u>λcI857</u>	
		30°C	42°C	30°C	42°C
DH5 α	none	+	+	+	+
VC4014	none	+	NA	+	NA
W3104	none	+	+	-	-
DH5 α	pDD51	+	+	-	-
VC4014	pDD51	+	+	-	-
DH5 α	pDD52	-	-	-	-
VC4014	pDD52	-	-	-	-

Results were scored after 14 h incubation: +, λ sensitive; -, λ immune; NA, not applicable, strain is ts.

because of the temperature sensitivity of the strain). Furthermore, strain W3104 (λ lysogen) was immune to λ_{cI857} at both 30°C and 42°C due to the provision of wild type CI by the resident λ prophage but was sensitive to λ_{vir} ; these results confirm the phenotypes of the λ derivatives. Table 14 also shows that DH5 α and VC4014 carrying pDD51 were sensitive to λ_{vir} (VC4014 could be tested at 42°C in this case because its lytD1 mutation was complemented by cro on pDD51). Significantly, both DH5 α and VC4014 pDD51 derivatives were immune to λ_{cI857} indicating that sufficient CI was being provided by the 7 kb fragment on pDD51 to repress the λ lytic cycles programmed by both the λ_{cI857} genome and the λ_{SE6} portion of pDD51. Furthermore, the fact that immunity was observed at 42°C suggested that the CI encoded on pDD51 was wild type. When the 7 kb BamHI fragment was present in the multicopy derivative, pDD52, DH5 α and VC4014 were not only immune to λ_{cI857} but also to λ_{vir} suggesting that the very high concentrations of CI suppress the vir mutation. Collectively, these results suggest the cI gene cloned on the 7 kb BamHI fragment provides sufficient CI protein to effectively repress the adjacent cro gene, and this has at least 2 possible ramifications. (i) It is possible that the expression of cro is unnecessary for its lytD1 complementing activity. (ii) On the other hand, the expression of cro may have been essential for lytD1 complementation, but in the case of the 7 kb BamHI fragment where cro expression was repressed by CI, it was

possible that the expression of an alternate gene was responsible for the observed lytD1 complementation; if so, the cI gene seemed to be the best candidate for this alternate gene. The second proposal suggests that the expression of either cro or cI could result in lytD1 complementation, and this is based on the fact that CI and Cro are functionally related; i.e., they are DNA-binding proteins which interact with identical recognition sequences. This proposal is tested in the next section.

4. Complementation of lytD1 by the λ cI Gene

Fig. 29 summarizes the strategy used to subclone the cI gene with a minimum amount of the cro gene present. A 2.4 kb BglII fragment derived from the 7 kb BamHI λ DNA insert in pDD52 contained the entire cI gene as well as a fragment of the cro gene encoding the N-terminal 20 amino acid residues. This fragment was ligated into the unique BamHI site of pUC19 (BglII and BamHI generate compatible cohesive ends). The ligation mixture was transformed into CSH66, a strain which is lysogenized by a λ derivative carrying a ts mutation in the cI gene; i.e., CSH66 is killed at 42°C because the mutant phage is thermoinduced. Transformants carrying recombinant plasmids with the cloned cI gene were readily selected by their ability to grow at 42°C. The plasmid from one such transformant, pDD65, was shown to carry the expected 2.4 kb insert (data not shown). Plasmids pDD65 and pUC19 were transformed into

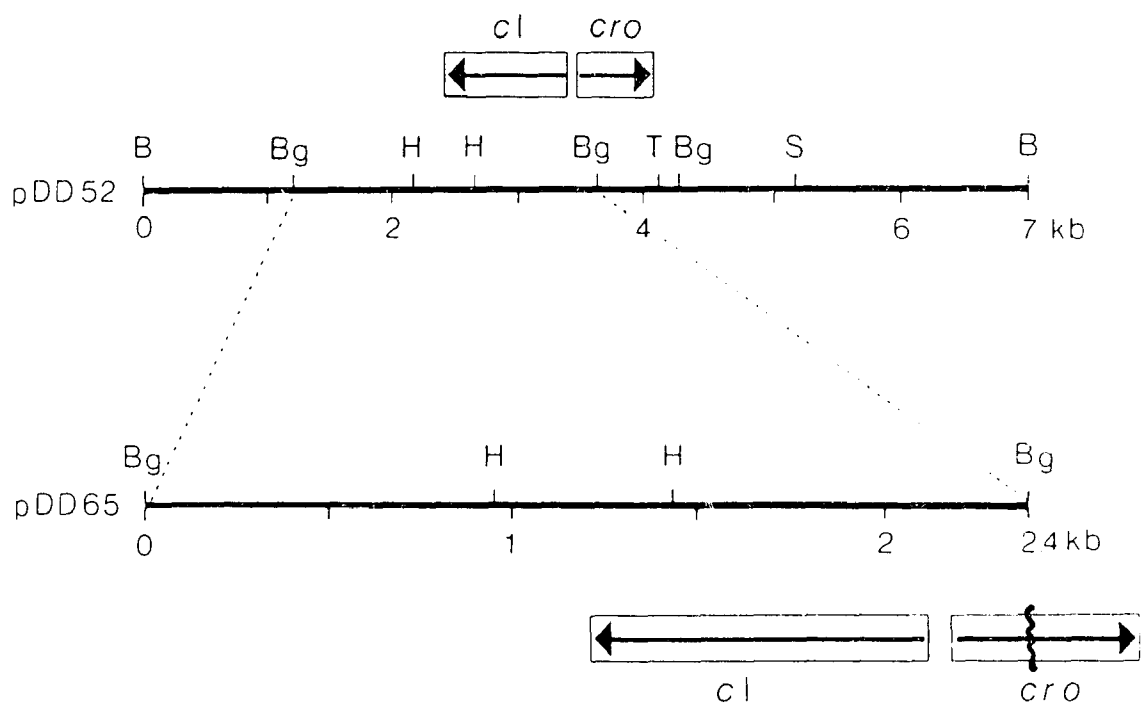


Fig. 29. Strategy for subcloning of the λ *cI* gene. Thick horizontal lines represent the fragments cloned into the corresponding vectors (see Table 12). The relevant restriction sites are: B, BamHI; Bg, BglII; H, HindIII; S, SmaI; T, TagI. The boxed arrows indicate the location and transcriptional direction of the open reading frames of the *cro* and *cI* genes. The vertical wavy line through the *cro* open reading frame denotes the position of the BglII site.

strain VC4014 (lytD1) to yield strains VC4211 and VC4214, respectively (in both cases, the selection was for ampicillin resistance at 30°C). VC4211 (pDD65) was immune to both wild type λ and to λ vir whereas VC4214 (pUC19) was sensitive to these phages. This result confirms that pDD65 contains the cI gene. Two results clearly indicate that cI complements lytD1. (i) Strain VC4211 was temperature-resistant and formed colonies at 42°C whereas VC4214 remained ts. (ii) Fig. 30 shows that, at 42°C, strain VC4214 exhibited a lysis phenotype whereas VC4211 was lysis-tolerant and actually grew normally.

5. Attempts to Complement Other Mutations with cI and cro

The cloned cI and cro genes failed to confer temperature resistance on the murH1, lytE1, and smhB1 mutants. Therefore, the complementation activities of cro and cI were apparently specific for the lytD1 allele.

IV. Discussion

The lytD1 complementing activities of all 4 clones isolated in this study were found to reside on a 7 kb BamHI fragment which was derived from the genome of phage λ . This DNA clearly did not originate from the E. coli strain W3110 chromosome, the source of the genomic library, as demonstrated by the Southern blot analysis using the 1.1 kb fragment carrying the cro gene as a probe. Thus, the clones obtained

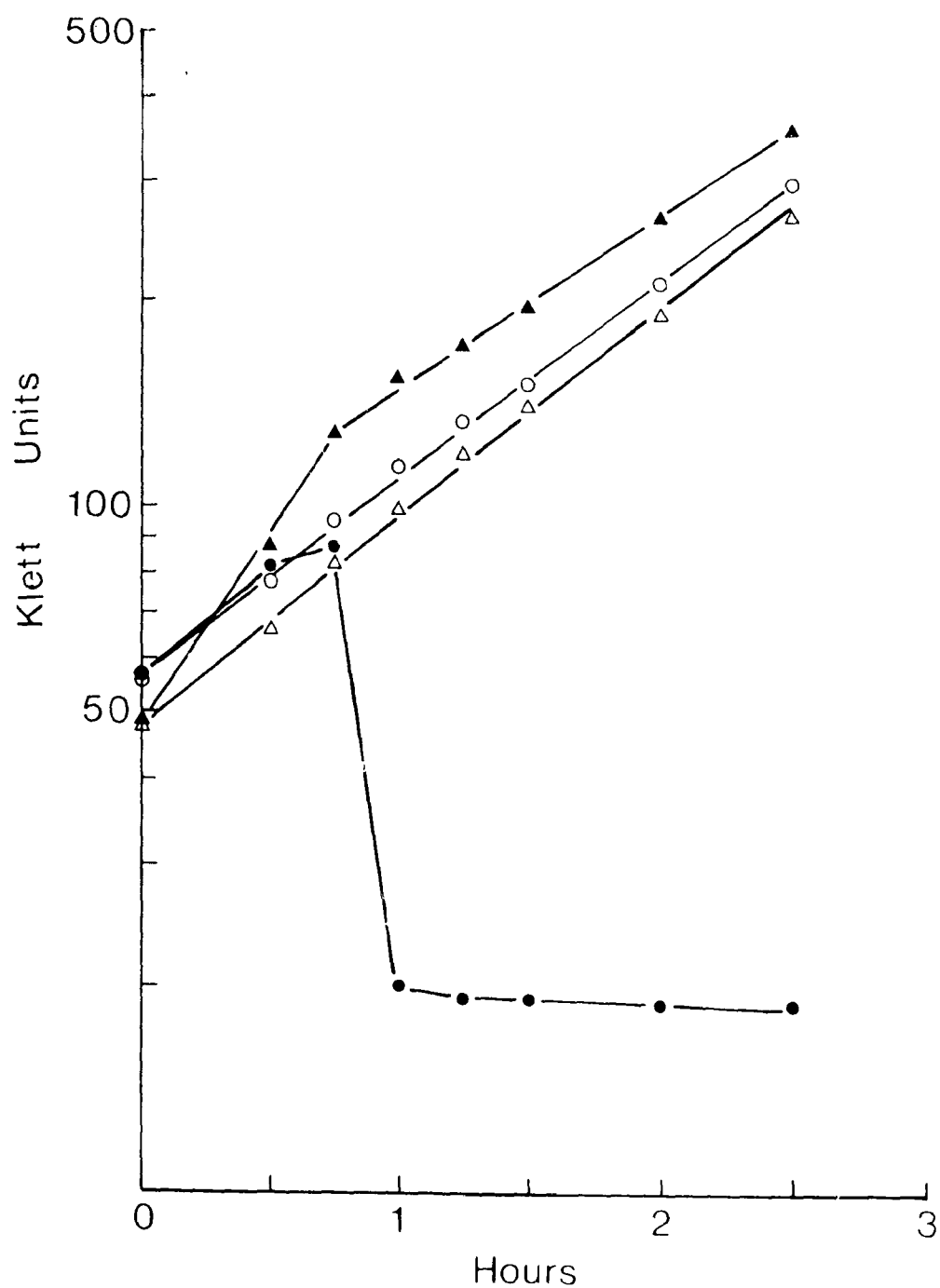


Fig. 30. Suppression of *lytD1*-mediated lysis by the λ *cI* gene. Growth of strain VC4214 (*lytD1*, *cI*⁻) at 30°C (O), and at 42°C (●) in TSB. Growth of strain VC4211 (*lytD1*, *cI*⁺) at 30°C (Δ) and at 42°C (▲) in TSB.

do not represent the E. coli lytD locus. The reason for our inability to isolate a lytD clone is not known, but it is worth noting again that 3 attempts to clone either the smhB or the lytE loci were also unsuccessful. Since clones complementing an assortment of auxotrophic markers were readily recovered from the gene library, it is unlikely that the library was defective. The 7 kb BamHI fragments found in all 4 positive clones were identical as determined by the Southern blot analysis, and they apparently originated from λ helper phage DNA which contaminated the λ SE6 vector DNA preparation. These fragments were probably generated when the vector preparation was digested with BamHI during the construction of the gene library. The restriction map of the BamHI fragment corresponded to the 35.3 to 42.3 kb segment of the phage λ physical map (166).

The lytD1 complementing activity was initially subcloned on a 2.5 kb HindIII-SalI fragment, and the deletion analysis of this fragment localized the activity to an area of about 0.6 kb. These results, combined with the nucleotide sequence of the segment, leave little doubt that complementing activity coincided with the λ cro gene. The subsequent demonstration of a functional cI gene on the BamHI fragment made it rather unlikely that the cro gene was being expressed in view of Ptashne's estimation that cro is 99.7% repressed by CI in λ lysogens (167). Therefore, one possible interpretation of these results is that the lytD1 complementing action of cro

required the intact cro gene, but complementation was not dependent on expression of the gene. However, this hypothesis requires further clarification on the number of copies of cro necessary for the observed lytD1 complementation, and this situation can be summarized by the following observations. (i) In lysogens carrying wild type λ (and therefore one copy of cro), the lytD1 mutation was still expressed in the form of a ts lysis phenotype. (ii) The λ SE6 cloning vector (which is a derivative of λ) contains a copy of cro, but only recombinants carrying the λ 7 kb BamHI fragment (i.e., carrying 2 copies of cro) complemented lytD1. Furthermore, since these clones were maintained in λ lysogens, which already have one copy of cro, lytD1 complementation apparently required a minimum of 3 copies of cro, none of which was being appreciably expressed due to the repressor action of CI. One problem with this hypothesis is that it is relatively difficult to think of a reasonable mechanistic basis for the complementation if gene expression was unnecessary. Nevertheless, it must be emphasized that there is no doubt about the lytD1 complementing activity of the cro gene which was subcloned on a multicopy vector in the absence of cI (e.g., pDD61). The fact that Cro and CI are DNA-binding proteins with identical target sequences suggested an alternative hypothesis, namely that both cro and cI could complement lytD1. In order to test this possibility, we had to clone the cI gene because we were unable to obtain the cloned cI gene previously constructed by

Backman et al. (168). The clone (pDD65) we constructed contained the complete cI gene as well as the 5'-end of cro which encoded the N-terminal portion of Cro consisting of 20 amino acid residues. Even if this truncated cro gene was expressed, the peptide product would not have any DNA-binding activity (153). This clone conferred both temperature-resistant colony-forming activity as well as lysis tolerance in the lytD1 mutant, indicating that cI could indeed complement lytD1.

We propose that the lytD1 complementing activity on the original 7 kb BamHI fragment was due to the expression of the cI gene rather than to the mere presence of the cro gene. On the other hand, it is clear that the cro gene by itself has lytD1 complementing activity, and we favour the idea that the expression of cI cro is absolutely essential for complementation. Collectively, the results presented here suggest that complementation is based on the common DNA-binding properties of the protein products of these genes, and this further suggests that the lytD gene product is a DNA-binding protein with a DNA target specificity similar to that of Cro and CI; i.e., LytD may bind to a sequence similar to the right operators of λ .

A minimum of 2 copies of the cI gene per cell were required to complement lytD1. A λ lysogenic lytD1 mutant strain such as strain VC4040 was still ts. Thus, the single copy of λ carried by these cells produced sufficient CI protein to confer immunity to λ infection, but this amount was

clearly insufficient to overcome the lytD1 mutation. On the other hand, the introduction of only a single additional copy of cI to these cells, e.g., on pDD51, conferred temperature resistance and lysis suppression. This result probably indicates that the binding affinity of CI for the proposed LytD DNA target sequence is lower than its affinity for the λ O_R sequences.

I will conclude this thesis with a consideration of a working hypothesis designed to serve as a guide for future investigations. It must be emphasized that the hypothesis is speculative but is consistent with the data. The key to the hypothesis is the finding that the LytD1 function can be replaced by either Cro or CI. LytD may regulate the expression of a gene(s) involved in a function which could lead either directly or indirectly to cellular autolysis. The identity of this gene(s) remains to be determined, and it is not known whether it is represented in the collection of mutants described in this work. In this respect, 2 points which have already been made are worth repeating. (i) The murH, smhA, smhB, and lytE loci are apparently functionally related to lytD. (ii) Our hypothesis predicts that the LytD-regulated gene(s) will have an operator sequence similar to the right operator sequences of λ . With regard to a mode of action, LytD may be a transcriptional repressor of this lysis gene(s), and the lytD1 mutation may produce a ts repressor which would result in the derepression of the lysis gene(s) at

the restrictive temperature. Although the possibility cannot be completely ruled out, it is more difficult to visualize LytD as a positive control element under these circumstances. Of course, it is possible that LytD acts both negatively and positively, for example, like CI.

The origin of the murH, smhA, smhB, lytD, and lytE genes is unknown, but 3 possibilities are worth considering. Firstly, they may represent examples of cryptic λ -related sequences which have been shown to exist in several regions of the E. coli chromosome by DNA hybridization with a λ DNA probe (110). In support of this observation, it is interesting that the cro probe used in this study detected several cro-related sequences in the chromosomes of our E. coli K-12 strains. Whether any of these sequences represents the chromosomal lytD locus remains to be determined. Phage λ is actually a member of a large family of similar temperate phages referred to collectively as the lambdoid family (reviewed in ref. 169). Other prominent members of this diverse group are the E. coli phages $\phi 80$, P21, and 434, and the Salmonella typhimurium phage P22. The concept of a family in this case is based primarily on the ability of these phages to exchange genetic information. The lambdoid phages possess a common genomic organization reflected by a modular arrangement of functional units; i.e., their genomic maps show an identical arrangement of sets of genes specifying such properties as immunity, recombination, replication, lysis, DNA integration, and phage

structural components. Furthermore, the mechanisms which control the expression of these modules is similar in all members of the family. However, the nucleotide sequences of the various modules may differ extensively in certain phages, and their regulatory mechanisms may involve elements which exhibit differences in specificities; e.g., the repressor from one phage may or may not interact with the operator sequences from other phages within the family. Nevertheless, their common genetic organization and mode of regulation permits exchange of whole genes or parts of genes and, sometimes, even groups of genes among the different phages. For example, nonhomologous genetic modules could be introduced into a lambdoid genome through recombination involving homologous flanking regions. This phenomenon could lead to a reshuffling of functional units, and this may be at least partially responsible for the diversity observed within the family. Therefore, it would appear that the lambdoid family constitutes a group of phages which have the potential for continually evolving by a process of interbreeding.

Campbell and Botstein (169) have proposed that the lambdoid phages evolved from a common primordial λ ancestor. It has also been hypothesized that most, if not all, viral genomes have originated from associations with their hosts, and, in the case of the lambdoid family, the similarities between certain genes in lambdoid phages and genes found in their bacterial host (e.g., chromosome replication origins)

support this proposal. Furthermore, as noted above, it is interesting and relevant here that cryptic λ -related DNA sequences have been demonstrated by DNA hybridization with λ probes in laboratory strains of E. coli and in other enterobacterial species (170). At least 3 of these sequences, designated as the Rac, Qsr', and Qin prophages, comprise modules which have been shown to recombine into the λ genome and to thereby functionally replace defective modules (171-173). Although the origins of these DNA elements are unknown, there are 2 interpretations worth considering. (i) Strathern and Herskowitz (109) have suggested that these cryptic sequences are "genetic debris" which had been introduced into cells during earlier virus infections and which were in the process of being eliminated. (ii) Campbell and Botstein (169) have proposed that some of these elements may in fact represent viral modules which have been recruited by the bacterial host to serve some cellular function. Perhaps both proposals are correct. It is known that the deletion of some of these sequences (e.g., Rac) has no effect on the host phenotype, and such sequences may therefore be genetic debris. On the other hand, it is clear that certain genes within these viral modules can be appropriated by the host under certain conditions. A relevant example here is the suppression of a mutation in the E. coli recB gene (which results in defects in recombination and DNA repair) by a mutation in the sbc gene which is part of the Rac module. Thus, as proposed by

Campbell and Botstein (169), it is unlikely that all genetic debris is simply discarded, and it is more likely that some debris is eventually salvaged for specific purposes by the host. They further point out that, from this viewpoint, it is difficult to distinguish between host modules which were appropriated by a primordial phage in the past from viral modules which are currently being appropriated by the host.

The 3 cryptic prophages which have been genetically mapped in E. coli are Rac at 29.6 to 30.1 min, Qin at 34.2 to 34.6 min, and Qsr' at 12.5 min (111). Interestingly, the location of Qsr' coincides with that of the smhB and lytD loci. Furthermore, the Qsr' module is composed of analogues of the λ Q, S, and R genes. The S and R genes encode a transmembrane channel-forming protein and a lytic peptidoglycan transglycosylase, respectively (174, 175). Together, these proteins carry out the lysis of the host cells during the terminal stage of the λ lytic cycle which is necessary to release the progeny phage. In view of the lysis phenotypes of the smhB1 and lytD1 mutations, it is tempting to propose that one or both of these mutations lie within the Qsr' module. If so, one limitation to our hypothesis is the fact that the regulation of gene expression is highly conserved within the lambdoid family. We have suggested that the lytD1 phenotype can be easily explained if LytD functioned as a transcriptional repressor of a lysis gene. Such a proposal seems unlikely in this case because the SR module is known to

be positively regulated (176).

On the other hand, it is very possible that the lysis gene(s) regulated by LytD are not related to lambdoid sequences; i.e., LytD could be a repressor of a native cellular gene(s) whose product(s) either directly or indirectly causes autolysis rather than genes which encode lambdoid lysins. In this case, LytD may still share structure-function features common to Cro and CI. An example of this nature has recently been reported. The angR gene of Vibrio anguillarum encodes a transcriptional activator which regulates the expression of an iron uptake system. Farrell et al. (177) have shown that the amino acid sequence of the AngR protein predicts a helix-turn-helix motif which is typical of prokaryotic DNA-binding proteins and is strikingly homologous to the DNA-binding domain of the phage P22 Cro protein. They have also demonstrated that the 2 putative operator sequences, representing the potential AngR binding sites, are similar to the O_R1 and O_R2 recognition sequences for the phage P22 Cro protein. Thus, it is possible to have lambdoid-like components involved in the regulation of nonlambdoid gene expression. We think this may evolve in one of 2 ways. (i) The lambdoid components may have been recruited, perhaps from the lambdoid "genetic debris" in the E. coli chromosome, to serve certain cellular functions such as the lysis functions dealt with here. (ii) Alternatively, the structure-function relationships of lambdoid-like components (e.g., DNA-binding

proteins and their specific recognition sequences) may have evolved fortuitously, independent of the lambdoid phage family.

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