The Characterization of *lytF*, a New Member of the *murH* Gene Family of *Escherichia coli.*

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Abstract

The characterization of a new gene, *lytF*, is described. The *lytF* mutation was mapped to 62.4 minutes on the *Escherichia coli* genetic linkage map, and was found to confer a temperature-sensitive colony forming and lysis phenotype. This mutant was of interest because its lytic phenotype resembled other previously described mutants that were defective in peptidoglycan metabolism.

To further characterize the role of *lytF*, two extragenic suppressors of *murH*, a mutant whose lysis phenotype is similar to that of *lytF* and which appears to be defective in peptidoglycan metabolism, were transduced into the *lytF* strain. Both of these suppressors, designated *smhA1* and *smhB1*, were found to suppress both the temperature-sensitive colony formation and lysis associated with the *lytF* mutation at high temperature. The *smhA1*- or *smhB1*-mediated suppression of the *lytF* temperature-sensitive colony formation was salt dependent, whereas the suppression of lysis was independent of the medium osmolarity. Because the ability of the *smhA1* and *smhB1* mutations to suppress lysis does not extend to mutational or antibiotic induced blockages in peptidoglycan biosynthesis, those mutations whose lytic phenotypes are suppressed by *smhA1* or *smhB1* have been proposed to be functionally related, and they have been designated as the *murH* family.

Three observations indicate that the *lytF* gene may belong to the *murH* family. (i) The temperature-sensitive colony forming and lysis phenotype associated with *lytF* is similar to that expressed by other mutants in the *murH* family (*murH*, *lytD*, *lytE*). (ii) The *lytF* phenotypes are suppressed by the presence of *smhA1* or *smhB1*, which are specific to this family in terms of their suppression phenotypes. (iii) It is possible to select spontaneous *smhA* or *smhB*-like mutations in a *lytF* genetic background, indicating that

the ability of *smhA* and *smhB* to suppress the *lytF* phenotype is more than just coincidence.

It was also observed that the phenotype associated with the *smhB* locus may be variable depending on the allele. All *smhB* alleles characterized in this study share a common genetic linkage to the *zbc-103* :: Tn*10* insertion, and are phenotypically dominant over the wild type *smhB*+ gene in complementation assays. However, the physical and suppression characteristics can differ substantially among alleles. It is currently unknown whether these differences in phenotype are due to different mutations in a single multifunctional gene, or different mutations in discrete, but closely linked genes.

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List of Abbreviations:

D-ala	D-alanine
L-ala	L-alanine
ATP	adensosine 5'-triphosphate
Bq	Becquerels
CFU	colony forming unit
DAP	diaminopimelic acid
DMA	Davis minimal agar
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis (β - aminoethyl ether) N,N,N',N'-tetraacetic acid
G	glycine
GCL	glycosyl carrier lipid
glcNAc	N-acetylglucosamine
D-glu	D-glutamic acid
H	L-histidine
K	L-lysine
λ	wavelength
LPS	lipopolysaccharide
meso-DAP	meso-diaminopimelic acid
MIC	minimum inhibitory concentration
murNAc	N-acetylmuramic acid
murNAc-tripeptide	N-acetylmuramyl - L-ala - D-glu - meso-DAP

List of Abbreviations (cont'd)

murNAc-pentapeptide	N-acetylmuramyl - L-ala - D-glu - meso-DAP - D-ala - D-ala
N	L-glutamine
NaCl	sodium chloride
NA	nutrient agar
NA + 1 % NaCl	nutrient agar containing 1 % NaCl
NB	nutrient broth
NB + 1 % NaCl	nutrient broth containing 1 % NaCl
NBN	nutrient broth containing 1 % NaCl
OD	optical density
PBP	penicillin binding protein
PG	peptidoglycan
ppGpp	guanosine 3'-diphosphate 5'-diphosphate
pppGpp	guanosine 3'-diphosphate 5'-triphosphate
S	L-serine
SDS	sodium dodecyl sulfate
T	L-threonine
ts	temperature-sensitive
TSA	tryptic soy agar
TSB	tryptic soy broth
UDP	uridine 5'-diphosphate
w/v	weight per volume
X	any L-amino acid
Y	L-tyrosine

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Introduction

This introduction will describe the current view of the structure of the stress bearing layer of the cell wall, peptidoglycan. The biosynthesis of this polymer, its regulation, and coordination with the potentially lethal activities of the peptidoglycan hydrolases will also be discussed. The activities of the peptidoglycan hydrolases will be discussed to lay the foundation for the discussion of the genes of the *murH* family, and the cellular autolysis which is conferred by many of the mutations in this group.

I. Cell Wall of *Escherichia coli*

Escherichia coli is a common inhabitant of the lower intestine of warm-blooded animals (52). Like other Gram negative organisms, *E. coli* has a cell wall that is made up of an external layer, the outer membrane, composed of phospholipids, lipopolysaccharide (LPS), and characteristic proteins, and an internal layer composed of peptidoglycan (51). The outer membrane of Gram negative bacteria is an asymmetric bilayer approximately 7.5 nm thick. The inner leaflet of this membrane is primarily phospholipid, whereas the outer leaflet is mostly LPS (41). Lipopolysaccharide molecules can be conveniently divided into three parts: lipid A, the core oligosaccharide, and the O-antigen. The hydrophobic lipid A portion of the molecule anchors the LPS into the hydrophobic core of the outer membrane. The core oligosaccharide connects the O-antigen to the lipid A anchor. The O-antigen consists of repeating units of three to six sugar residues, and may be up to 40 repeat units long. Because growth conditions can alter the appearance of the O-antigen and the number of the repeats in the O-antigen can vary within a culture, O-antigen provides the cell with the ability to alter its molecular appearance to evade the host immune response. The structure of the O-antigen is the basis of the serotyping used to identify subspecies of *E. coli* (41).

In addition to these lipid components, the outer membrane also contains a characteristic set of proteins, many of which are involved in nutrient acquisition. The porin family is a major group of proteins involved in maintaining the limited permeability of the outer membrane. The porins form water-filled protein channels through the outer membrane that limit passage to small, hydrophilic molecules of less than 600 Daltons molecular weight. This limited entry serves to protect the inner membrane of the cell and its overlying peptidoglycan (PG) from the hazards of the cell's environment, which are generally larger than the exclusion limit of the porin channels. These hazards may include digestive enzymes, bile salts, antibiotics and host defensive proteins, such as the PG hydrolytic enzyme, lysozyme (51).

Unlike the porin proteins, lipoprotein is not involved in the selective permeability functions of the outer membrane. Lipoprotein is one of the most abundant proteins in the cell (7.5×10^5 copies / cell), and exists in both peptidoglycan-bound (1/3 of lipoprotein) and free forms (2/3 of lipoprotein) (53). Lipoprotein is anchored into the hydrophobic core of the outer membrane by its N-terminal cysteine residue which is substituted with a thioether-linked diglyceride and an amide-linked fatty acid (41). A covalent bond between the carboxy terminal lysine of the lipoprotein and the free carboxyl group of the diaminopimelic acid (DAP) binds lipoprotein to approximately every tenth DAP residue (41). Although lipoprotein deletion mutants appear to grow and divide normally, they leak periplasmic enzymes into the external environment. This observation suggests that the lipoprotein molecules themselves or the physical connection between the outer membrane and the peptidoglycan created by the lipoprotein may have a role in stabilizing the structure of the outer membrane (23). Further stabilization of the membrane may be accomplished through noncovalent interactions between PG and outer membrane proteins such as OmpA, OmpF, and OmpC (51).

The underlying peptidoglycan, or murein (from the Greek for "wall" (61)), forms a porous enclosed sac surrounding the cytoplasmic membrane. This "sacculus" provides the inner membrane with mechanical support against the cell's internal turgor pressure, thus preventing osmotic lysis, and also maintains the cell's shape, i.e., rod-shaped for *E. coli* (27). The relative ease with which the intact sacculi can be isolated from cells has facilitated its physical characterization. The protocol generally involves boiling the cells in sodium dodecyl sulfate (SDS) to remove the membranes, followed by washing steps and protease treatment to remove the peptidoglycan associated outer membrane proteins and lipoproteins (61).

Although a direct physical linkage between the cytoplasmic membrane and the peptidoglycan analogous to the lipoprotein linkage between the outer membrane and the peptidoglycan has not been characterized, "zones of adhesion", or Bayer bridges, have been observed by conventional thin section electron microscopy of normal and plasmolyzed cells. At these Bayer bridges the outer and inner membranes appear to be fused and the state of the peptidoglycan is unknown (41). However, these areas have been proposed to be (i) sites of bacteriophage DNA penetration, (ii) sites of translocation for newly synthesized LPS (41) and PG (53), (iii) sites of synthesis and translocation of some outer membrane proteins, or (iv) involved in the production of sex pili (41), or (v) an artifact of the method of sample preparation for electron microscopy (32).

II. *E. coli* Peptidoglycan Structure

1. General Features

The peptidoglycan net is composed of glycan chains cross-linked by short peptides. Figure 1 shows a highly simplified view of an idealized peptidoglycan net. The glycan chains are composed of alternating residues of N-acetylmuramic acid and N-acetylglucosamine connected by β (1,4)-glycosidic linkages (27). These glycan

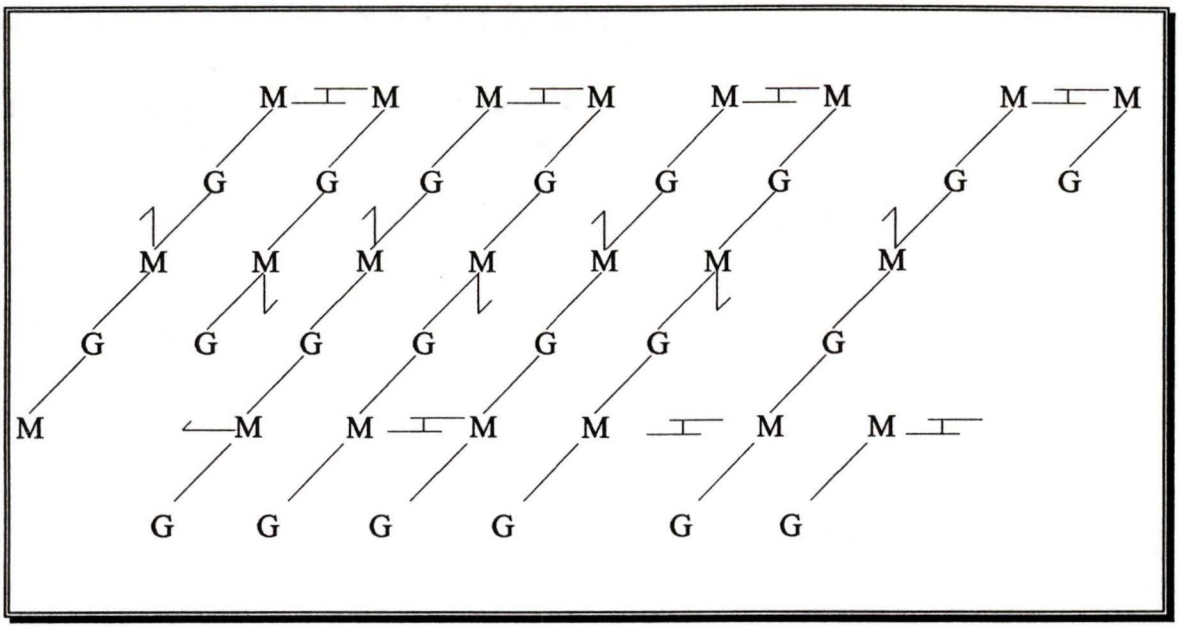


Figure 1. General structural features of *E. coli* peptidoglycan. G, glcNAc; M, murNAc. Lines connecting G and M residues represent glycosidic bonds. ——\ , peptide side chains. ⊤⊥— , between two M residues represents cross-linked peptides. Model building experiments indicate that peptide cross-links can only be formed between every fourth peptide side chain on each side. Modified from reference 53.

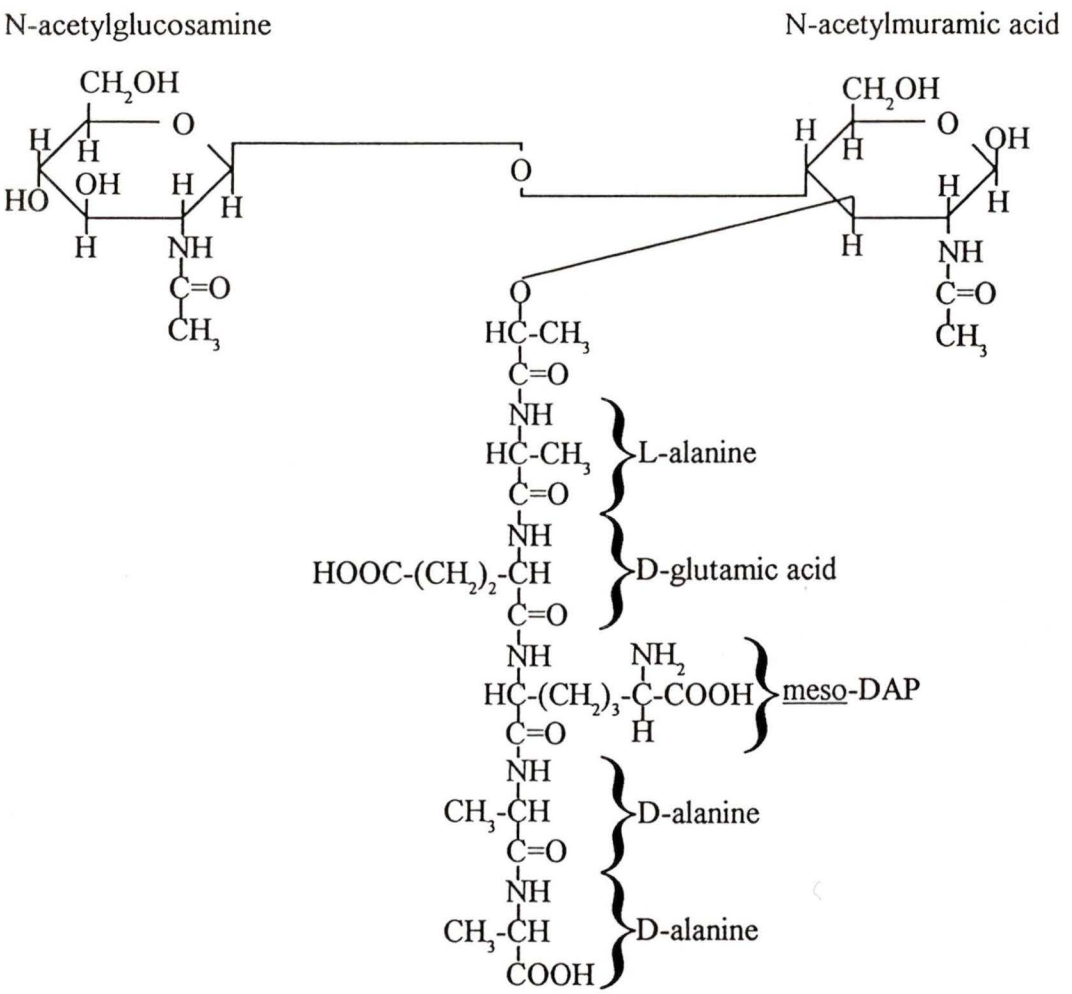


Figure 2. Chemical structure of the disaccharide-pentapeptide monomeric subunit of E.coli P.G. Modified from "Developmental Biology of the Bacteria", (Dworkin, M.ed.), p.164, Menlo Park, California, U.S.A.

chains average approximately 33 disaccharide units long (~33 nm) (27). Recent work has shown that while some of the glycan chains are quite long (30% of the chains are greater than 30 disaccharide units long), most are only five to ten disaccharides in length (22). Each chain terminates in a nonreducing 1,6-anhydromuramic acid (18) and appears to be oriented such that it runs perpendicular to the long axis of the cell (27). Each N-acetylmuramic acid residue is substituted with a peptide of defined sequence , which for *E. coli* is L-alanine - D-glutamic acid - meso-diaminopimelic acid (DAP) - D-alanine - D-alanine (figure 2); however, these peptides may also be shorter (61). Figure 2 represents the basic disaccharide-pentapeptide subunit. The peptides cross-link the various glycan chains by forming peptide bonds between the carboxylic acid group of the fourth residue, D-alanine, of the donor peptide and the free amino group of the meso-DAP residue of the acceptor peptide (27). During the creation of this linkage, the terminal D-alanine residue of the donor peptide is lost. Approximately 50% of the peptides are involved in cross-link formation (53). The appearance of N-acetylmuramic acid and diaminopimelic acid is unique to peptidoglycan (61), whereas the appearance of D-amino acids in bacteria is restricted to peptidoglycan, certain bacterial cell wall polymers such as teichoic acids, and some peptide antibiotics (50).

2. Recent Observations on the Chemical Composition of Peptidoglycan

The original monolayered net-like model seen in figure 1 was based on early work in which purified peptidoglycan was digested with egg white lysozyme, and the fragments were fractionated by paper or thin layer chromatography. This process yielded only eight fragments, of which the two major components were the disaccharide-tetrapeptide and a dimer of this component connected through cross-linked peptide chains (27).

However, recent work has indicated that the structure of peptidoglycan is considerably more complex than the original experiments indicated. Using a high pressure

liquid chromatography-based separation system, approximately 80 different muropeptide products were identified in a β-N-acetylmuraminidase digest of purified peptidoglycan (18). Three major observations emphasized the complexity of peptidoglycan. (i) Peptide cross-linkages can be formed between adjacent DAP residues. (ii) Glycine may be incorporated at the terminal position of the peptide chain rather than D-alanine.
(iii) Trimers and tetramers of disaccharide-peptide units connected by peptide bonds between their peptide side chains are present in normal peptidoglycan, and these multimers may be joined to other monomers or dimers in a variety of combinations (18). The presence of DAP-DAP bonds implies that the cell has the ability to increase the cross-linking of mature peptidoglycan, which because of the action of carboxypeptidases, is much less likely to retain the terminal D-alanine residue of the pentapeptide needed for the formation of a conventional D-alanine - DAP cross-link. For example, stationary phase cells have 27% more total cross-linking and 70% more bound lipoprotein than exponential phase cells. This ability to cross-link mature peptidoglycan may be important in adapting the cell to life in different environmental conditions and in stabilizing the peptidoglycan and cell wall under conditions (e.g. nutrient deprivation) in which continued synthesis may be difficult (18).

The amount of glycine in the peptide substituents of peptidoglycan appears to depend on the growth medium and the growth phase of the cells. Cells grown in complex media show much higher levels of glycine incorporation into their peptidoglycan than do cells grown in minimal media lacking glycine. The peptidoglycan of stationary phase cells contains more than twice as much glycine as does the peptidoglycan of exponential phase cells. These data indicate that the composition of PG may be modified by the cell in response to its environment (18).

The observation that trimers and tetramers of disaccharide-peptide units linked through peptide cross-links can be isolated from *Chalaropsis* β-N-acetylmuraminidase

digested peptidoglycan suggests that *E. coli* peptidoglycan structure is considerably more complex than is indicated by figure 1. In a monolayered structure, disaccharide-peptide trimers can only exist at the ends of the glycan chains; however, in cell wall peptidoglycan 80% of these oligomers are found within the glycan chain. Therefore, because of steric considerations, at least one of the disaccharide-peptide units of the trimer must be outside the plane of the other two units, suggesting that a multilayered peptidoglycan is present in the cell, with peptide cross-bridges connecting the various layers. The idea of a multilayered structure is also supported by pulse-chase experiments with [^{3}H]DAP, which demonstrated that the peptide cross-links in new and old peptidoglycan have different halflives. Cross-links between new strands are stable, whereas those between new and old peptidoglycan are broken and reformed extensively as the peptidoglycan matures. Together these two results suggest that the growth of *E. coli* peptidoglycan may occur by means of an inside-to-outside mechanism similar to that used by Gram positive organisms, such as *Bacillus subtilis*. Because there is sufficient peptidoglycan in an *E. coli* cell to form three layers, it has been suggested that this may be the minimum number of layers necessary to guarantee the integrity of the cell wall (18).

The concept of a multilayered peptidoglycan is further supported by the studies of Hobot *et al* (24), whose work was based on the development of less disruptive fixation and embedding techniques for electron microscopy (progressive lowering of the temperature in embedding and freeze substitution). When thin sections of the cells prepared by this method were examined, the peptidoglycan appeared to be a thick gel-like material filling the entire periplasmic space (~10 nm thick). This highly hydrated material is proposed to contain an aqueous solution of periplasmic proteins, small solutes and various sugars , in addition to the peptidoglycan "gel" matrix. Because these investigators found that only the outer half of the periplasmic gel is recovered as purified peptidoglycan sacculi, they proposed that the cross-linking of the peptide substituents of the glycan

chains increases closer to the outer membrane, and decreases or disappears close to the cytoplasmic membrane (24).

The concept of a periplasmic gel is supported by the fact that the rate of lateral diffusion through the periplasm is approximately 1000-fold lower than diffusion through an aqueous solution (2). This indicates that the periplasm is very viscous, although the contribution of peptidoglycan to this viscosity is unknown (2). Hobot *et al* also suggest that the glycan chains do not lie parallel to one another in discrete layers, but traverse layers, creating a cross-linked gel that is capable of elasticity (24). This would be in agreement with the work of Koch and Woeste whose study of purified peptidoglycan indicated that the cell wall peptidoglycan was capable of stretching to three times its relaxed size, and that the sacculus is 45% larger under normal growth conditions than it is in the absence of turgor pressure (36). The implications of this stretched condition for cell wall expansion will be dealt with below. However, parallel glycan chains arranged in discrete layers could also have this property of elasticity because although the glycan chains are quite inelastic, the charge state of the peptide chains determines the relative extension of their conformation and thus the relative elasticity of the cell wall. Therefore, based on the elastic properties of the sacculus no distinction between these two models is possible. Hobot *et al* believe that, due to the highly hydrated condition of their peptidoglycan, there is sufficient peptidoglycan to form two layers (24).

The number of layers of peptidoglycan in the cell is the source of much debate, although recent work suggests that at least parts of the sacculus are likely to be multilayered. Much of the evidence is indirect and is based on electron microscopy studies that indicate that the peptidoglycan is ~10 nm thick (39, 24) and contains enough material to form two to three layers (18). The studies of Leduc *et al* (40) relied on heavy metal staining with phosphotungstic acid, the progressive lowering of temperature during fixation technique designed by Hobot *et al* (24), and relatively thick sections (which will

reduce the resolution of the electron micrographs). Additionally, there is the caveat that not all the material that electron stains in the periplasmic space is peptidoglycan (61). Leduc *et al* noted that their detection method was not sensitive enough to determine whether the peptidoglycan layers present are of uniform thickness (40). Other evidence for a multilayered peptidoglycan is the presence of oligomers of disaccharide-peptide in lysosomal digests of purified peptidoglycan (18).

Direct proof of a multilayered peptidoglycan was generated by Labischinski *et al* (39). This group measured the thickness of purified sacculi with a low angle neutron scattering technique. The results indicated that the maximum thickness of the sacculi was 7 nm, but that this thickness was not uniform. Further work in this system with deuterated sacculi demonstrated that approximately 25% of the sacculi is triple-layered (~7.5 nm thick) and 75% is monolayered (~2.5 nm thick). These measurements are in reasonable agreement with the thickness of the peptidoglycan measured by other investigators (24, 40). These triple-layered areas may represent either the polar caps of the cell, or they may be multilayered "growth zones" in which the peptidoglycan is growing by an inside-to-outside growth mechanism (39). This model of sacculus growth will be further discussed below, in conjunction with the peptidoglycan hydrolases. Therefore, although the results of Labischinski *et al* support the indirect evidence for a multilayered structure, they also indicate that the majority of the peptidoglycan exists as a monolayer (39).

In support of the idea of a primarily monolayered peptidoglycan is the work of Wientjes *et al* (62). This group measured the number of DAP molecules per sacculus (3.5×10^6) and compared this value to the surface area of cells. This comparison generated an average area per disaccharide unit of 2.5 nm^2. Comparison to a theoretical value of approximately 2.2 nm^2 per peptidoglycan subunit led the authors to the conclusion that the peptidoglycan must be primarily a monolayer. They note; however,

that the sensitivity of their assay is not sufficient to discount a "patched " arrangement (62) as proposed by Labinchinski *et al* (39).

In summary, peptidoglycan structure is considerably more complex than is suggested by the structure shown in figure 1. The most recent view is that the peptidoglycan is mainly a monolayer, with triple-layered areas (39). This highly hydrated polymer appears to form a gel-like structure which fills the periplasmic space, and which is thicker than was suggested by previous electron microscopic specimens that were dehydrated during their preparation (24).

3. Three Dimensional Structure of Peptidoglycan

The model shown in figure 1 gives the impression that peptidoglycan has a regular crystalline structure. However, X-ray data, conformational energy calculations and model building indicate that the glycan strands are arranged as helices, with approximately four disaccharide units per helical turn. This model further supports the concept of a multilayered peptidoglycan because the peptide chains will point in different directions from the helical glycan strands (25). However, only some of the peptides would be in the correct orientation for cross-link formation, in agreement with experimental observations that 50% of peptides are involved in cross-link formation (53).

III. Biosynthesis of Peptidoglycan

1. The Biosynthetic Pathway

Peptidoglycan synthesis is catalyzed by a large group of soluble and particulate enzymes and is divided into three stages based on the cellular compartment in which the reactions occur. The basic steps in *E. coli* peptidoglycan synthesis are outlined in figure 3. In the first stage, the cytoplasmic reactions, the UDP-activated precursors, UDP-N-

acetylglucosamine (UDP-glcNAc) and UDP-N-acetylmuramic acid-pentapeptide (UDP-murNAc-pentapeptide), are synthesized by the soluble enzyme system.

Synthesis of UDP-N-acetylglucosamine is not shown in figure 3 because the synthetic reactions in *E. coli* have not yet been elucidated. UDP-glcNAc serves as a precursor for synthesis of both UDP-murNAc (reactions 1,2), and for LPS, a major component of the outer membrane of *E. coli.* The pentapeptide side chain is synthesized by the sequential addition of L-alanine, D-glutamic acid, meso-DAP, and D-alanyl-D-alanine to the UDP-murNAc in ATP-dependent reactions catalyzed by specific amino acid ligases (reactions 3 - 8) (27).

In stage two, the membrane bound reactions, the phosphate-activated murNAc-pentapeptide and glcNAc are sequentially translocated to undecaprenylphosphate, a membrane carrier lipid (reactions 9,10), designated as GCL, glycosyl carrier lipid, in figure 3. This lipid translocates glycan intermediates involved in LPS, peptidoglycan, and capsule synthesis across the inner membrane. Therefore, disruption of the synthesis of any of these heteropolysaccharides will cause disruption of the synthesis of the others because of a shortage of membrane carriers caused by the precursors of the stalled system being trapped on the membrane carriers (53). The dephosphorylation reaction (reaction 12) which recycles the membrane carrier is thus critical not only to peptidoglycan synthesis but also to cell wall synthesis in general.

The final stage of PG synthesis occurs at the outer face of the cytoplasmic membrane, and involves the transfer of the disaccharide-pentapeptide subunit from the lipid carrier to an acceptor site in the nascent peptidoglycan (reaction 11). This reaction is catalyzed by the penicillin binding proteins (PBP's) (27), which are named to reflect the fact that they are the targets for the action of penicillin. The covalent binding of penicillin to this group of enzymes is characteristic of penicillin binding proteins (16). Because of their complexity, the functions of the PBP's, their role in peptidoglycan metabolism, and

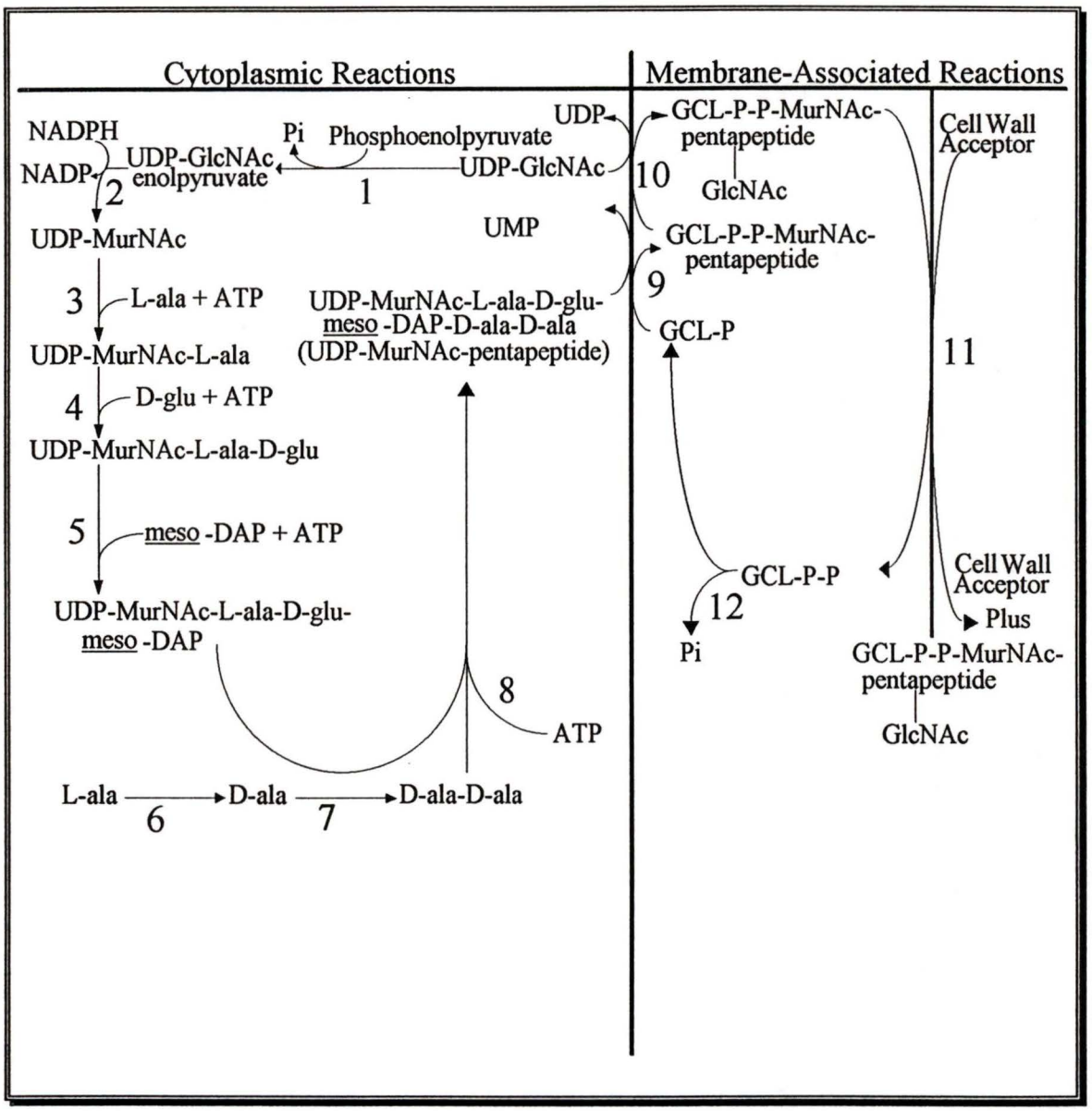


Figure 3. Major steps in *E. coli* peptidoglycan biosynthesis. The enzymes catalyzing the indicated reactions are: 1, phosphoenolpyruvate: UDP - glcNAc - pyrophosphorylase; 2, UDP - glcNAc -enolpyruvate reductase; 3, L-alanine adding enzyme; 4, D-glutamate adding enzyme; 5, meso-DAP adding enzyme; 6, 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 (PBP's); 12, undecaprenol pyrophosphate pyrophosphatase.

Modified from reference 5.

the coordination of their activity with that of the PG hydrolases will be considered separately.

2. Penicillin Binding Proteins

There are at least eight PBP's present in *E. coli* (66), which can be divided into two functional groups based on their molecular weight (16). There have been two approaches used to attempt to determine the functions of the PBP's: the characterization of cells carrying mutations in the various PBP's and characterization of cells grown in the presence of β-lactams which specifically inhibit the different PBP's (16). The first group, the high molecular weight PBP's, includes PBP 1A, 1B, 2 and 3. These are bifunctional enzymes containing both transpeptidase and transglycosylase activity (16), although the transglycosylase activity of PBP2 may not be expressed under all conditions (66). The high molecular weight PBP's are directly involved in the insertion of nascent peptidoglycan into the cell wall, that is, the polymerization of peptidoglycan. These proteins are anchored to the cytoplasmic membrane by an N-terminal hydrophobic sequence that acts as an uncleaved signal sequence and causes the translocation of the bulk of the protein into the periplasm (16). These proteins have two functional domains. The amino terminal transglycosylase domain creates β (1,4)-glycosidic linkages between the murNAc-pentapeptide of the acceptor subunit and the glcNAc residue of the incoming donor subunit (16). The carboxy terminal transpeptidase domain, or penicillin binding domain, catalyzes the formation of the peptide cross-linkages between the nascent polymerized peptidoglycan and the new subunit (16). Penicillin inhibits transpeptidation by binding directly to the active site of the enzyme; however, binding of penicillin to the active site in the amino terminus has no effect on the transglycosylation reaction (59). The transglycosylation reaction may be inhibited with the antibiotic moenomycin (59). Inhibition of transglycosylation also strongly inhibits peptide cross-linking, implying that

transpeptidation requires prior or concomitant elongation of the glycan chain, and thus both activities are required for sacculus growth (16). It should be noted that the exact details of how new subunits are incorporated into the existing peptidoglycan are unknown.

Because peptidoglycan shape determines the shape of the cell, rod shaped for cells such as *E. coli*, it is helpful to think of the cell as a cylinder with hemispherical caps on either end of the cell representing the septal caps formed during division. This analogy is useful because it also describes the division of labor for the high molecular weight PBP's. Physiological and genetic experiments have shown that PBP 1A and PBP 1B are involved in the process of elongating the cylindrical peptidoglycan (55, 56). The PBP 1A dimer (67), can functionally replace or be replaced by PBP 1B, and therefore, single mutants in either gene can grow and elongate (56). The double mutant of PBP 1A and PBP 1B is unable to elongate, and consequently the double mutation is lethal to the cell. Therefore, these two proteins are required for cell elongation, and have an essential function in peptidoglycan synthesis (56).

The role of PBP 2 may involve maintainance of the cell's shape, as cells with mutant PBP 2 or in which PBP 2 has been inactivated with mecillinam treatment grow as osmotically stable ovoid cells (55). Thus, PBP 2 appears to be essential in maintaining the rod shaped morphology typical of *E. coli* and in controlling the elongation of cylindrical peptidoglycan (55).

At the time of cell division, PBP 3 is specifically activated and is responsible for the formation of septa during cell division (55). Cells which do not make PBP 3 or whose PBP 3 is inactivated by β-lactam treatment grow as long septa-less filaments, which eventually lyse (55).

The second group of penicillin binding proteins, the low molecular weight PBP's, includes PBP 4, 5, 6, and 7. These membrane bound proteins are anchored into the membrane by a C-terminal signal peptide-like sequence. Because the bulk of the proteins

are soluble in the periplasmic space, proteolytic cleavage or site directed mutagenesis to remove the membrane anchor has made purification and characterization of these proteins somewhat easier, although the exact roles for many of these proteins are unknown. These proteins have no transglycosidase activity, and are D,D-carboxypeptidases that catalyze acyl transfer reactions from D-ala-D-ala terminated peptides and peptide analogs to the active site serine of the enzyme with the subsequent loss of the carboxy terminal D-alanine residue. The short lived enzyme-acyl intermediate is subsequently attacked by water to form the tetrapeptide hydrolysis product. However, transpeptidation may also be catalyzed by these enzymes, depending on the presence of a suitable amino acceptor group in the active site at an appropriate position. An amino acceptor may also influence the binding of the D-ala-D-ala peptide to the enzyme and the formation of the acyl intermediate, thereby influencing whether the enzyme will have predominantly D,D-carboxypeptidase or transpeptidase activity (16). Because β-lactam antibiotics resemble the structure of the D-alanyl-D-alanine peptide bond, the natural substrate of these enzymes, the low molecular weight PBP's can also bind and be inactivated by β-lactams regardless of whether the enzymes have transpeptidase or carboxypeptidase activity (16). The relatively high stability of the penicilloyl acyl enzyme intermediate makes the enzyme appear to be irreversibly inactivated (16).

The low molecular weight PBP's are thought to help to control the extent of cellular cross-linking. During exponential phase, PBP 5 is expressed and catalyzes the cleavage of the D-ala-D-ala peptide bond to yield the tetrapeptide product (46, 58). This is proposed to help the cell to regulate cross-linking because the pentapeptide side chain is required to act as a donor for the formation of the D-ala - meso-DAP cross-link that is generally used by the cell during elongation (46). Although PBP 6 has a three to four-fold lower level of D,D-carboxypeptidase activity than does PBP 5, the expression of PBP 6 rises during stationary phase while the expression of PBP 5 remains steady (58).

Moreover, because nongrowing cells (stationary phase or amino acid starved cells) have been found to stabilize their peptidoglycan by increasing the level of DAP-DAP cross-links (17) (these cells do not contain pentapeptide donors for cross-link formation), it has been suggested that PBP 6 may have a role in this stabilization of the peptidoglycan (58).

Although *in vitro* PBP 4 has both D,D-endopeptidase and carboxypeptidase activity, *in vivo* PBP 4 is a transpeptidase engaged in the processing of new peptidoglycan (11). This enzyme appears to cause the increase in D-ala - meso-DAP cross-links which is characteristic of the maturation of peptidoglycan. Cells with mutant PBP 4's show only the loose cross-linking characteristic of new peptidoglycan (11). In contrast to the high molecular weight PBP mutants, mutants in the low molecular weight PBP's do not have striking morphological defects, and they grow quite normally (27). In all PBP's and β-lactamases there are several conserved sequences which are associated with the penicillin binding active site. Two of these sequences, (K/H)T(S/G) and (S/Y)XN, appear to be on the sides of the binding site cavity of the enzyme. The most important conserved sequence, SXXN, includes the reactive serine residue which is acylated by either penicillin or the penultimate D-alanine residue of the pentapeptide (16).

The ability of all PBP's to bind radioactive penicillin allows for rapid detection and quantification of these proteins without the need for extensive purification (55). The availability of this assay has led investigators to ask whether a full complement of PBP's is necessary for normal levels of peptidoglycan synthesis to occur (57). These experiments involved acylating all cellular PBP's using a quantity of penicillin which was 50-fold larger than the minimum inhibitory concentration (MIC), under conditions of amino acid starvation. Under these conditions, the cells are not actively growing, thus they cannot lyse, and no new PBP's can be made to replace the acylated enzymes. This phenomenon of penicillin tolerance will be discussed further below. The excess unbound penicillin was removed with penicillinase treatment, and the cells were returned to complete penicillin-

free medium to resume growth. Cells were characterized with respect to the synthesis of peptidoglycan, RNA, cellular protein and the production of new PBP's. This study found that although the old PBP's were not appreciably deacylated during the experiment (40-50 minutes) the cells did not lyse, and within 5 minutes of transferring the cells to drug-free medium, the RNA, protein, and peptidoglycan synthesis had recovered to 70% of the pre-treatment levels. Cellular macromolecular synthesis had completely recovered after 25 minutes. In contrast, after 20 minutes in penicillin-free medium, the level of new, unacylated PBP's was barely detectable, indicating that near normal levels of peptidoglycan synthesis can be catalyzed by a small fraction of the number of the PBP's that are normally present in *E. coli.* Cells subjected to the treatment outlined above were found to be hypersensitive to the effects of penicillin before recovery of the full complement of PBP's, and lysed after exposure to one-tenth the MIC. In comparison, cells not subjected to pre-acylation with benzylpenicillin were not affected by exposure to one-half the MIC. This indicates that acylation of these new PBP's is sufficient to cause bacteriolysis. Tuomanen suggested that PBP's may have a finite functional lifetime during which they are integrated with the activities of the peptidoglycan hydrolases, and when their inactivity can potentially cause lysis (57). This suggestion was based on the observation that penicillin-inactivated PBP's were unable to cause lysis when the acylation event and the lysis-permissive conditions were temporally dissociated (57). It is known that *E. coli* incorporates its nascent peptidoglycan into discrete growth zones (10). Based on the presence of these growth zones, Tuomanen suggests that new PBP's may make their first appearance within a growth zone, and with age, may gradually move out of it (57). If the PBP's are acylated while they are in the growth zone, peptidoglycan biosynthesis would be disrupted and uncontrolled autolytic activity may result (57). Therefore, the lethal effects of penicillin would be due to acylation of new PBP's, which are responsible for peptidoglycan synthesis (57). The functional role for the old PBP's

may be to compete with the new PBP's for the binding of β-lactams, thus preventing disruption of peptidoglycan synthesis by levels of antibiotic that are not sufficient to acylate very large numbers of PBP's (57).

In summary, PBP's can be functionally divided into two classes, the high molecular weight enzymes responsible for the polymerization of peptidoglycan, and the low molecular weight enzymes responsible for modifying the peptide chain length and cross-linking of the peptidoglycan following polymerization (55). All PBP's and β-lactamases have a conserved sequence, SXXN, which contains the active site serine, which becomes acylated during activity (16). Peptidoglycan appears to be synthesized by a very small subset of the total cellular PBP's, which may have a finite functional lifetime during which their activity is coordinated with that of the PG hydrolases (57).

3. Peptidoglycan Hydrolases

For almost every bond present in peptidoglycan, there appears to exist at least one enzyme capable of breaking that bond (27). Those enzymes are known as peptidoglycan hydrolases and they occur throughout the bacterial world (15). In *E. coli*, there are at least nine enzymes capable of cutting the various bonds in peptidoglycan (figure 4) (28). Based on their sites of action, (figure 4), these proteins can be divided into two groups - glycosidases, which cleave the glycosidic bonds between the N-acetyl sugar residues; and the peptidases, which cleave the various peptide bonds in the peptide side chain of the peptidoglycan (28).

The glycosidases include the soluble lytic transglycosylase, the membrane lytic transglycosylase and β-N-acetylglucosaminidase (28). The S or soluble lytic transglycosylase is found primarily in the periplasm, where it appears to be tightly bound to the outer surface of the peptidoglycan (60). The M or membrane lytic transglycosylase

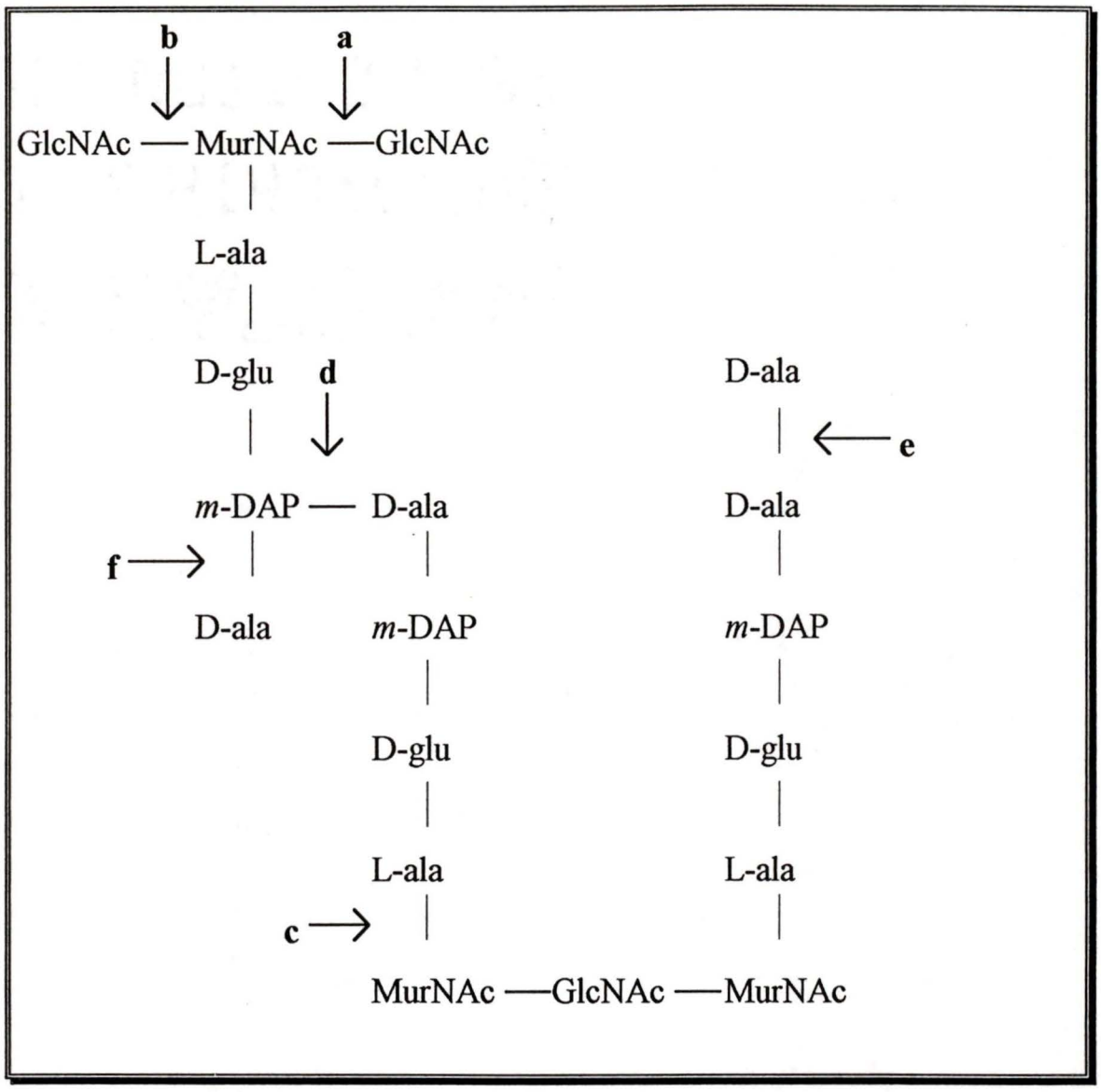


Figure 4. Cleavage Sites of the *E. coli* Peptidoglycan Hydrolases. **a**, lytic transglycosylase; **b**, β-N-acetylglucosaminidase; **c**, N-acetylmuramyl - L-alanine - amidase; **d**, D,D-endopeptidase; **e**, D,D-carboxypeptidase; **f**, L,D-carboxypeptidase.

Modified from reference 28.

appears to be associated with the outer membrane and shows no immunological cross-reactivity with the soluble enzyme (27). Both lytic transglycosylases catalyze the cleavage of the β (1,4)-glycosidic linkage between murNAc-peptide and glcNAc. Following this cleavage, the enzymes catalyze an intramolecular rearrangement - the transfer of the O-muramyl group onto its own C-6 hydroxyl group to form 1,6-anhydromuramic acid-peptide (26, 28). Roles proposed for these enzymes include : (i) creation of the non-reducing end which is characteristic of glycan chain ends in peptidoglycan (26), (ii) conservation of the glycosidic bond energy in the 1,6-anhydro bond may allow sacculus remodelling or repair by storing the bond energy needed to form new glycosidic linkages without further energy input (24, 26), and (iii) involvement in the removal and disassembly of peptidoglycan fragments to be recycled from the cell wall peptidoglycan (27).

Both lytic transglycosylases are able to accept isolated sacculi as substrates. In contrast, the third enzyme in this group, β-N-acetylglucosaminidase, is unable to cleave bonds in polymerized peptidoglycan. β-N-acetylglucosaminidase is a cytoplasmic enzyme (27) which cleaves the β (1,4)-glycosidic linkage between glcNAc and murNAc-peptide to generate a reducing end N-acetylglucosamine from low molecular weight peptidoglycan fragments (28). This enzyme may have a role in the disassembly of the glycan moieties of the peptidoglycan fragments removed from the cell wall during the process of turnover (27, 28). The process of peptidoglycan turnover will be discussed further below.

The second group of PG hydrolytic enzymes is the peptidases, which include N-acetylmuramyl-L-alanine amidase, penicillin-sensitive D,D-endopeptidase (PBP 4), penicillin-insensitive D,D-endopeptidase (MepA), D,D-carboxypeptidase 1B (PBP 4), D,D-carboxypeptidase 1A (PBP 5), D,D-carboxypeptidase 1A (PBP 6), and L,D-carboxypeptidase (28). N-acetylmuramyl-L-alanine amidase is a periplasmic enzyme which cleaves the peptide bond formed between the carboxyl group of the lactyl moiety of the muramic acid and the amino group of the N-terminal L-alanine residue of the peptide

side chain (28). Because this enzyme does not cleave these bonds in high molecular weight peptidoglycan, it is thought to be involved in the recycling of low molecular weight peptidoglycan fragments removed from the sacculus during the process of turnover (27, 28). The low molecular weight PBP's 4,5, and 6 are included as PG hydrolases because of their D,D-carboxypeptidase activity, which can result in cleavage of the terminal D-alanyl-D-alanine bond of the peptide (27, 28). The L,D-carboxypeptidase cleaves the bond between meso-DAP and the penultimate D-alanine residue of the side chain. The penicillin-insensitive D,D-endopeptidase and PBP 4 endopeptidase catalyze the cleavage of the D-ala - meso-DAP peptide cross-links between peptide side chains, and can use high molecular weight peptidoglycan as a substrate (28). Because only some of these enzymes can accept murein as a substrate, only the lytic transglycosylases, and the endopeptidases (PBP 4 and MepA) are considered to be autolytic under conditions in which their activity is not controlled (28, 33).

Peptidoglycan hydrolases are thought to have two general roles in peptidoglycan metabolism. First, they are thought to be needed to break bonds in the covalently closed sac of peptidoglycan to allow insertion of new subunits during growth and remodelling, although exactly how this is accomplished is unknown (61). The Koch model of sacculus growth proposes that new subunits are added as bridges under old subunits. The covalent cross-linkages between the old subunits are then cleaved, and the tension on the sacculus pulls the new subunits into the wall, allowing it to enlarge (35). Second, PG hydrolases are thought to be involved in the process of cell wall peptidoglycan turnover (20) and subunit recycling (19).

One of the remodelling roles, that of peptidoglycan "maturation" appears to involve the hydrolytic activities of the low molecular weight penicillin binding proteins 4 and 5. Maturation of peptidoglycan involves increasing the D-ala - meso-DAP cross-

linking of the loosely linked nascent peptidoglycan and the quantity of covalently linked lipoprotein, and decreasing the number of pentapeptide side chains (11). PBP 4 appears to catalyze the transpeptidation reaction between the nascent pentapeptide D-alanine donors and the meso-DAP acceptors of the other side chain, as evidenced by the decreased cross-linking and increased pentapeptide side chains seen in PBP 4 deletion mutants (11). As mentioned previously, the carboxypeptidase activity of PBP 5 appears to regulate cross-linking of the sacculus by removing the terminal D-alanine residue from the pentapeptide side chain, thus removing the ability of the side chain to act as the donor for the most commonly used D-ala - meso-DAP cross-linkage. The overexpression of PBP 5 leads to a decrease in the number of pentapeptide side chains, likely as a result of the increased carboxypeptidase activity (46). However, these PBP 5 overexpression mutants had increased cross-linking within the nascent peptidoglycan, and grow as cocci rather than as rods. This suggests that PBP 5 may have a role in maintaining cell shape, but the details of its role in the cell remain unknown (46).

Peptidoglycan hydrolases are thought to have a role in separating the septa of the daughter cells during cell division. Indirect evidence for this role is that cells treated with low concentrations of penicillin show splits in the sacculus in the center of the cell (the division site) indicating a localized area for hydrolytic action (28). Two studies of chain forming mutants, *envA* (64) and *envC* (31), provide the only evidence in *E. coli* for a role in cell separation for the PG hydrolases. The *envA* mutant shows considerably reduced activity of the N-acetylmuramyl-L-amidase (64), while the *envC* mutant is deficient in L,D-carboxypeptidase activity (31). Although these results suggest that these two enzymes are involved in septal splitting and cell separation, it should be noted that firm conclusions on this point cannot be drawn because both *envA* and *envC* have pleiotrophic effects (64, 31).

Peptidoglycan turnover is an enzymatic process that involves excision of peptidoglycan fragments from the existing high molecular weight peptidoglycan of the sacculus (13). To conform to this definition, the enzymes excising the fragments must be produced by the organism undergoing peptidoglycan turnover, and the cell must remain viable (13). It was previously thought that the *E. coli* peptidoglycan net was able to enlarge itself without losing peptidoglycan fragments to the surrounding medium (25). This idea stood in contrast to the situation in Gram positive cells, such as *Bacillus* sp., in which growth of the murein via an inside-to-outside mechanism is accompanied by the loss of peptidoglycan fragments from the external layer to the medium (13). However, experiments by Goodell and Schwarz (20) demonstrated that *E. coli* was losing 6 to 8% of its murein as fragments to the external medium (20). Further work established that *E. coli* cells actually turnover about 50% of the cell wall peptidoglycan per generation, a rate comparable to that seen in Gram positive cells (19). The loss of these peptidoglycan fragments is largely prevented by the presence of the outer membrane in *E. coli* (13, 19). Cleavage of these fragments from the sacculus appears to involve the activity of the N-acetylmuramyl-L-alanine amidase to release the peptide fragments from the glycan subunits (20). The lytic transglycosylase is also proposed to be involved because there is no evidence for the presence of murNAc residues lacking peptide side chains, and 1,6-anhydromuramic acid residues are formed as a result of the turnover process (20). The free tetrapeptides may be converted to tripeptides by carboxypeptidases (19), and the peptides are then taken across the inner membrane via the oligopeptide permease (13). The peptides are apparently not degraded, but are recycled by linkage to UDP-murNAc to form UDP-murNAc-tripeptide (19).

In summary, peptidoglycan hydrolases appear to have a variety of roles in the cell, including involvement in septal separation (31, 64), growth and remodelling of the sacculus (25, 35, 61), peptidoglycan turnover and fragment recycling (19, 20).

IV. Cellular Autolysis

1. Role of the PG Hydrolases

The growth of *E. coli* in hypotonic media requires an intact peptidoglycan layer to provide mechanical support for the membrane and to prevent osmotic lysis from occurring (4, 27, 61). In growing *E. coli* cells, it is possible to induce PG hydrolase-mediated autolysis by disrupting any of the biosynthetic steps involved in peptidoglycan synthesis, consequently temperature-sensitive mutants with defects in any of the biosynthetic genes undergo lysis at the restrictive temperature (12, 42 - 45, 47, 49, 54 - 56, 63). Similarly, disruption of the various biosynthetic steps through the use of antibiotics also results in PG hydrolase-mediated lysis (15, 28, 55, 59), suggesting that the bactericidal effects of many of these antibiotics may be attributable to the induction of uncontrolled hydrolase activity resulting in osmotic fragility of the cell (28). Hydrolysis of the stress-bearing layer of the peptidoglycan is thought to be caused by deregulation of the lytic transglycosylases and an endopeptidase (33, 37). Because of the relationship between synthetic blockage and autolysis, it has been suggested that insertion of new subunits by the biosynthetic enzymes must be tightly coupled to the opening of new acceptor sites in the existing peptidoglycan by the hydrolases (61). This idea implies that blockage of the biosynthetic pathway uncouples the system and leads to uncontrolled activity on the part of the PG hydrolases, resulting in cellular lysis (27, 61). The lack of autolysis under normal growth conditions indicates that the activity of the hydrolases is tightly controlled, but exactly how this is accomplished is not understood (28).

One of the requirements for cellular lysis in the presence of PG biosynthesis inhibitors is that the cells must be in a growing state. Non-growing cells, such as those that are amino acid starved, cannot be lysed by penicillin treatment, a condition known as tolerance (27). Cells that are suspended in a non-nutrient buffer solution are stable, and do not lyse. This suggests that a barrier may exist to prevent hydrolase activity under non-

growing conditions because active hydrolases can be isolated from these cells (21). However, this barrier may be disrupted by treatment with sodium chloride, sucrose, Triton X-100, EGTA, EDTA, or trichloroacetic acid, causing the cells to lyse (21). It has been suggested that this barrier may involve sequestration of the hydrolases away from their substrate or that the location of the hydrolases within a growth zone may be necessary for activity (25).

2. Interaction of the Stringent Response with the PG Biosynthetic and Hydrolase Systems.

The product of the *relA* gene, (p)ppGpp synthetase I, is a ribosome associated protein that catalyzes the transfer of pyrophosphate from ATP to the 3' position of guanosine 5'-triphosphate (GTP) in response to a conformational change that occurs in the ribosome when an uncharged tRNA binds (3). Under conditions of amino acid starvation, this leads to the accumulation of guanosine 3'-diphosphate 5'-triphosphate (pppGpp) and guanosine 3'-diphosphate 5'-diphosphate (ppGpp) (3). This accumulation acts as a global signal to shut down macromolecular synthesis (peptidoglycan, phospholipids, stable RNA's, etc.) under conditions of nutritional deficiency (29). This global shut down can be "relaxed" by treatment with ribosome inhibitors, such as chloramphenicol, which block RelA activity and prevent ppGpp accumulation (38). This relaxed control when cells are nutritionally deprived can also be seen in *relA-* cells, which are incapable of making ppGpp, and thus continue macromolecular synthesis in the absence of growth (29).

The stringent response is thought to interact with peptidoglycan biosynthesis at two points: (i) at an early step in the synthesis of UDP-glcNAc and UDP-murNAc-pentapeptide and (ii) at a late step in peptidoglycan polymerization, likely the PBP-dependent transpeptidation reaction (30). Thus the stringent response prevents the build-up of unnecessary intermediates when peptidoglycan polymerization has been stopped.

Although the mechanism by which peptidoglycan synthesis is stopped by the stringent response is not understood, it is thought to be dependent on phospholipid synthesis. Therefore, during nutrient deprivation, the stringent response is activated, thus preventing the accumulation of both peptidoglycan and excess membrane by controlling phospholipid synthesis (29).

As discussed above, non-growing (amino acid deprived) cells are tolerant to the effects of penicillin treatment, and thus do not lyse. It has also been shown that the stringent response results in the inhibition of penicillin-induced lysis. However, it is possible to lyse such penicillin-tolerant cells in the presence of penicillin by relaxing the stringent response, either by introducing mutations into the *relA* gene, or by treating the cells with a ribosome inhibitor that will antagonize *relA* function, such as chloramphenicol (38).

V. The *murH* Gene Family

The interest in this laboratory has focussed on the mechanisms of the regulation of peptidoglycan biosynthesis and hydrolysis and has led to the search for *E. coli* mutants that are defective in peptidoglycan metabolism. Previous research by D. Dai in this laboratory led to the isolation of a temperature sensitive (ts) mutation, *murH* (6). The phenotype of the *murH* mutant strain is similar to that of other mutants with defects in peptidoglycan biosynthesis, (11, 42-45, 47, 49, 54-56, 63) in that peptidoglycan hydrolase-mediated autolysis occurs at the restrictive temperature (6). Extragenic (second-site) mutations which suppressed the ts phenotype of the *murH* mutant occurred spontaneously at a relatively high frequency of 3×10^{-6} (6). These suppressor mutations were represented by two loci which were designated *smhA* (9) and *smhB* (7) (suppressor of *murH*) which were located at 24.5 minutes (9) and 12.5 minutes (7), respectively, on the *E. coli* genetic map (1). The *smhA* mutant was found to have no obvious phenotype

apart from the suppression of *murH* (9). In contrast, the *smhB* mutant was temperature sensitive in a salt dependent manner, and did not form colonies at the restrictive temperature on low salt medium (7). Routine laboratory maintenance of the *smhA* and *smhB* strains resulted in the isolation of a new extragenic suppressor from each strain. The mutation appearing in the *smhA* strain was designated *lytE* (9), and that in the *smhB* strain was named *lytD* (7). The *lytD* and *lytE* mutants possessed a similar phenotype to *murH*, in that both strains showed ts growth and lysed at the restrictive growth temperature (7,9).

Analysis of the patterns of suppression for these new alleles showed that although the *lytE* phenotype could be suppressed by both *smhA* and *smhB*, the *lytD* allele was suppressed by *smhB* only. The proposed interactions of these mutants are summarized in figure 5. The lysis suppression mediated by *smhA* and *smhB* is quite specific, in that neither mutation is capable of preventing lysis caused by antibiotic or mutational blockages (*murE* or *murF* mutations) in peptidoglycan biosynthesis (9). Based on the specificity of their interactions with each other, it has been proposed that these loci may be a family of functionally related genes involved in peptidoglycan mediated autolysis (9).

The *lytD* gene serendipitously was shown to be complemented by cloned copies of either the *cI* or *cro* genes from phage λ (8). The CI and Cro proteins of phage λ are well known DNA binding proteins which bind a common set of 17 base pair DNA sequences found in the right operator region of the phage λ genome. These results suggest that the *lytD* gene may encode a DNA binding protein resembling CI and Cro. It has been further proposed that the LytD target sequence should possess a 17 base pair sequence resembling the λ right operator sequences. The function of LytD may be to repress a gene that is directly or indirectly involved in lysis (8).

The purpose of this thesis is to report the discovery and characterization of the *lytF* mutation, and its interaction with the other mutations of the *murH* family. Based on

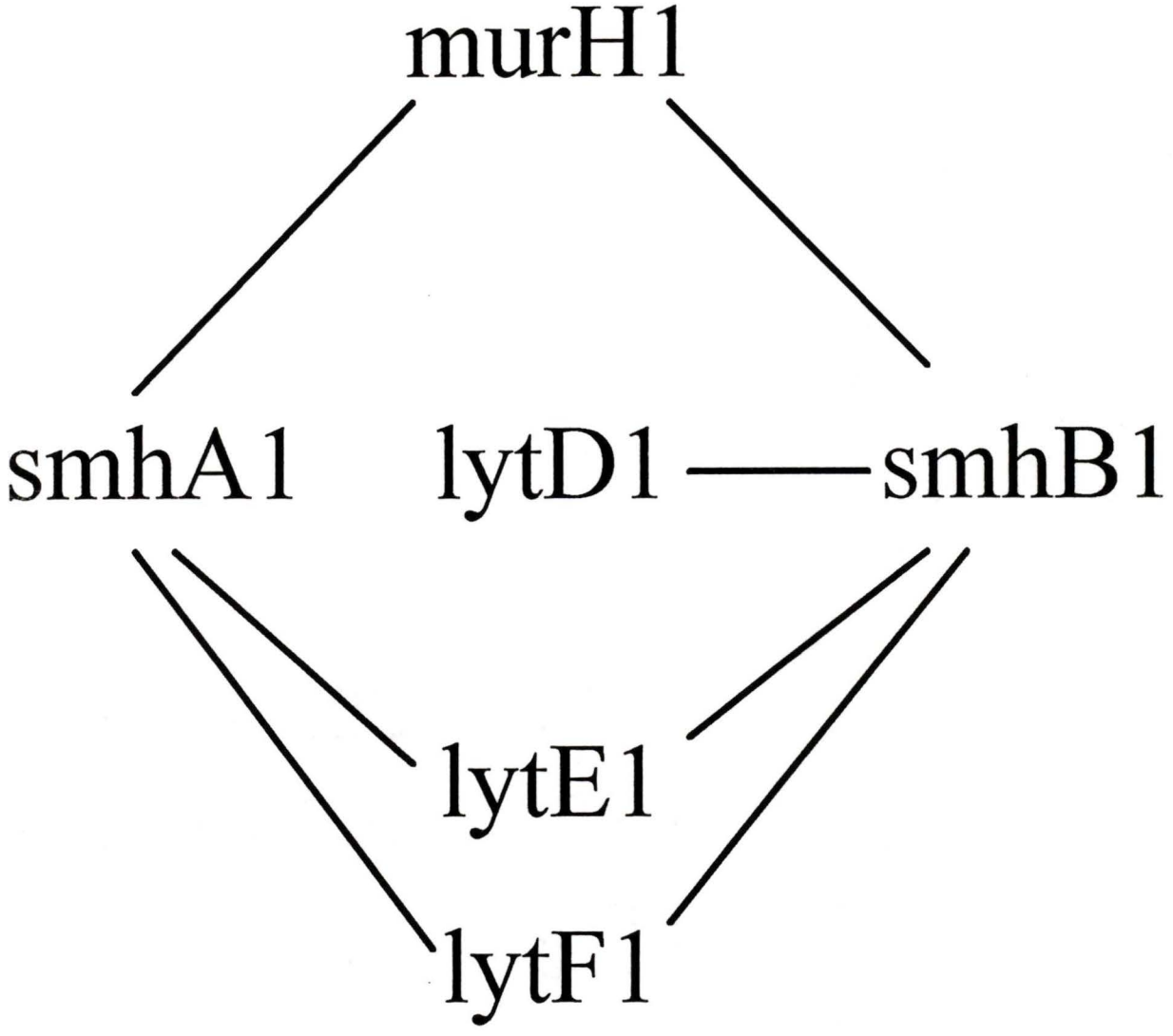


Figure 5. Proposed relationships between the members of the *murH* gene family based on suppression studies. These interactions may be direct or indirect. The *smhA* allele interacts with all the other alleles except *smhB* and *lytD*. In contrast, the only allele *smhB* cannot interact with is *smhA*. There is no interaction detected between alleles not connected by lines.

Modified from D.Dai's Ph.D. thesis, University of Victoria (1990).

the interaction of *lytF* with both *smhA* and *smhB*, it is proposed that *lytF* may belong to the *murH* family, the members of which are proposed to be functionally related.

Materials and Methods

I. Bacteria

The *E. coli* K-12 strains used in this study are listed in table one. The *zbc-103* :: Tn*10* insertion, used to manipulate the *smhB* locus (7), and the *smhA*-linked *zce-102* :: Tn*10* (9) and the *zce-1* :: Tn*10kan* insertions (9) have all been previously described. The origins of the *lysA* :: Tn*10* and the *serA*+ :: Tn*10* insertions will be described below.

II. Culture Conditions

Bacteria were grown in Tryptic Soy Broth (TSB, Difco Laboratories, Detroit, Mich., USA); or in Nutrient Broth (NB, Difco Laboratories). The solid versions of these media, TSA and NA, contained 1.5% agar. Sodium chloride (NaCl) was added to these media to a final concentration of 1% (w/v) where indicated. For genetic experiments involving auxotrophic markers the medium was Davis Minimal Agar (DMA, Difco Laboratories) containing 0.2% (w/v) glucose as a carbon source, 0.5 μg/ml thiamine, and 50 μg/ml amino acid supplements. Cultures in liquid media were incubated in waterbath shakers at the indicated temperatures, and the growth of the culture was monitored either with a green-filtered Klett-Summerson colorimeter, or with a Beckmann DU-64 spectrophotometer (λ = 600 nm). Where antibiotics were required, they were added to the following final concentrations: tetracycline, 20 μg/ml and kanamycin, 50 μg/ml.

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

Strain	Genotype	Source
JC158	*serA6 thi-1*	
VC7	*thi-1 lysA23 rpsL109*	Laboratory Collection
VC460	VC7 *zaa-1* :: Tn*5 murH*	Dai and Ishiguro, 1988
VC1100	JC158 *serA+ lytF*	This study
VC1110	VC1100 *lysA* :: Tn*10*	This study
VC1112	VC1100 *serA+* :: Tn*10*	This study
VC1119	VC7 *serA6 lysA+*	This study
VC1137	VC1100 *zce-102* :: Tn*10*	This study
VC1138	VC1100 *zce-102* :: Tn*10 smhA1*	This study
VC1140	VC1100 *zbc-103* :: Tn*10 smhB1*	This study
VC1142	VC1100 *zbc-103* :: Tn*10*	This study
VC1144	VC7 *zbc-103* :: Tn*10 smhB1*	This study
VC1146	VC1100 *zbc-103* :: Tn*10 smhB3*	This study
VC1148	VC7 *zbc-103* :: Tn*10 smhB3*	This study
VC1149	VC1100 *zce-102* :: Tn*10 smhA8*	This study
VC1157	VC7 *zbc-103* ::Tn*10 smhB5*	This study
VC1166	VC1157 *zce-1* ::Tn*10kan smhA1*	This study
VC1188	VC460 *zce -1*:: Tn*10kan*	This study

III. Genetic Techniques

Conjugation and bacteriophage P1*vir* mediated generalized transduction were performed according to the methods of Miller (48). Genetic linkages were calculated as described by Wu (65). To facilitate the mapping of *lytF*, Tn*10* insertions linked to *serA* and *lysA* were obtained by screening random Tn*10* insertion pools prepared on a protrophic strain, W3110, with the phage lambda derivative, λNK55, as described by Kleckner *et al* (34). Screening these pools involved P1*vir*-mediated transduction of the random transposon insertions into strains JC158 and VC1100, and the subsequent selection of appropriate transductants on TSA containing 20 μg/ml tetracycline at 30°C.

IV. Assessment of Temperature Sensitivity

To allow the assessment of the temperature sensitivity of strains with respect to their colony forming capability, serial dilutions of over night cultures were plated in duplicate on TSA, NA, and NA + 1% NaCl. One set of plates on each type of media was incubated at 30°C, whereas the other set was incubated at 42°C. After 24 h (TSA) or 48 h (NA, NA + 1%) of incubation, plate counts were determined. The plating efficiency was expressed as the ratio of the plate counts, in terms of colony forming units (CFU) per ml plated at 42°C to the plate count obtained at 30°C.

The temperature sensitivity of cell growth in liquid cultures was assessed by comparing the optical density of the culture at high temperature to that of the culture at low temperature. The culture was grown for two to three doubling times (~2h) to a density of about 50 Klett Units (or O.D. ~ 0.5, λ = 600 nm). The culture was then split into two portions : a control culture, maintained at 30°C, and a test culture which was incubated at 42°C. The optical densities of each culture were then followed during the next two to three hours.

V. Solubilization of Radiolabelled Peptidoglycan

Peptidoglycan was pre-labelled with [G - ^{3}H] diaminopimelic acid ([^{3}H]DAP, Amersham Canada Ltd., Oakville, Ontario, Canada.) as described by Dai and Ishiguro (6). Strain VC1110 (*lytF*) was grown in TSB for two doubling times to a density of 2 x 10^8 cells/ml (~2h), and [^{3}H]DAP was then added to a final concentration of 0.6 μg/ml (8.14 x 10^4 Bq). After one doubling (~1h), the addition of unlabelled DAP to 25 μg/ml terminated the labelling. The labelled culture was split into two portions, with one portion remaining at 30°C, whereas the other portion was shifted to 42°C. At the indicated times, 0.5 ml samples of each culture were added to an equal volume of boiling 4% sodium dodecyl sulfate (SDS), and boiled for 30 minutes. The SDS-insoluble fraction of each sample was collected on Millipore filters (pore size 0.45 μm). The filters were rinsed with distilled water, dried, and counted in a Beckmann LS3145T liquid scintillation counter in a toluene-based scintillation cocktail.

VI. *SmhA* and *smhB*-like Mutations

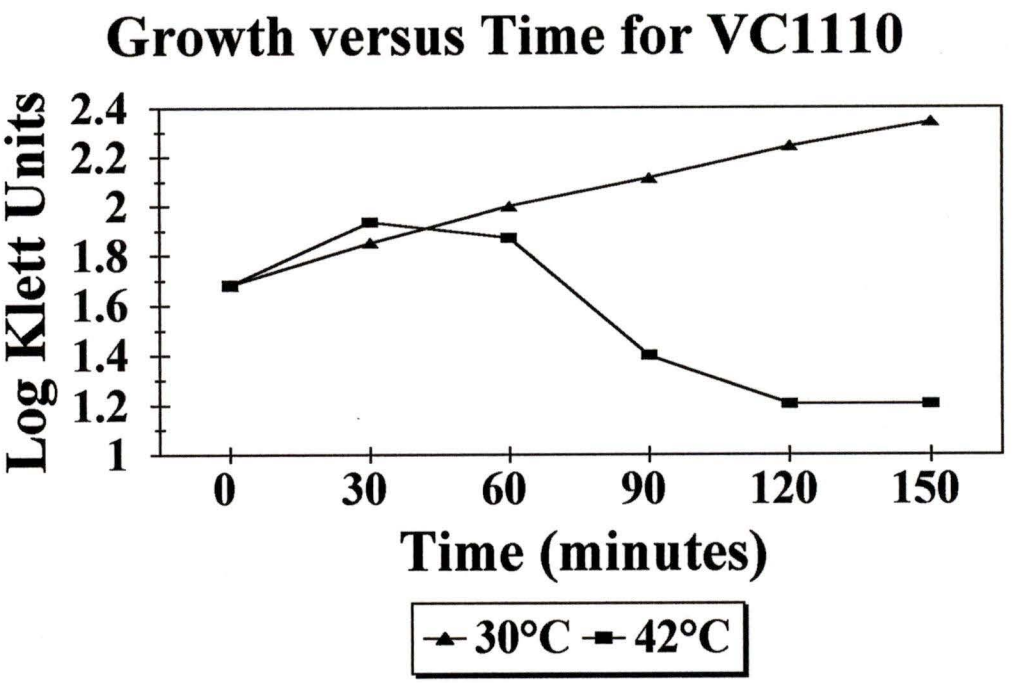
To determine whether *smhA*-like mutations could occur spontaneously in a *lytF* genetic background, VC1137 (*zce-102* :: Tn*10* *lytF*) cells were plated on TSA plates containing 20 μg/ml tetracycline and incubated at 42°C for 48 h. P1*vir* generalized transducing phage were grown on the survivors which successfully formed colonies. This P1*vir* preparation was then used as the donor for P1*vir* mediated transduction into VC1100 (*lytF*) and VC460 (*murH1*), using the Tn*10* insertions of the donors as the selectable marker. The transductants were plated on TSA containing 20 μg/ml tetracycline, and screened for temperature resistance to determine whether the putative extragenic suppressors were *smhA*-like with respect to genetic linkage to *zce-102* :: Tn*10* and suppression phenotype. Plating efficiencies of the suppressed strains were determined on TSA, NA, and NA + 1% NaCl as above.

To examine the possibility of the spontaneous occurrence of *smhB*-like mutations in a *lytF* genetic background, VC1142 (*zbc-103* :: Tn*10* *lytF*) was plated on TSA containing 20 μg/ml tetracycline, and incubated at 42°C. After 48 h, the colonies were scored, and the plates were incubated for an additional 24 h at 30°C. The surviving colonies which appeared at the lower temperature were used to grow P1*vir* generalized transducing phage, which were subsequently used to transduce the Tn*10* insertions of the survivors into VC1100 (*lytF*) and VC460 (*murH*), to determine whether the putative *smhB*-like mutants were *smhB*-like with respect to map position, growth and suppression phenotype.

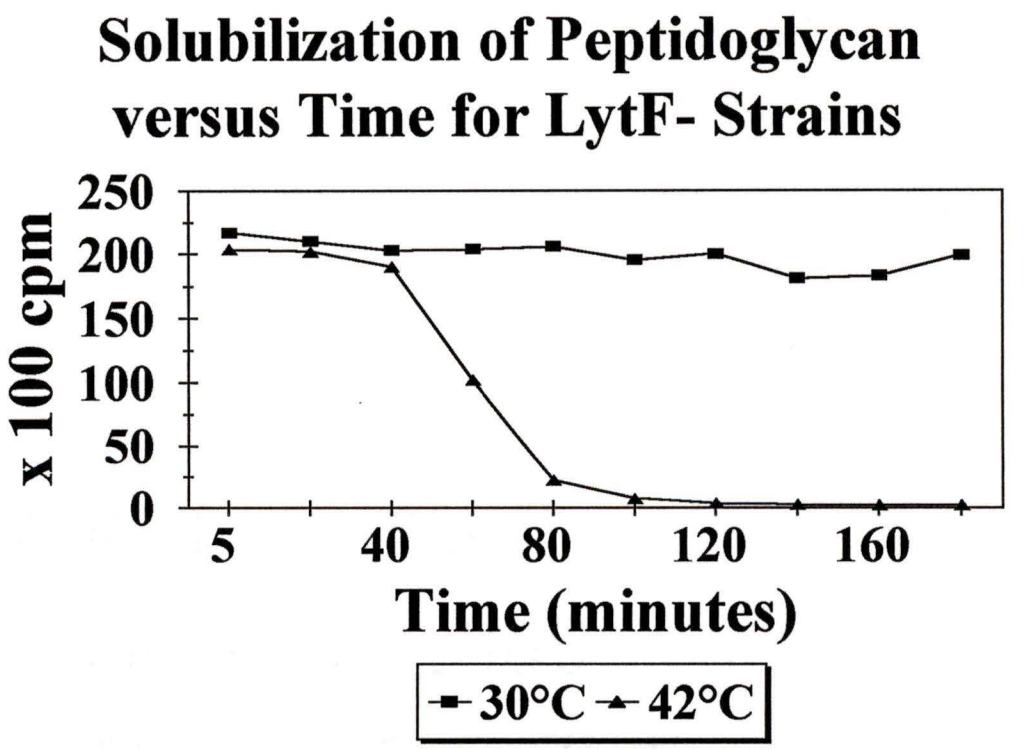
Results

Phenotypic properties: The *lytF* mutant was fortuitously discovered during the screening of a collection of EMS-mutagenized, temperature-sensitive derivatives of VC7 that were apparently defective in peptidoglycan metabolism. The *lytF* mutation conferred temperature-sensitive growth and a lysis phenotype at the restrictive temperature of 42°C. As seen in figure 6A, the lysis of VC1110 (*lytF*) cultures growing in complex medium began 30 minutes after the culture was shifted to 42°C. This strain, VC1110, also underwent lysis upon temperature up-shift when grown in minimal media (data not shown) As demonstrated by figure 6B, the lysis of VC1110 cells which began 30 minutes after temperature up-shift was associated with the solubilization of [^{3}H]DAP labelled high molecular weight peptidoglycan from the cell wall. Although the quantity of label in the 30°C control culture remained relatively constant, in the 42°C culture, a 20-fold decrease in the amount of [^{3}H]DAP in the insoluble wall occurred between 30 and 90 minutes after the temperature shift to 42°C (figure 6B). This decrease (figure 6B) correlated well with the time course of lysis of the 42°C culture as demonstrated by figure 6A, in which the

Figure 6. Temperature sensitive autolysis of strain VC1110 (*lytF*).



A) Turbidimetric measurement of lysis as a function of time.



B) Measurement of the solubilization of cell wall peptidoglycan (prelabelled with [^{3}H]DAP as a function of time and growth temperature.

optical density dropped from 1.9 to 1.2 log Klett Units over the same time period, suggesting that cellular lysis may be attributable to the PG hydrolase-mediated solubilization of the cell wall.

Mapping data: Mapping strains were generated by P1*vir*-mediated transduction of Tn*10* insertions linked to *serA* and *lysA* into VC1100 as described in Materials and Methods. The *lytF* mutation was mapped to 62.4 minutes (figure 7) on the *E. coli* genetic linkage map (1). This did not correspond to a known locus for a gene encoding any peptidoglycan biosynthetic enzyme or hydrolase (12, 41-47, 49, 53-56, 63).

The similarity of the ts growth and lysis phenotypes expressed by the *lytF* mutant and the previously described *murH* mutant (6) suggested that the *murH* extragenic suppressors, *smhA* (9) and *smhB* (7), might also be effective in suppressing the phenotype conferred by *lytF*. Table 1 lists the strains that were used to compare the influence of *smhA* and *smhB* on the ability of the *lytF* strain to grow at the restrictive temperature. These strains were isogenic combinations of the mutant and wild type alleles of either *smhA1* and *lytF* or *smhB* and *lytF*.

These strains were constructed by P1*vir*-mediated cotransduction of either the *zce-102* :: Tn*10* and *smhA*, or *zbc-103* :: Tn*10* and *smhB*, respectively, into appropriate recipient strains. The genotypes of all constructions were checked by backcrossing the appropriate genetic markers into VC7, using the linked Tn*10* insertions as the selectable marker in each cross.

***SmhA*-mediated suppression of the *lytF* phenotypes:** Transduction of the *smhA* mutation into the *lytF* genetic background resulted in the suppression of the ts growth phenotype conferred by *lytF* (table 2). The growth of the isogenic pair, VC1137 (*smhA+ lytF*) and VC1138 (*smhA1 lytF*), on three different media is shown in table 2. The plating

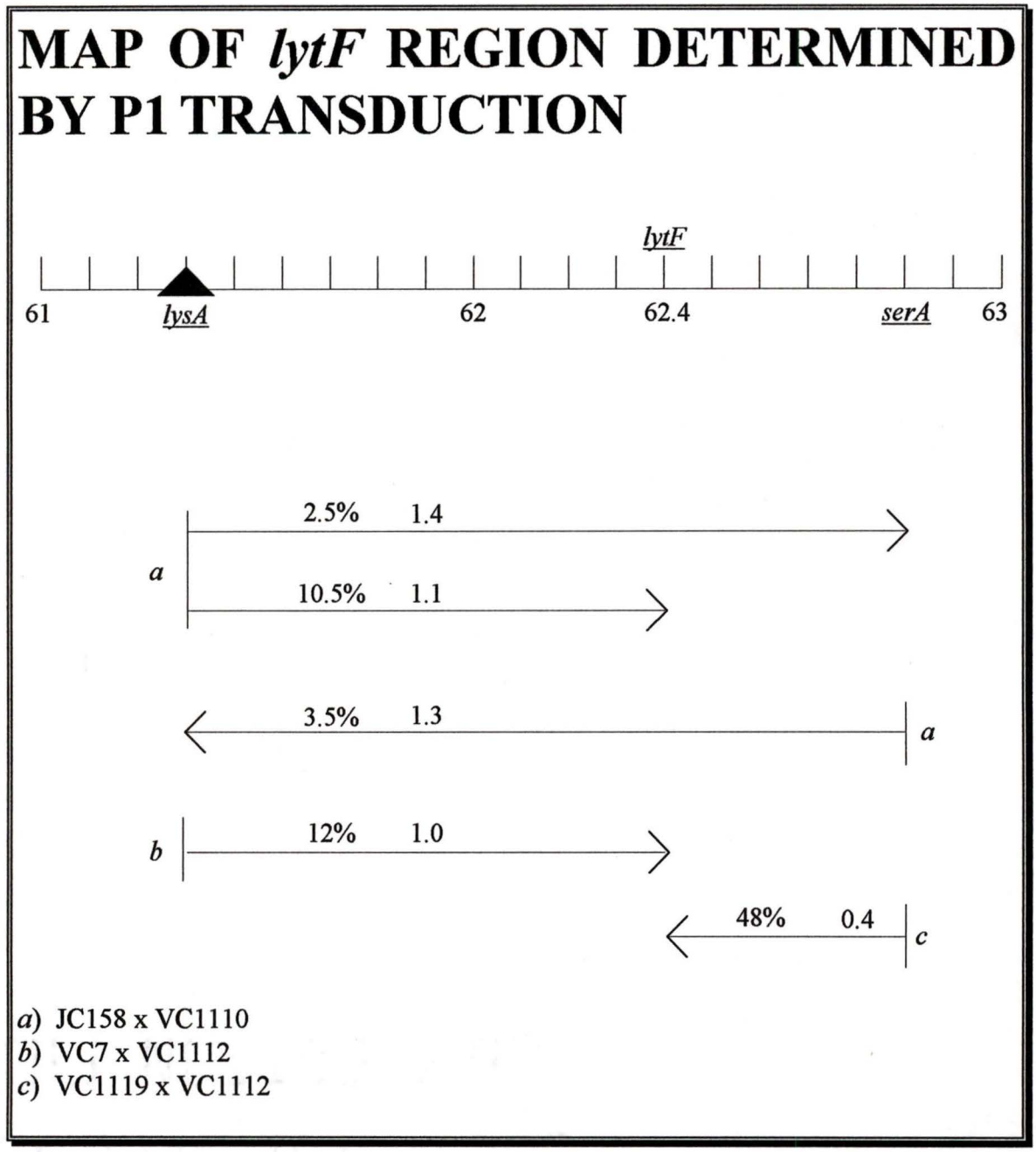


Figure 7. Genetic linkage map of the *lytF* region. The results of the following crosses (recipient x donor) are shown with the number of recombinants scored in each case indicated in parentheses: a) JC158 x VC1110 (285); b) VC7 x VC1112 (276); c) VC1119 x VC1112 (300). For each set of arrows, the vertical line represents the position of the selected marker, while the arrowheads represent the positions of the unselected markers in each cross. The numbers over the arrows indicate the cotransduction frequency (%) and the genetic map distance.

Table 2. Colony formation by isogenic *smhA lytF* and *smhA murH* strains as a function of growth media and temperature (CFU per ml at 42C / CFU per ml at 30C).

Strain	Relevant Genotype	NA	NA + 1% NaCl
VC1138	*smhA1 lytF1*	2.5×10^{-5}	0.85
VC1137	*smhA+ lytF1*	2.1×10^{-8}	6.4×10^{-5}
VC1149	*smhA8 lytF1*	1.4×10^{-5}	0.95
VC1188	*smhA8 murH1*	1.0	1.0

efficiency of VC1137 (*lytF*) was only 2.1 x 10^{-8} on NA and 6.4 x 10^{-5} on NA + 1% NaCl, which indicated that this strain was temperature sensitive and unable to form colonies at 42°C on any of these media, regardless of the medium osmolarity. The growth rate of VC1137 in nutrient broth and nutrient broth + 1% NaCl prior to cellular lysis is approximately the same. However, although the turbidity of the cultures is approximately the same, the extent of cellular lysis is less when 1% NaCl is added to the medium. This difference in survival may reflect the 1000 fold anhancement of the plating efficiency (table 2) seen for nutrient agar plates + 1% NaCl compared to that of cells grown on nutrient agar.In contrast, VC1138 (*smhA1 lytF*) with a plating efficiency of 0.9 on TSA and 0.85 on NA + 1% NaCl was able to form colonies at 42°C on media containing a high concentration of salt (TSA, NA + 1% NaCl), (table 2).

Therefore, the *smhA*-mediated suppression of the ts colony forming phenotype conferred by *lytF* was salt dependent - that is, the addition of 1% NaCl to the medium allowed the *smhA lytF* double mutant, VC1138, to form colonies at 42°C. Although the *smhA* mutation restored temperature resistant colony formation to both the *murH* and the *lytF* mutant strains, the *smhA1*-mediated restoration of growth was independent of the medium osmolarity in the *murH* mutant strain (8). As shown by the comparison of the growth and lysis curves of VC1137 (*lytF smhA+*) and VC1138 (*lytF smhA1*) in nutrient broth + 1% NaCl at 30°C and 42°C, in figure 8, the lytic phenotype conferred by the *lytF* at 42°C can be suppressed by the presence of the *smhA* mutation in the same cell. Growth and lysis curves similar to figure 8 were also obtained for VC1137 (*lytF*) and VC1138 (*lytF smhA*) cells grown in TSB at 30°C and 42°C (data not shown). Although restoration of colony formation was dependent on the osmolarity of the medium, the prevention of cellular lysis at the restrictive temperature was not. VC1138 cells (*lytF smhA*) grown at 42°C in both high and low osmolarity media failed to lyse (figure 8 and 9). The inability of the *lytF smhA* double mutant, VC1138, to form discernible colonies on nutrient agar (table 2) may

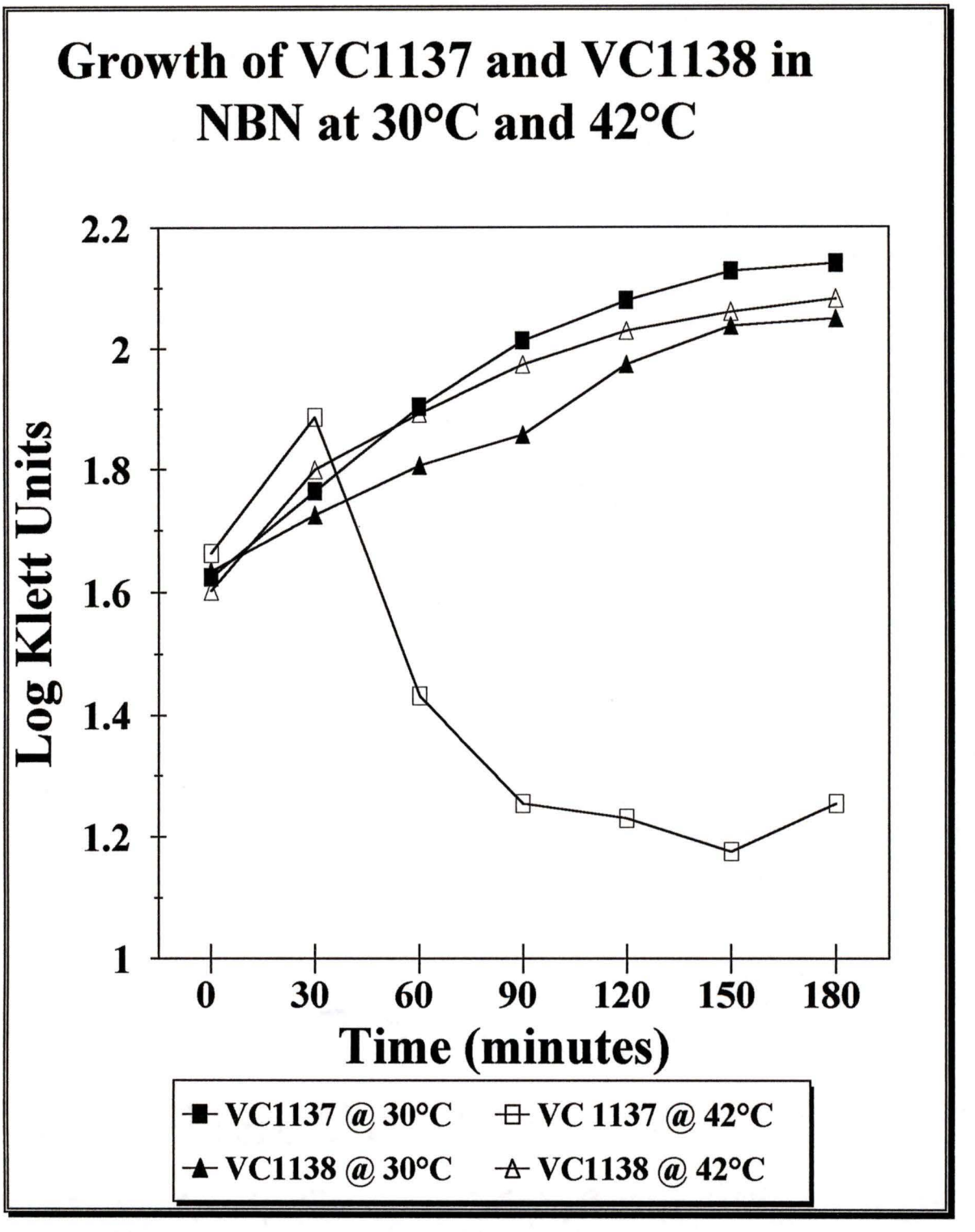


Figure 8. Suppression of *lytF* by *smhA1*: growth of strains VC1137 (*lytF smhA+*) and VC1138 (*lytF smhA1*) in NB + 1% NaCl at 30°C and 42°C.

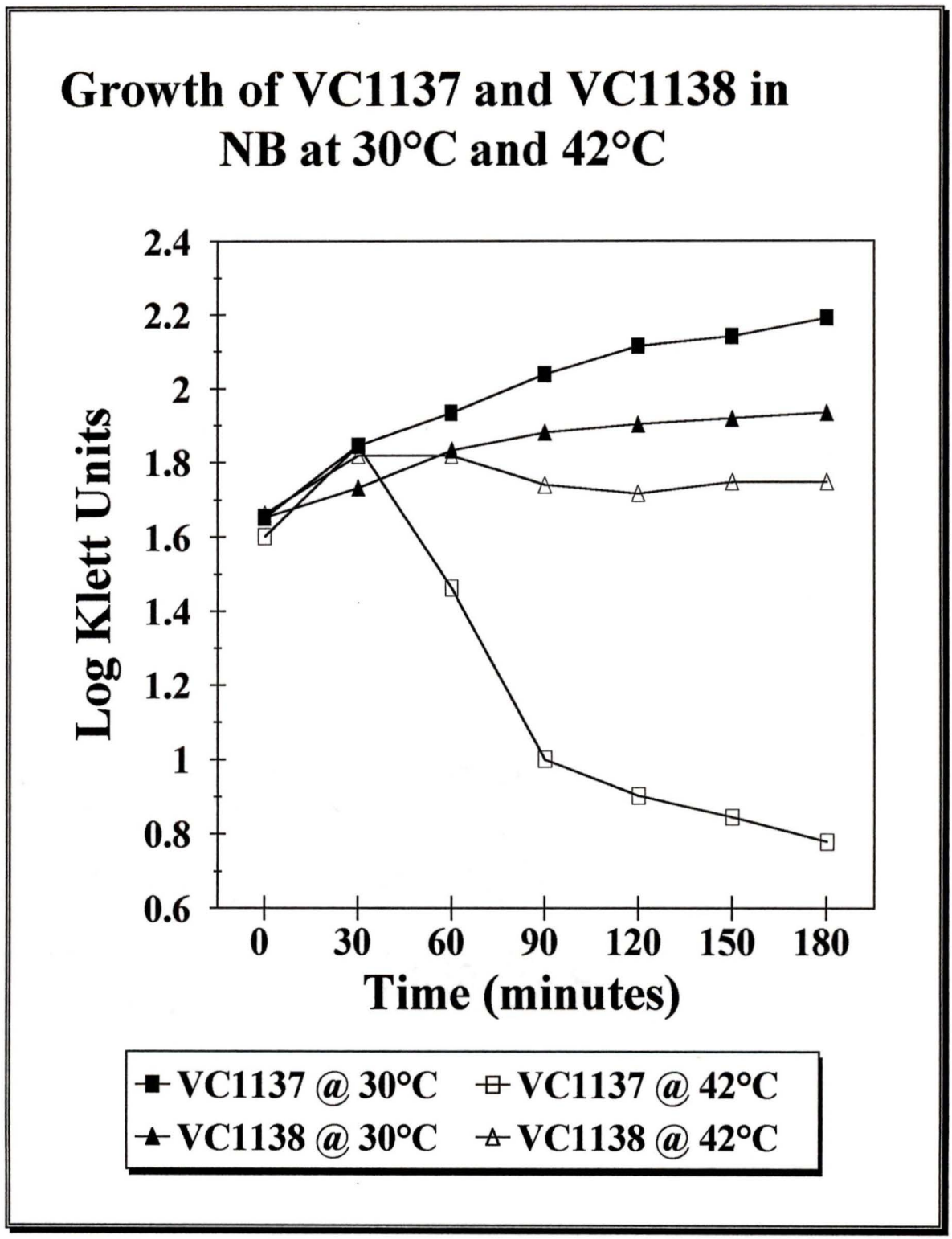


Figure 9. Suppression of *lytF* by *smhA1*: growth of strains VC1137 (*lytF smhA+*) and VC1138 (*lytF smhA1*) in NB at 30°C and 42°C.

be due to the cessation of growth which appears to occur in nutrient broth at the restrictive temperature (figure 9).

The phenotypic similarity between *murH* and *lytF* implied that the ability of the *murH* suppressor, *smhA*, to interact with *lytF* was more than a fortuitous event. In addition, the spontaneous appearance of *smhA1* in an *murH* mutant strain indicated the possibility that *smhA*-like mutations could occur spontaneously in a *lytF* genetic background. The techniques outlined in Methods resulted in the isolation of an *smhA*-like mutation capable of suppressing the ts growth and lysis phenotypes of *murH* and *lytF*. This mutation was designated as *smhA8* because it resembled *smhA1* with respect to several key phenotypic features. (i) The genetic linkage between the *zce-102* :: Tn*10* insertion was approximately 50% for both *smhA1* (9) and *smhA8* (data not shown). (ii) *SmhA8* suppressed the lytic phenotype of *lytF* mutants at 42°C in a very similar manner to the suppression of lysis seen in *lytF smhA1* mutants (data not shown). (iii) Like *smhA1*, *smhA8* restored the ability to form colonies at the restrictive temperature to *lytF* mutants in a salt dependent manner (table 2, VC1149), whereas restoration of colony formation at 42°C of the *murH* mutant is salt independent (table 2, VC1188). Thus, the salt dependence of the *smhA*-mediated suppression of the ts colony forming phenotype of *lytF* mutants appears to be a function of the *lytF* genetic background rather than a peculiarity of the interaction between *lytF* and the *smhA1* allele. Based on the evidence outlined above, the *smhA*-like mutation has been designated *smhA8*. To confirm the allelic relationship, an F' complementation study will be necessary. This awaits further study.

***SmhB*-mediated suppression of the *lytF* phenotypes:** As noted above, isogenic constructions of the mutant and wild type alleles of *lytF* and *smhB* were generated (table 1) and used to determine the influence of the *smhB* mutation on the phenotype of the *lytF* mutant. The comparison of the ability of these strains to form colonies on three different

Table 3. Colony formation by isogenic *smhB1 lytF* and *smhB3 lytF* strains as a function of growth media and temperature (CFU per ml at 42C / CFU per ml at 30C).

Strain	Relevant Genotype	TSA	NA	NA + 1% NaCl
VC1140	*smhB1 lytF1*	0.7	5.8×10^{-7}	0.6
VC1142	*smhB+ lytF1*	3.9×10^{-5}	1.0×10^{-5}	5.2×10^{-5}
VC1144	*smhB1 lytF+*	0.98	1.18×10^{-6}	1.1
VC1146	*smhB3 lytF1*	0.12	5.1×10^{-9}	1.0
VC1148	*smhB3 lytF+*	0.99	5.8×10^{-7}	0.93

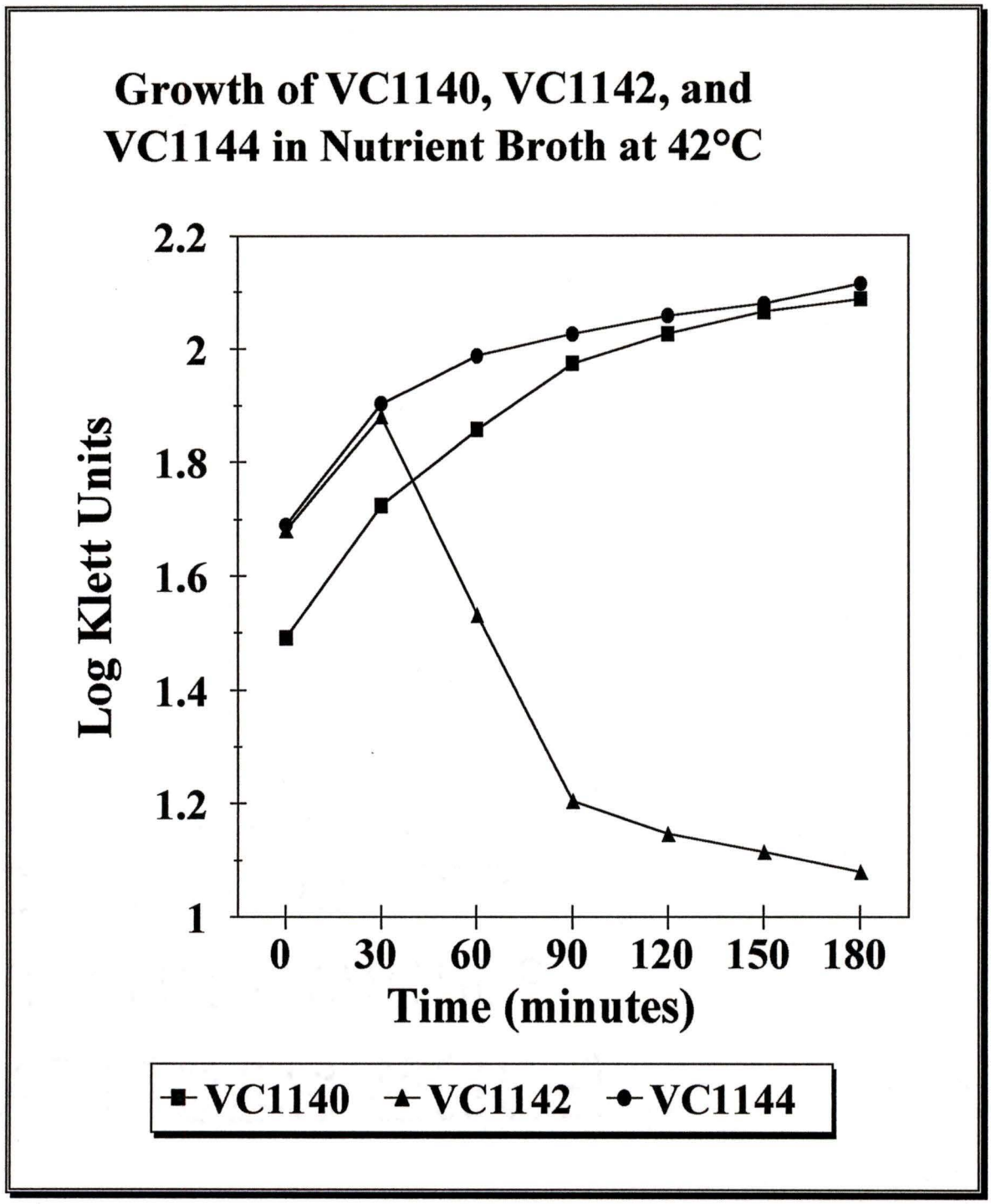


Figure 10. Suppression of *lytF1* by *smhB1* : Growth of Strains VC1140 (*lytF1 smhB1*), VC1142 (*lytF1 smhB*+) and VC1144 (*lytF*+ *smhB1*) in NB at 42°C.

media at 42°C (table 3) indicated that the addition of 1% NaCl to the medium allowed colony formation by VC1140 (*smhB1 lytF*) and VC1144 (*smhB1*) but not by VC1142 (*lytF*). This indicated that suppression of the ts growth phenotype of the *lytF* mutant by the *smhB* mutation was salt dependent. In contrast, the *lytF* mutant was unable to make colonies at 42°C regardless of the medium osmolarity, confirming that *lytF* was not an osmoremedial mutation (table 3). The growth of these three strains in NB at 42°C is shown in figure 10. The lack of colony formation on low salt medium at 42°C by VC1142 (*lytF*) was apparently due to the lysis of these cells at the restrictive temperature (table 3, figure 10). Although neither VC1144 (*smhB*) nor VC1140 (*smhB lytF*) strains lysed in low osmolarity medium (NB) at 42°C, the growth curves of both strains were biphasic and appeared to level off at approximately 105 Klett Units, indicating that growth of these strains may cease after a limited number of generations. This limited growth on low osmolarity medium may be responsible for the lack of discernible colonies formed by VC1144 and VC1140 at 42°C on the solid version of this medium (NA).

The idea that *smhB*-like mutations may occur spontaneously in the *lytF* genetic background was suggested by three observations. (i) *LytF* and *murH* are phenotypically similar. (ii) *LytF* is suppressed by the *murH* suppresssor, *smhB*. (iii) *SmhB* appeared spontaneously in the *murH* mutant strain. The techniques outlined in Methods resulted in the isolation of a mutation that was *smhB*-like with respect to the ability to suppress the ts growth and lysis phenotypes of *murH* and *lytF*. This mutation was designated *smhB3* because of its phenotypic resemblance to the *smhB1* allele, and because like *smhB1*, the *smhB3* allele is over 95% linked to the *zbc-103* :: Tn*10* insertion. Isogenic pairs of the mutant *smhB3* allele with the mutant and wild type *lytF* alleles were constructed and used to assess the influence of *smhB3* on the phenotype of the *lytF* mutant strain. In table 3, the colony forming characteristics of this pair of *smhB3* strains on three different media were demonstrated. Comparison of the colony forming phenotype of VC1148 (*smhB3*)

and VC1144 (*smhB1*) in table 3 shows that both strains exhibited the ability to form colonies at 42°C on nutrient agar following the addition of 1% NaCl. Therefore, like VC1144 (*smhB1*), the VC1146 (*smhB3*) strain appears to be dependent on high medium osmolarity for colony formation at the restrictive temperature. Comparison of the ability to form colonies at the restrictive temperature by VC1146 (*smhB3 lytF*) and VC1140 (*smhB1 lytF*) demonstrated the requirement for high medium osmolarity to allow colony formation at 42°C (table 3). Therefore, the *smhB3*-mediated and the *smhB1*-mediated suppression of the ts growth phenotype of *lytF* are similarly salt dependent.

The growth curves of VC1146 (*smhB3 lytF*) and VC1148 (*smhB3*) were similar to those of their *smhB1* counterparts seen in figure 10. Although neither strain underwent osmotic lysis at 42°C, even in a low osmolarity medium such as NB, the growth curves of the *smhB3* strains were similar to those of the *smhB1* strains in that they also appeared to plateau after a limited number of generations. This observation indicates that the lack of visible colony formation on low salt medium at 42°C by both the *smhB1* and the *smhB3* strains may be due to the cessation of growth by these strains after a limited number of generations.

Based on the evidence outlined above, the *smhB*-like mutation has been designated *smhB3*, although the F' complementation assay needed to demonstrate this relationship conclusively has not yet been done.

Variability in the phenotype conferred by the *smhB* locus: During the construction of new *smhB* mutant strains from the original *smhB1* parent strain isolated by D. Dai, it was noted that occasionally the resulting strain construction did not behave as anticipated. One such strain was further characterized with respect to the phenotype conferred by its *smhB* locus. The *smhB* allele in this strain was designated *smhB5*, to reflect the differences in the phenotype conferred by this mutation compared with the phenotype

Table 4. Colony formation by isogenic smhB5 murH and smhB5 *smhA*1 strains as a function of growth medium and temperature. (CFU at 42 C / CFU at 30 C).

Strain	Relavent Genotype	NA	NA + 1% NaCl
VC1157	*smhB5*	3.17×10^{-9}	9.29×10^{-6}
VC1166	*smhB5 smhA1*	1.1	0.92
VC1186	*smhB5 murH*	4.29×10^{-9}	6.71×10^{-6}

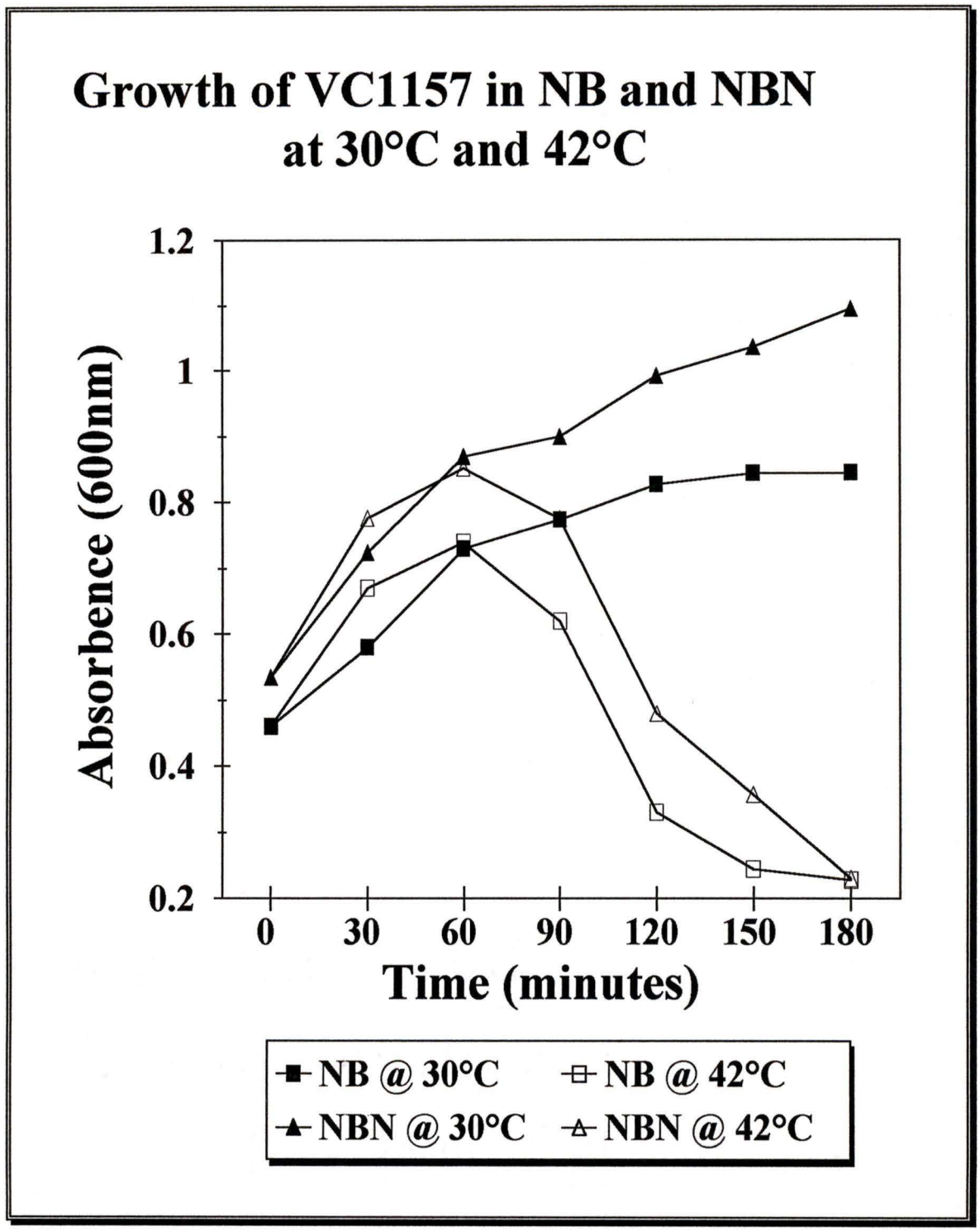


Figure 11. Temperature-sensitive lysis of strain VC1157 (*smhB5*) in NB and NB + 1% NaCl at 30°C and 42°C.

conferred by the *smhB1* allele. In order to demonstrate that the observed phenotype was due to the *smhB5* marker, and not to the presence of an unlinked extragenic suppressor of the *smhB1* allele, the *smhB5* locus from this strain was backcrossed into the VC7 parent strain using the *zbc-103* :: Tn*10* insertion as the selectable marker. The *smhB5* mutation was similar to the *smhB1* mutation in two respects. First, both mutations were 99% cotransducible with the *zbc-103* :: Tn*10* insertion. And second, during preliminary experiments using F' complementation assays to determine whether the putative alleles of *smhB* were allelic, it was found that *smhB1* and *smhB5* were phenotypically dominant over the wild type *smhB*+ allele. This phenotypic dominance was expressed regardless of whether the wild type allele in the merodiploid was carried on the F' plasmid (a derivative of F' 254) or on the chromosome.

However, the phenotype conferred by the *smhB5* mutation was also distinct from that conferred by the *smhB1* mutation in several respects. The inability of VC1157 (*smhB5*) to form colonies at 42°C on either of the tested media, regardless of their osmolarity (table 4) demonstrates that the addition of 1% NaCl to the medium is not sufficient to suppress the ts colony formation conferred by *smhB5*. Additionally, VC1157 underwent osmotic lysis at 42°C in media containing both high (NB + 1% NaCl) and low (NB) concentrations of salt (figure 11). Based on this evidence, the ts growth and lysis phenotypes conferred by the *smhB5* mutation do not appear to be osmoremedial, and the lack of colony formation at 42°C appears to be due to the cellular lysis which occurs at this temperature. Therefore, the phenotype conferred by *smhB5* was distinct from that conferred by *smhB1,* in that *smhB1* was an osmoremedial mutation (table 3) and was not lytic, even when the cells were grown in medium of low salt concentration (figure 10).

SmhB5 and *smhB1* appear to interact differently with *lytF*, *murH*, and *smhA1*. The combination of *smhB5* and *lytF* in a double mutant strain does not result in suppression of the ts growth and lysis phenotype conferred by *lytF* (data not shown). This observation is

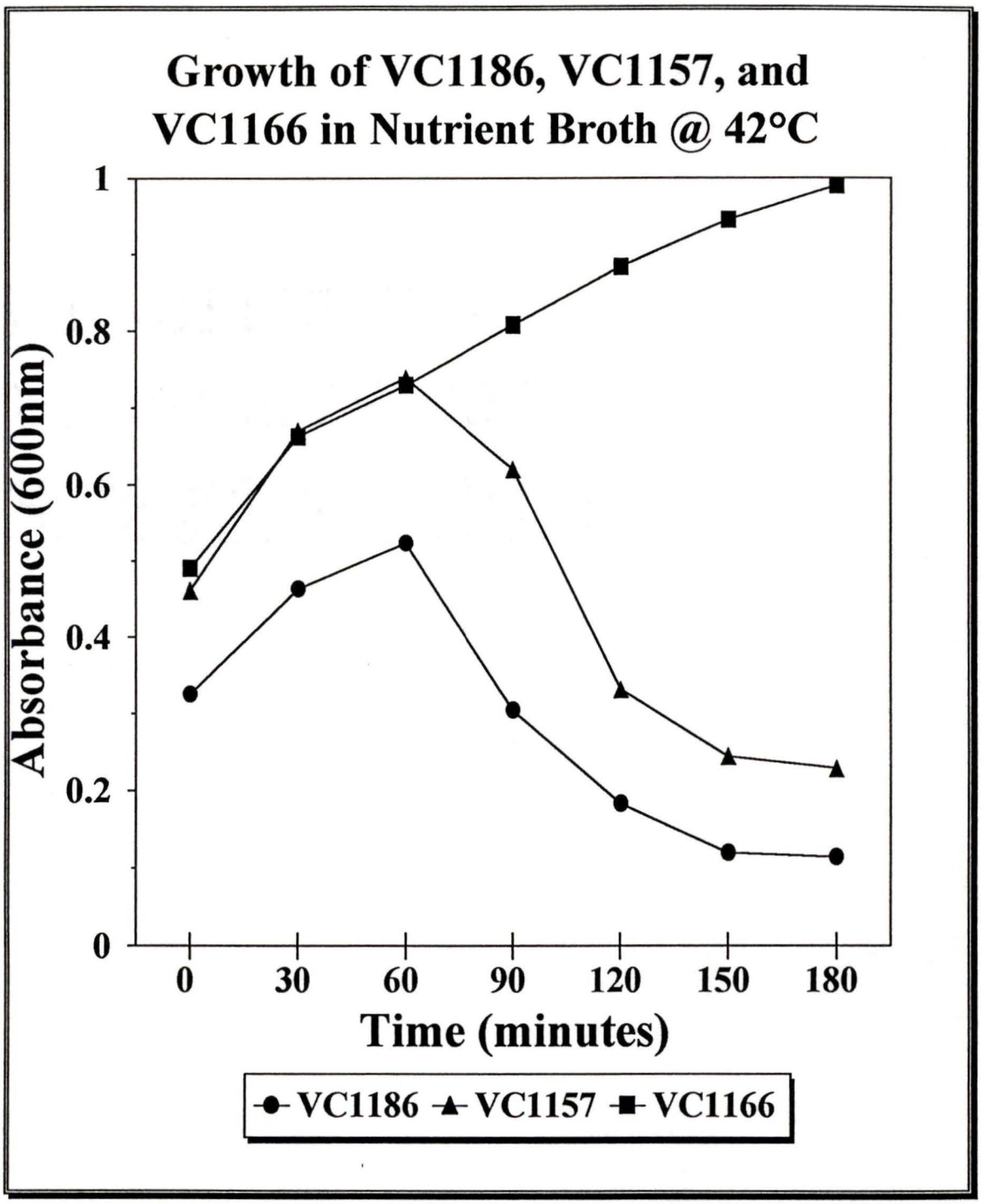


Figure 12. Suppression of *smhB5* by *smhA1* in strain VC1166, and temperature-sensitive lysis of strain VC1157 (*smhB5*) and VC1186 (*murH smhB5*) in NB at 42°C.

in contrast with the restoration of colony formation and suppression of the lysis of the *lytF* strain that occurs in the *smhB1 lytF* double mutant, VC1140 (table 3, figure 10). The plating efficiency at 42°C for isogenic combinations of *smhB5* with *murH*, and *smhA1* on NA and NA + 1% NaCl is shown in table 4. As demonstrated by table 4, the VC1186 (*smhB5 murH*) strain retained the temperature sensitive colony forming phenotype characteristic of *smhB5* strains (table 4, VC1157) and *murH* strains (7). The lack of colony formation by VC1186 at 42°C appeared to be due to cellular autolysis, which occured at the restrictive temperature in both high and low osmolarity media (data not shown, figure 12). Thus, *smhB5* is not capable of interacting with *murH* to cosuppress the ts growth and lysis phenotypes associated with the individual mutations. This contrasts with the ability of *smhB1* mutation to suppress the phenotypic characteristics associated with the *murH* mutation.

Unlike the *smhB1* mutation, the *smhB5* mutation was able to interact productively with *smhA1* to produce suppression of the ts colony forming phenotype associated with *smhB5* (table 4, VC1166). As shown by the plating efficiencies of VC1166 on NA and NA + 1% NaCl at 42°C, the *smhA1*-mediated suppression of the ts growth phenotype conferred by *smhB5* was not dependent on the osmolarity of the media, because the addition of 1% NaCl to the media had no effect on the ability of VC1166 to form colonies at the restrictive temperature. Similarly, *smhA1* suppressed the lysis associated with the *smhB5* allele at 42°C in a salt independent manner (figure 12). Thus, although *smhB1* was previously shown not to exhibit cosuppression with *smhA1*, *smhA1* was able to suppress the ts growth and lysis phenotypes associated with the *smhB5* mutation.

In summary, *smhB5* and *smhB1* were similar with respect to map position and the phenotypic dominance that they exhibited over the wild type *smhB+* allele. However, *smhB5* and *smhB1* also differed in several respects. (i) The ts colony forming phenotype of *smhB5* mutants was not osmoremedial, whereas that associated with *smhB1* mutants

was. (ii) *SmhB5* cells underwent lysis at 42°C regardless of the medium osmolarity, whereas *smhB1* cells did not lyse even in media with a low salt concentration. (iii) The ts growth and lysis phenotype associated with *smhB5* can be suppressed by the presence of the *smhA1* allele in the same cell, whereas *smhA1* had no effect on *smhB1* mutants. (iv) Unlike *smhB1*, *smhB5* had no effect on the ts growth and lysis phenotypes associated with the presence of *murH* or *lytF* in *E. coli* cells.

Discussion

Peptidoglycan synthesis appears to require the coordinated activities of the biosynthetic enzymes, which synthesize the nascent subunits, and the PG hydrolases, which open acceptor sites in the established peptidoglycan of the cell wall (61, 27). It has been proposed that when the biosynthetic pathway becomes blocked at any step, the PG hydrolase activity becomes deregulated and cellular autolysis results (61, 27); however, the mechanism by which this deregulation occurs is unknown. The deregulation of the PG hydrolase activity at 42°C in strain VC1110 (*lytF*) is confirmed by the solubilization of the mature cell wall and the subsequent lysis that occurs. Thus the lytic phenotype displayed by the *lytF* mutant (VC1110) at the restrictive temperature resembled the phenotype of other previously characterized mutants with defects in peptidoglycan metabolism.

The ts lytic phenotype conferred by the *lytF* mutation was suppressed by the presence of *smhA* or *smhB*, two suppressor mutations originally isolated from a previously characterized mutant strain, *murH*. The *murH* strain appeared to have a defect that was directly or indirectly involved in peptidoglycan metabolism, and resulted in lysis at 42°C (5, 6). Because the ability of *smhA* and *smhB* to suppress lysis does not extend to autolysis caused by mutational or antibiotic-induced blockages in peptidoglycan synthesis, the genes whose mutation-induced lysis are suppressed by *smhA* and *smhB* (*lytD*, *lytE*, and *murH*) were proposed to be functionally related to each other and to *smhA* and *smhB*

(5, 7). The *lytF* gene is thought to belong to this group because (i) it has a ts lytic phenotype similar to that associated with the *murH, lytE* and *lytD* mutations, (ii) the ts lytic phenotype conferred by *lytF* is suppressed by the presence of *smhA* or *smhB*, whose suppression phenotypes are specific to the members of the *murH* family, and (iii) *smhA* and *smhB*-like mutations appear spontaneously in the *lytF* genetic background, indicating that their presence may somehow be beneficial to the *lytF* mutant strain. Both *smhA* and *smhB* appear to have two separable suppressor functions in *lytF* cells - restoration of temperature resistant colony formation (dependent on medium osmolarity), and prevention of lysis at 42°C (osmolarity independent). This contrasts with the situation in *murH* cells, where the *smhB* mutation appears to have two separable suppressor functions, whereas the suppressor functions of *smhA* remain inseparable. The cause of the difference in the behavior of *smhA* in a *lytF* cell compared with its behavior in an *murH* cell is unknown.

In the *lytF* genetic background, colony formation is temperature sensitive regardless of the medium's osmolarity. In the presence of *smhA* or *smhB*, however, temperature resistant colony formation is restored in the presence of 1% NaCl. The salt-dependent factor in the osmolarity dependent suppression of *lytF* by *smhA* and *smhB* is unknown. Although the presence of 1% NaCl sauses a 1000-fold increase in the plating efficiency of VC1137, the strain remains temperature sensitive. Thus, the increased salt concentration must be primarily affecting the *smhA* and *smhB* gene products to allow the suppression of the *lytF* phenotype, although the cause of this effect is unknown. Therefore, in the *lytF* background, *smhA* and *smhB* may be classified as osmoremedial mutations (4), while *lytF* itself is not. Although it was originally thought that an osmoremedial phenotype reflected a fragile cell envelope that could be stabilized by increasing the external salt concentration, it is now known that there are many osmoremedial temperature sensitive mutations that do not involve the cell envelope (4). Therefore, it should not be assumed that *smhA* and *smhB* must be involved in cell

envelope integrity (4). Both temperature sensitive and osmoremedial mutations are associated with slight amino acid changes in the protein product, which may then become destabilized at high temperature or under hypotonic conditions (4). Compatible solutes, such as potassium ions or glutamate, may provide the mutant protein with salt bridges to stabilize its structure under hypertonic conditions (4). However, not all temperature sensitive mutations are osmoremedial (4), and it should be noted that although *lytF* is temperature sensitive, unlike *smhB1, lytF* is not osmoremedial.

SmhA is not osmoremedial in all genetic contexts. The *smhA1* mutation suppresses the ts colony forming phenotypes associated with both *murH* and *lytF*, with *smhA1* interacting with *lytF* in a salt-dependent manner, whereas the *murH-smhA1* interaction is independent of the medium osmolarity (9). Because the above observation is consistent when the *smhA8* allele is used instead of the *smhA1* allele, it appears that the source from which the *smhA* allele was isolated is not a factor in the osmolarity dependence of certain genetic backgrounds (*smhA1* originated in an *murH* strain, while *smhA8* originated from a *lytF* strain). Therefore, the salt-dependence of the *lytF-smhA* interaction and the salt-independence of the *murH-smhA* interaction appear to reflect differences between how the *smhA* product and the *lytF* or *murH* products interact, and not the source of the *smhA* allele. However, the cause of these differences is unknown.

Unlike *smhA1*, the *smhB1* mutation confers an osmoremedial phenotype in both the *murH* (7) and the *lytF* genetic backgrounds. However, an anomaly in the behavior of the *smhB1 murH* double mutant was noted by D. Dai (5). He noticed that although the *smhB1* mutation was isolated from an *murH* strain as a ts extragenic suppressor of *murH* (that is, one that was unable to form colonies at 42°C), that the *smhB1 murH* double mutant was subsequently found to form colonies at the restrictive temperature during plating efficiency assays (5). A similar observation was made for the *smhB3 lytF* double mutant, which was selected from a *lytF* culture using a modification of the protocol

designed by Dai and Ishiguro (5, 7, 9) to isolate ts suppressors of *murH*. This protocol resulted in the isolation of VC1146 (*smhB3 lytF*) as a strain that failed to make colonies at 42°C, but whose colonies did develop during subsequent incubation at 30°C. However, like the *murH smhB1* double mutant, when VC1146 was tested in a plating efficiency assay to determine the degree of its temperature sensitivity, it was found to make colonies at 42°C. This trait appears to be characteristic of *smhB* alleles, although there is currently no explanation for the initial failure of these strains to make colonies at 42°C; the feature which distinguished them from *smhA*-like mutations, nor the subsequent salt-dependent restoration of this trait.

The *smhB* locus appears to be associated with a variable phenotype, which depends on the *smhB* allele in question. All these putative *smhB* alleles appear to have a similar genetic linkage to the *zbc-103* :: Tn*10* insertion (97-99%) and are phenotypically dominant over *smhB*+. However, their physical and suppression phenotypes may be quite distinct. For example, *smhB1* is a ts osmoremedial mutation capable of suppressing the lysis and ts colony forming phenotypes associated with *lytF, lytE, lytD* and *murH*, but which does not interact with *smhA1*. In contrast, *smhB5* is a ts lytic mutation which is insensitive to the medium osmolarity, and which interacts with *smhA1*, but does not interact with either *lytF* or *murH*. These markers have been given *smhB* allele numbers based on the similarities outlined above; however, it should be noted that P1 transduction using the *zbc-103* :: Tn*10* insertion as the selectable marker is not a sensitive enough technique with which to determine the source of the differences in the observed phenotype. Two possible explanations are that the phenotypic differences are a result of different mutational alleles of a single gene, which may be multifunctional, or the differences are caused by different mutations in closely linked genes. These options also cannot be distinguished based on the use of an F' or plasmid complementation assay because of the phenotypic dominance of the mutant *smhB* alleles over the wild type

smhB+ allele. The phenotypic dominance of the mutants makes it impossible to distinguish between the noncomplementation characteristic of alleles with mutations in the same gene and a complementary gene pair with a dominant mutant phenotype. Thus, it appears that the final proof of an allelic relationship between *smhB1* and *smhB5* may need to await the sequencing or elucidation of the role of the gene product in the cell. If *smhB1* and *smhB5* are indeed allelic, it appears that the sphere of genes with which *smhB* may be able to interact should be expanded to include *smhA1*.

Although the mechanism by which *murH, lytD, lytE,* and *lytF* induce lysis at the restrictive temperature is unknown, it is anticipated that if these genes are functionally related, then the mechanism of induction should be similar. Because *smhA1* and *smhB1* are unable to suppress lysis triggered by mutational or antibiotic-induced blockages (5), it is unlikely that the *murH* family of genes is directly involved in the synthesis of peptidoglycan. There are two other possibilities to consider with regard to the mechanism of lysis. First, these mutations may have pleiotropic effects that directly interfere with the control of the PG hydrolases (while blocking PG synthesis), leading to deregulated hydrolase activity and cellular lysis. Second, lysis may be induced by a mechanism that is independent of the pathway of lysis induction used by the cell when peptidoglycan biosynthesis is blocked by mutations or antibiotics. One possibility is that the genes of the *murH* group may represent cryptic integrated prophages which become activated at high temperature, and subsequently cause cellular lysis to allow their release from the host cell (66). However, the mechanism of lysis or how lysis is suppressed by the *smhA* and *smhB* gene products is unknown. Answers to these questions will require the identification of the cellular functions of the gene products of the *murH* gene family.

In summary, the *lytF* mutation is associated with a ts colony forming and lytic phenotype. The *lytF* mutation is proposed to belong to the *murH* family of genes (figure 5) based on: (i) its interaction with *smhA1* and *smhB1*, and the relative specificity of their

suppression phenotype, (ii) the appearance of spontaneous *smhA* and *smhB*-like mutations in *lytF* cells, and (iii) the similarity of the *lytF* lytic phenotype to that associated with *lytE*, *lytD*, and *murH*. The phenotype associated with the *smhB* locus may be variable depending on the allele in question, although it is not known whether the different phenotypes are representative of different mutations in a multifunctional gene or mutations in separate, closely linked genes.

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