The Characterization of Two Functionally Related Genes, *murH* and *lytG*, in *Escherichia coli*

by

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ABSTRACT

The characterization of two distinct but apparently functionally related genes in *E. coli*, *murH* and *lytG*, is described. *lytG* represents a newly identified genetic locus, whereas the identification of *murH* was previously reported (85). The apparent relationship between these two genes is based on common suppression by the *smhA* locus and the similarities between *murH* and *lytG* mutant phenotypes. Both mutants exhibited temperature-sensitive growth and autolysis, characteristic of that mediated by peptidoglycan hydrolases, at the restrictive temperature.

Attempts to subclone DNA fragments containing either *murH* or *lytG* complementing activity from phasmid-derived clones resulted in deletions within the fragments which indicated that, in both cases, some component of the complementing fragment was unstable when removed from the original phasmid clone.

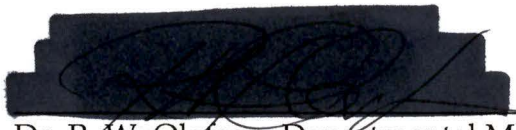
The *murH* deletion derivatives had lost the ability to restore growth at the restrictive temperature in *murH* mutants strains, suggesting that the deletion had encompassed at least a portion of the sequence from which the *murH* activity originated. The cloned fragment containing *murH* complementing activity was mapped to 16.0 minutes on the genetic map of the *E. coli* chromosome. This location was distinct from the 99.2 minute area to which the mutation in the *murH* mutant was previously mapped, indicating that a suppressor was likely responsible for the complementing

activity found in the *murH* clone. DNA hybridization studies indicated that the cloned fragment harbouring *murH* activity contained DNA sequences similar to sequences within the 0-23 kb and 37.5-44.1 kb regions of the bacteriophage λ genome.

The *lytG* complementing activity was subcloned into the multicopy number vector pUC19. Restriction mapping analysis indicated that the resulting *lytG* subclones contained deletions and possible rearrangements. Neither the *lytG* subclones, nor the original *lytG* phasmid-derived clone, could be detected on the *E. coli* K-12 chromosome by DNA hybridization, even though the mutation in the *lytG* mutant was previously mapped to the 25 minute position. This indicated that, similar to *murH*, a suppressor could be involved in restoring the ability of the *lytG* mutant to grow at the restrictive temperature. The cloned fragment containing *lytG* activity was shown, by hybridization studies and sequencing, to have significant identity to a DNA sequence within the 25.9-37.6 kb region of the phage λ genome. The possible basis for the instability, in the form of spontaneous deletions, of the cloned DNA fragments containing either *murH* or *lytG* activity, an explanation for the DNA sequence similarity with phage λ DNA, and the source of the presumptive suppression in the *lytG* and *murH* clones are discussed.

Examiners:

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



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ABBREVIATIONS

Abs	absorbance
Amp	ampicillin
Amp^R	ampicillin resistant
AMPPD®	3-(2'-Spiro-adamantane)-4-methoxy-4-(3''-phosphorloxy)-phenyl-1,2-dioxetane
anhydroMur*N*Ac	1,6-anhydro-*N*-acetylmuramic acid
ATP	adenosine 5'-triphosphate
CFU	colony forming units
Cm	chloramphenicol
D-ala	D-alanine
DAP	*meso*-diaminopimelic acid
DIG	digoxigenin
D-glu	D-glutamic acid
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
E.O.P.	efficiency of plating
Glc*N*Ac	*N*-acetylglucosamine
HPLC	high performance liquid chromatography
h	hour
IPTG	isopropyl β-D-thiogalatoside
IS	insertion sequence
kb	kilobase pairs

kan^R	kanamycin resistant
λ	lambda
LA	Luria agar
LB	Luria broth
μg	microgram
μl	microlitre
mCi	milliCurie
min	minute
ml	millilitre
mM	millimolar
Mur*N*Ac	*N*-acetylmuramic acid
Mur*N*Ac-pentapeptide	*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-DAP-D-alanyl-D-alanine
MW	molecular weight
NaCl	sodium chloride
nm	nanometre
PBPs	penicillin binding proteins
PG	peptidoglycan
RT	room temperature
Spc^R	spectinomycin resistant
Str^R	streptomycin resistant
Tn	transposon
Tris	tris-(hydroxymethyl)aminomethane
ts	temperature sensitive
TSA	tryptic soy agar

TSB	tryptic soy broth
UDP	uridine 5'-diphosphate
UV	ultraviolet
v/v	volume per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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DEDICATION

"...faith is being sure of what we hope for
and certain of what we do not see."

Hebrews 12:1 (NIV)

"...those who hope in the Lord will renew their strength,
They will soar on wings like eagles;
they will run and not grow weary,
they will walk and not be faint."

Isaiah 40:31 (NIV)

I give thanks to the Lord for my faith, which has been strengthened, and, perhaps, has matured during the course of this work. I give thanks to Him for the desire and the ability to run, a gift which has sustained me and which has provided for me a sanctuary.

This work is dedicated to the memory of the restless wanderer, desiring peace, striving for unity, and seeking a glimpse of the Infinite.

1 INTRODUCTION

1.1 Chapter Overview

The introduction of this thesis covers two general topics which, although they appear to be relatively unrelated, are both necessary for understanding the results of this work. The first of these topics is peptidoglycan (PG) metabolism in *Escherichia coli* with an emphasis on PG biosynthetic and hydrolytic enzymes. An understanding of this topic is important because the mutant phenotypes herein may involve PG hydrolytic enzyme activity.

The second topic is a discussion of the lambdoid family of bacteriophages with a focus on bacteriophage lambda (λ) and cryptic λ-related DNA sequences present in the *E. coli* chromosome. This topic is discussed for two main reasons, firstly because the use of a λ-derived vector had a key role in the cloning of the complementing activity of the two genes described in this thesis, and secondly because there is a possible relationship between the mutant loci described in this work and the cryptic lambdoid DNA sequences that reside within the *E. coli* genome.

1.2 Peptidoglycan Metabolism in *E. coli*

1.2.1 The *E. coli* cell wall

Escherichia coli, like other Gram-negative bacteria, are organisms with a three-layered coat consisting of an inner membrane, a layer of peptidoglycan (PG), and an outer membrane. The outer membrane and the

PG together constitute the bacterial cell wall and these two layers are associated though both covalent and non-covalent interactions. PG is covalently linked to the outer membrane though a small, relatively abundant molecule called PG lipoprotein which is embedded in the outer membrane. Approximately one-third of the PG lipoprotein molecules are involved in forming a physical bridge between PG and the outer membrane (1). It has been proposed that these covalent bonds assist in holding the outer membrane to the PG during cell division, enabling the outer membrane to follow the ingrowth of the bacterial septum prior to cell separation (2). The outer membrane also contains proteins, such as OmpA, and trimeric permeation molecules called porins, such as OmpC and OmpF, which are involved in non-covalent interactions with PG (3).

PG is a polymer composed of long glycan strands crosslinked by short peptide bridges (4). It forms a large, semi-rigid, net-like structure, called a sacculus, which encloses the inner membrane and serves as an exoskeleton. The main function of the PG sacculus is to maintain the integrity of the cell by holding it together against the existing large internal turgor pressure. Without the stabilizing strength and rigidity of PG, the cell would rupture as the cytoplasmic membrane is subjected to stress caused by the entry of water into the cell to equalize the osmotic pressure (4, 5). This function of PG is revealed by observations that enzymatic digestion of PG results in cell lysis unless the cells are suspended in a hypertonic medium, *i.e.*, high concentrations of sucrose or glycerol (6). Another role of the PG sacculus involves determining the characteristic rod shape of *E. coli* (7). As a

result of this function, alterations in PG metabolism often lead to changes in the shape of the *E. coli* cell (4).

In order to maintain the shape of the bacterial cell and to protect the cell from lysis the sacculus must be mechanically stable, but it is not biochemically inert after its synthesis (8). As depicted in Figure 1, the *E. coli* sacculus may be viewed as a dynamic structure, subject to both biosynthetic and hydrolytic processes. These processes allow for PG biosynthesis, rearrangements and modifications of PG, turnover and muropeptide recycling, and PG degredation leading to cellular autolysis.

The continuous structural modification of PG during synthesis is necessary for growth and division of the cell, and it has been attributed to the coordinate activities of a variety of PG biosynthetic and hydrolytic enzymes (10, 11). During growth of the cell, peptide cross bridges are cleaved and reformed, apparently to allow the incorporation of newly synthesized material and thus expansion of the sacculus. In addition to insertion of nascent glycan chains, the sacculus undergoes remodeling and rearrangements of existing PG, with release and subsequent reuse of aged material in the form of muropeptides (8, 12). This process of PG turnover and muropeptide recycling involves multiple steps which occur in an apparently controlled manner, distinct from the uncontrolled breakdown of PG leading to cellular autolysis (11).

E. coli is able to reuse a significant amount of the degradation products; all but approximately 7% are recycled rather than lost to the surrounding growth medium (8). This metabolic short cut saves the cell energy since ATP is consumed in creating the PG precursors.

Approximately 50% of PG in the sacculus is degraded to peptides and recycled each generation (8, 9). The rate of recycling is about one-half as fast as *de novo* synthesis of PG (8). Considering the potential autolytic consequence to the cell of uncontrolled degradation of PG, the mechanism(s) involved in introducing changes in the PG polymer must be highly sophisticated in order to allow the cell to make essential modifications while maintaining its integrity.

1.2.2 PG structure and chemical composition

As previously mentioned, PG is composed of subunits consisting of linear polysaccharide chains inter-linked by short peptide substitutions. The polysaccharide chains are composed of alternating units of two sugars, *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac). Figure 2 diagrammatically shows the glycan chains forming a network interconnected by peptide bridges linking Mur*N*Ac residues. It should be emphasized that this represents a simplified version of the structure of PG. Each strand carries a nonreducing 1,6-anhydromuramic acid (anhydroMur*N*Ac) residue and this feature has been used in estimating the average glycan chain length (approximately 33 disaccharides) by comparing the ratio of anhydroMur*N*Ac to total Mur*N*Ac. (14). All linkages between the Glc*N*Ac and Mur*N*Ac sugars are β(1->4). Mur*N*Ac residues each carry a peptide substitution usually composed of L-alanine, D-glutamic acid, *meso*-diaminopimelic acid (DAP), and D-alanine as shown, although a novel substitution involving the replacement of D-alanine with glycine has also been reported (10, 14).

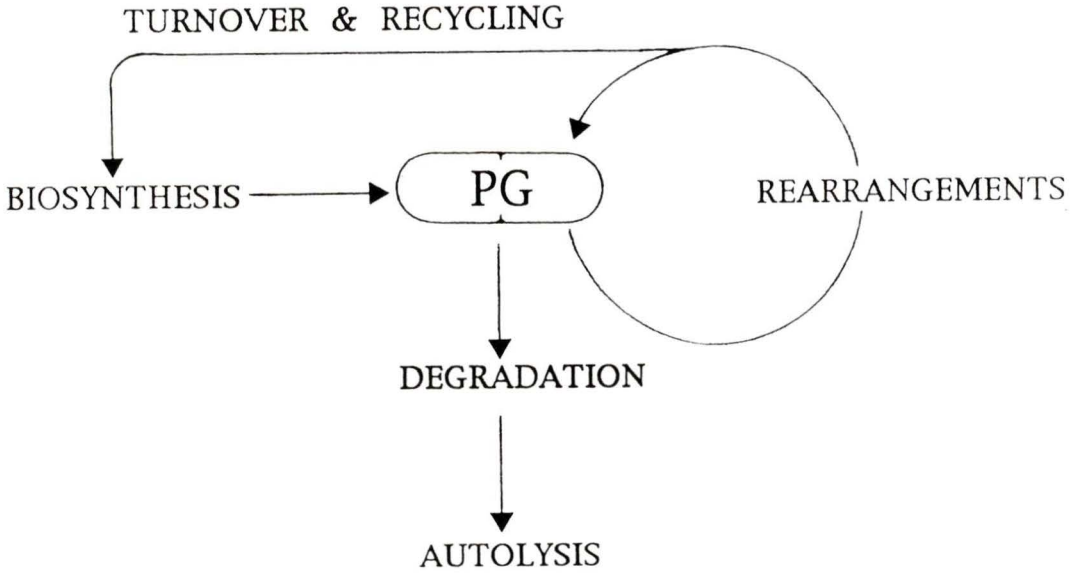


Figure 1. Overview of peptidoglycan metabolism in *E. coli*.

PG is a dynamic structure subject to biosynthesis, modifications and rearrangements, turnover and muropeptide recycling, and hydrolysis leading to cellular autolysis. (Modified from reference 9).

Approximately one-half of the peptide substitutions in *E. coli* are involved in crosslinking adjacent glycan strands. The number of peptide cross-bridges varies with the growth state of the cell, with newly incorporated peptidoglycan approximately two-thirds less crosslinked than existing PG (10, 15, 16). A typical peptide bridge occurs between the D-alanine of one strand and the *meso*-DAP of another, but bonds between two *meso*-DAP residues of neighboring strands are also found.

HPLC analysis has shown that crosslinks between glycan strands can involve tetrapeptide-tetrapeptide, tetrapeptide-tripeptide, and tetrapeptide-pentapeptide subunits (10, 11, 14). In addition, approximately 10% of the peptides are covalently linked though the meso-DAP residue to PG lipoprotein in the outer membrane (10), with newly incorporated PG less covalently bound to PG lipoprotein than mature PG (14).

The glycan chains in the sacculus are arranged mainly in parallel rather than randomly (9, 17). Since the average length of a glycan chain is 30-33 subunits, which is about 30 nm, approximately 80 chains arranged in parallel to each other and perpendicular to the long axis of the cell would be required to cover the circumference of the cell (4).

The earlier model of PG as a single monolayer encompassing the cell is no longer sufficient to explain more recent evidence obtained from PG structural studies. This model evolved from earlier electron microscopic studies in which dehydration of the relatively loose and strongly hydrated PG, apparently caused by ethanol used in the procedure, led to a smaller appearance of the PG than what is the case in the actual cell. More recent electron microscopic observations based on less disruptive cryoscopic

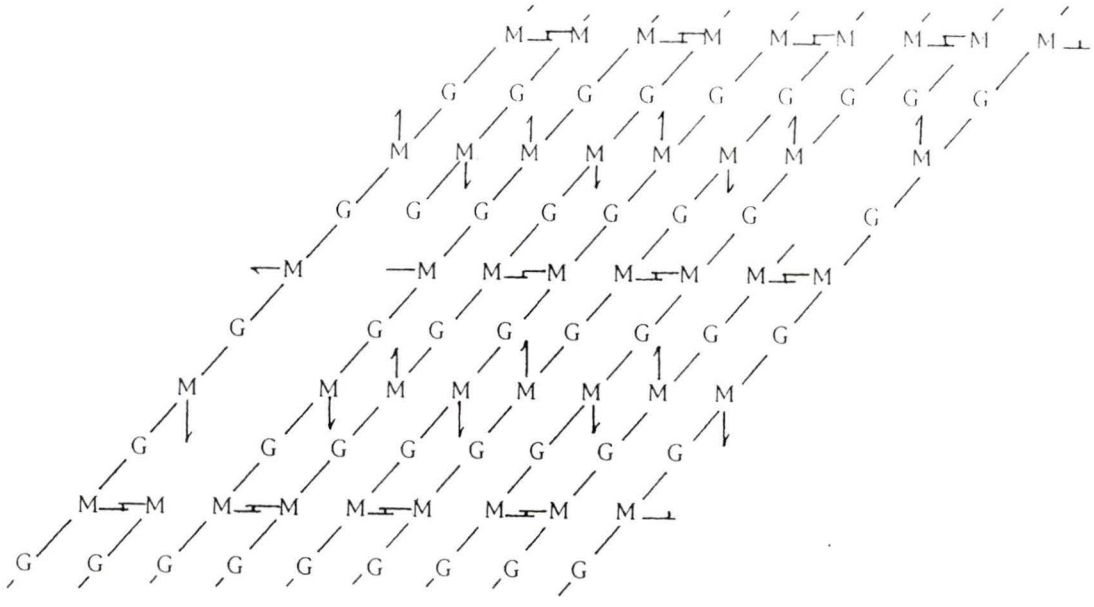


Figure 2. Peptidoglycan structure in *E. coli*.

Linear chains of Glc*N*Ac (G) and Mur*N*Ac (M) run parallel to each other and are crosslinked though peptide side chains on M to form a network. The lines connecting G and M represent glycosidic bonds (—). Peptide side chains are represented by →. Peptide crossbridges between M residues are represented by ⊥⊤.

techniques such as low-temperature embedding and freeze substitution have shown instead that the periplasm is filled with PG (18). What was originally thought to be a monomolecular film is now proposed to be a multilayered, highly hydrated gel-like material occupying the entire periplasm. This gel-like substance is thought to be composed mainly of periplasmic proteins, polysaccharides and several layers of highly hydrated PG, with an estimated thickness of approximately 7-10 nm (18). Viscosity studies support this gel-like concept of PG, since the rate of lateral diffusion of a periplasmic protein is 1000-fold lower in the periplasm than in free aqueous medium (19). The degree to which PG is involved in the viscosity of the periplasm is undetermined.

Indirect support for a multilayered structure of PG has been found in the results of studies employing digestion of PG by muramidases, which were used to degrade PG into muropeptides for subsequent chemical analysis (20). The release of trimeric and tetrameric subunits from the digested PG indicated that three or four glycan chains must be linked together in more than one geometric plane; such results cannot be accounted for by a completely monolayered PG structure (21).

Direct evidence from neutron small-angle scattering studies supports a 75-80% single-layered (thickness of 2.5 nm) and 20-25% triple-layered (thickness of about 7.5 nm) structure of PG in exponentially growing cells (22). This technique uses neutrons as the primary exciting beam to measure the thickness and scattering density distribution across the deuterium-labeled sacculi under fully hydrated conditions. Labischinski *et al.* (22) proposed that if the distribution of the single- and triple-layered structures

occurs in two distinct regions of the sacculus, single-layered PG may be found in the cylinder and triple-layered PG at the polar caps. Alternately, growth zones consisting of multilayered regions across some areas of the cylinder may exist (22).

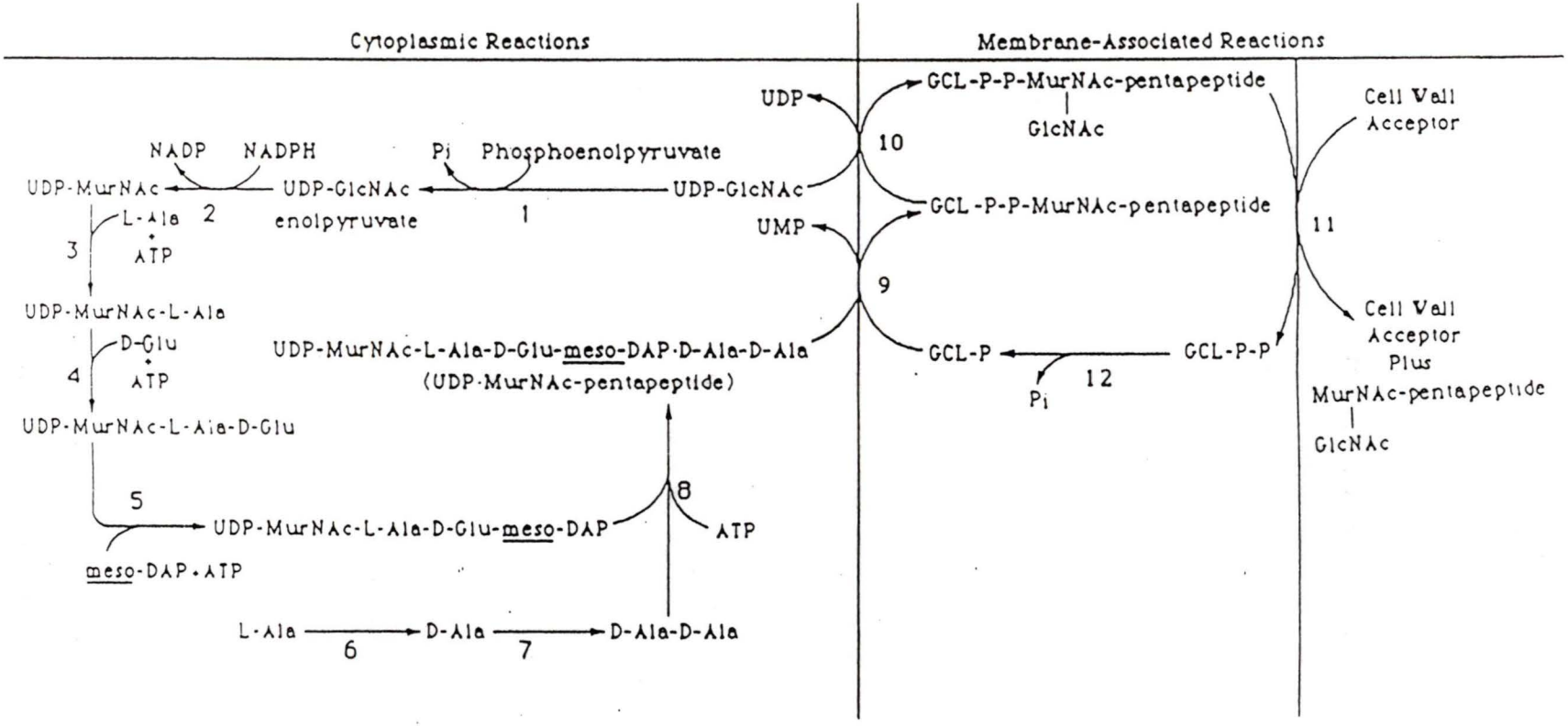
1.2.3 PG biosynthetic pathway

Research involved in elucidating the steps involved in the synthesis of PG has included a variety of genetic and physiological studies. The biosynthetic pathway of PG occurs in three main steps, each of which takes place in a separate compartment of the cell, including the cytoplasm, the cytoplasmic face of the membrane, and the periplasmic face of the cytoplasmic membrane (10). An overview of the steps involved in PG biosynthesis in *E. coli* is presented in Figure 3.

In the first step, cytoplasmic enzymes synthesize the two nucleotide-activated PG precursors, uridine diphosphate UDP-Glc*N*Ac and UDP-Mur*N*Ac-pentapeptide. The formation of UDP-Mur*N*Ac-pentapeptide involves condensation of UDP-Glc*N*Ac and phosphoenolpyruvate followed by the sequential addition of L-alanine, D-glutamic acid, and *meso*-DAP creating Mur*N*Ac-tripeptide. A D-alanyl-D-alanine dipeptide, produced from two L-alanine residues by a racemase and a ligase, is added to the tripeptide to form Mur*N*Ac-pentapeptide. The formation of the tetrapeptide subunit of UDP-Mur*N*Ac-pentapeptide is catalyzed by specific amino acid ligases and involves the hydrolysis of one molecule of ATP for each amino acid added (4).

Figure 3. Peptidoglycan biosynthesis in *E. coli*.

The three main stages of PG biosynthesis are compartmentalized into reactions occurring in the cytoplasm, on the face of cytoplasmic membrane and on the periplasmic membrane face. The enzymes catalyzing the biosynthetic reactions are numbered from 1 to 12 as follows: 1, phosphoenolpyruvate:UDP-Glc*N*Ac-pyrophosphorylase; 2, UDP-Glc*N*Ac-enolpyruvate reductase; 3, L-ala adding enzyme; 4, D-glu adding enzyme; 5, meso-DAP adding enzyme; 6, ala racemase; 7, D-ala-D-ala synthetase; 8, D-ala-D-ala adding enzyme; 9, UDP-Mur*N*Ac-pentapeptide translocase; 10, UDP-Glc*N*Ac translocase; 11, peptidoglycan translocase and/or transpeptidase (PBPs); 12, undecaprenol pyrophosphate pyrophosphatase. (From reference 23).


Cytoplasmic Reactions
Membrane-Associated Reactions
UDP-GlcNAc
enolpyruvate
NADP
NADPH
Pi
Phosphoenolpyruvate
UDP-GlcNAc
UDP-MurNAc
L-Ala
ATP
UDP-MurNAc-L-Ala
D-Glu
ATP
UDP-MurNAc-L-Ala-D-Glu
meso-DAP + ATP
UDP-MurNAc-L-Ala-D-Glu-meso-DAP
ATP
UDP-MurNAc-L-Ala-D-Glu-meso-DAP-D-Ala-D-Ala
(UDP-MurNAc-pentapeptide)
L-Ala
D-Ala
D-Ala-D-Ala
UDP
UMP
GCL-P-P-MurNAc-pentapeptide
GlcNAc
GCL-P-P-MurNAc-pentapeptide
GCL-P
GCL-P-P
Pi
Cell Wall Acceptor
Cell Wall Acceptor Plus MurNAc-pentapeptide
GlcNAc

The second biosynthetic step involves the transfer of the UDP-activated precursors to a lipid carrier, called undecaprenol pyrophosphate, which is embedded in the cytoplasmic membrane. The UDP-activated precursors are assembled into the disaccharide-pentapeptide repeat unit of PG on the lipid carrier and translocated from the cytoplasmic face to the periplasmic face of the cytoplasmic membrane.

The third step of PG synthesis occurs in the periplasm and involves the translocation of the repeat unit from the lipid carrier to an acceptor site in the PG of the growing sacculus by membrane-bound enzymes known as penicillin binding proteins (PBPs). In *E. coli*, there are at least two distinct modes for insertion of newly polymerized PG into the existing sacculus, one for elongation and another for septation (9, 24). The polymerization reactions directly involve several proteins, including the PBPs (25). The roles of the PBPs and their involvement in PG biosynthesis will be discussed in more detail in the next subsection of this thesis.

After releasing the disaccharide-pentapeptide unit, the lipid carrier is recycled for reuse in further PG biosynthetic reactions, or for use in the synthesis of other heterologous polysaccharides (4). Due to the limited amount of this lipid in the cell, the recycling of the lipid carrier is an important step in the transport of molecules with hydrophilic glycan chains across the cytoplasmic membrane. Regeneration of the lipid carrier involves the activity of a membrane bound pyrophosphatase to complete the cycle (4).

1.2.4 Enzymes involved in PG biosynthesis and metabolism

(i) Penicillin binding proteins

As mentioned, PBPs are enzymes which are involved in the polymerization of PG via insertion of nascent PG repeat units into the sacculus (10). These proteins have specific roles in the processes of cell division and elongation, and in the maintenance of cell shape (26). PBPs were named for their ability to covalently bind the antibiotic penicillin. The D-alanyl-D-alanine bond of the pentapeptide in PG is a structural analogue of the β-lactam bond found in β-lactam antibiotics such as penicillin and its derivatives. Thus, penicillin competes with PG to acylate the active site of the PBPs (10). PBPs are irreversibly inactivated by penicillin though the formation of a stable penicilloylated intermediate (10).

In *E. coli*, seven PBPs have been characterized and the genes encoding them have been mapped, cloned and sequenced (27, 28, 29, 30). Studies based on the characterization of mutants and the effects of β-lactam antibiotics on viability and cellular morphology have aided in elucidating the functions of these proteins (10). The seven PBPs have been subdivided into two classes based on molecular weight (MW), a division which also conveniently separates them according to function. The high MW PBPs, designated PBP1A, PBP1B, PBP2 and PBP3 are involved in PG biosynthesis. The functions of the low MW PBPs, designated PBP4, PBP5, and PBP6 are uncertain; they appear to be primarily involved in PG hydrolysis. As well, there is evidence for the existence of at least one additional member PBP7, but little is known about this protein at present except that it is involved in

the synthesis of autolysis-resistant PG characteristic of non-growing cells, a subject to be further discussed in a later section of this thesis (4, 31).

PBPs 1A and 1B are bifunctional enzymes which are involved in the polymerization of cylindrical PG during the elongation stage of the cell cycle. Two processes in which these enzymes are involved are glycosylation, leading to elongation of the glycan strand, and transpeptidation, resulting in the crosslinking of adjacent amino acid side chains. Both enzymatic activities are required in order to get incorporation of the disaccharide-pentapeptide units into the sacculus as determined by *in vitro* studies using the antibiotics moenomycin to inhibit the glycosylation reaction and penicillin to inhibit the transpeptidation reaction (32, 33). In addition, it has recently been shown that PBP1B is also involved in the initiation of septation (34, 35).

PBP2 has a transpeptidase activity which functions in synthesizing cylindrical PG (33). Along with another membrane-bound protein called RodA, it is involved in maintaining the rod shape of the cell during elongation.

PBP3 is involved in PG synthesis and remodeling, and it is essential in the formation of septal PG (9, 26). PBP3 was originally thought to be a bifunctional enzyme, with both transglycosylase and transpeptidase activities, but recently the presence of the transglycosylase activity has been questioned by Spratt *et al.* who claim that it does not exist based on studies using PBP3 mutants (26). Consistent with this claim, a recent study by van Heijenoort *et al.* has found no transglycosylase activity *in vitro* in PBP3 using disaccharide-pentapeptide and -tripeptide as substrates (35). Instead,

based on evidence that PBP1B is involved in septation, van Heijenoort *et al.* speculate that the transglycosylase activity of PBP1B is coupled to the transpeptidase activity of PBP3 (35). In addition, a penicillin-insensitive transglycosylase has been isolated which is able to polymerize uncrosslinked PG (36), but its role *in vivo* is still in question.

It has recently been proposed that during the cell cycle of *E. coli*, the alternating processes of cell elongation and septation depend on a regularly shifting balance between the activities of two competing morphogenetic systems (37). During growth by elongation, transpeptidation would be carried out preferentially with pentapeptide side chains by the PBP2 and RodA-dependent system. During septum formation, tripeptide subunits would be the preferred acceptors for transpeptidation carried out by PBP3 and other proteins (37, 38). Although still speculative, recent studies have indicated that the balance between the PBP2-dependent and PBP3-dependent systems, thus the switch from cell elongation to cell division, may involve a change in the relative availabilities of the different peptide side chains on the disaccharide-peptide repeat unit of newly synthesized PG (37, 39). The switch into and out of cell division is governed by a complex control system which is not yet fully understood, but appears to involve the coordinate action of both the PBPs and PG hydrolases.

Recent evidence suggests that a penicillin-insensitive PG synthesizing system is involved in the initiation of division, acting prior to the PBP3-dependent system (9), but the functional significance *in vivo* has not yet been determined.

Since PBPs 4, 5 and 6 are usually considered to be involved in the hydrolysis of PG, they will be discussed further in the following section on PG hydrolases.

(ii) PG hydrolases

The PG hydrolases are a diverse group of enzymes which cleave covalent bonds in the high MW PG comprising the intact sacculus, or in PG muropeptides which have been previously acted upon by muramidases (10). In *E. coli*, a total of nine PG hydrolases have been described thus far, to which at least eight distinct hydrolytic activities may be attributed. These activities fall into one of two categories of enzymes, either glucosidases or peptidases. The glucosidases are involved in cleaving interglycan linkages whereas the peptidases cleave either interpeptide or glycan-peptide bonds. The PG hydrolases and their cleavage sites are summarized in Figure 4.

Two distinct types of glucosidases have been described, the lytic transglycosylases of which there are two, and a β-*N*-acetylglucosaminidase. The lytic transglycosylases are lysozyme-like enzymes which cleave the β-1,4 glycosidic bond between Mur*N*Ac and Glc*N*Ac (Figure 4, site 1), and catalyze an intramolecular transglycosylation which results in the formation of 1,6-anhydromuramic acid (10, 11, 40). The 1,6-anhydro bond retains a portion of the chemical energy from the cleaved β-1,4-gycosidic bond, and it has been suggested that this energy may be used in reactions involving rearrangements of the sacculus or in its repair (11). One lytic transglycosylase is a soluble cytoplasmic protein, the product of the *slt* gene, while the other is membrane bound and encoded by the *mlt* gene. The

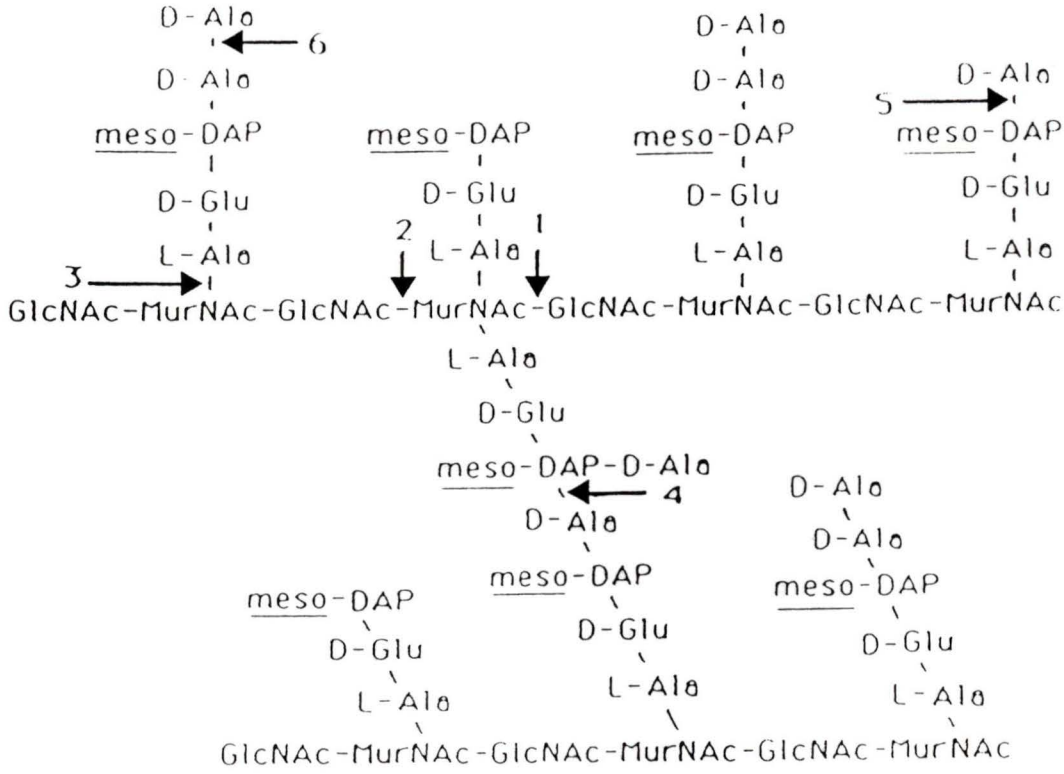


Figure 4. Cleavage sites of the peptidoglycan hydrolases in *E. coli*.

The hydrolases are numbered from 1 to 6 as follows: 1, lytic transglycosylases S and M; 2, β-*N*-acetylglucosaminidase; 3, *N*-acetylmuramyl-L-alanine amidase; 4, PBP4 endopeptidase and penicillin-insensitive endopeptidase; 5, L,D-carboxypeptidase (carboxypeptidase II); 6, D,D-carboxypeptidases (PBP4 carboxypeptidase IB activity and PBP 5, and possibly PBP6, carboxypeptidase IA activity). (Modified from reference 23).

β-*N* acetylglucosaminidase (Figure 4, site 2) acts only on PG fragments rather than on the intact sacculus (41), thus it has been implicated in the process of PG muropeptide recycling.

The peptidases include a group of enzymes with amidase, carboxypeptidase and endopeptidase activities which cleave peptide bonds in PG. *N*-acetylmuramyl-L-alanine amidase acts on the bond which connects Mur*N*Ac and the first peptide of the side chain, L-alanine (Figure 4, site 3). Like the β-*N*-acetylglucosaminidase, this enzyme is thought to be involved in the recycling of PG precursor, as it accepts only muropeptide subunits as substrate rather than intact sacculi (11).

The low MW PBPs are generally believed to be D,D-carboxypeptidases which cleave the terminal D-alanyl-D-alanine bond of the pentapeptide side chain in newly synthesized PG. Cleavage of this peptide bond results in an intermediate which reacts with H_2O to release a product which is one D-alanine shorter that the original substrate. PBP5 has D-alanine carboxypeptidase IA activity (Figure 4, site 6) which converts the pentapeptide side chains of uncrosslinked PG to tetrapeptides by removing the terminal D-alanine (37, 42). The proposed biological role for PBP5 is the regulation of peptide crosslinkages in PG (42). It has also been suggested that carboxypeptidase IA is involved in the control of the level of pentapeptide side chain in the growth zone of the cell and in the switch between cell elongation and division (37). It has generally been believed that PBP6 fulfills a function comparable to PBP5, as described above, but recent evidence suggests that the roles of these two enzymes may be distinct. In contrast to previously reported findings (43),

van der Linden *et al.* were not able to detect D,D-carboxypeptidase activity for PBP6 (44). Instead, they suggest that PBP6 may be involved specifically in the stabilization of PG during the stationary phase of growth (44). Observations which support this proposal include a significant increase in expression level of PBP6 in stationary-phase cells compared with exponentially growing cells (45), and stabilization of PG in non-growing cells by increased levels of DAP-DAP crosslinkages (46).

PBP4 has a biological role distinct from that of carboxypeptidase IA and its activity is referred to as carboxypeptidase IB (11, 47). It is a transpeptidase, linking adjacent strands though peptide bridges (Figure 4, site 6), and it appears to be involved in the maturation of PG (48). This process involves a conversion of "new" PG to "old" PG though a secondary transpeptidation reaction with pentapeptide side chains serving as donors, and results in an increase in PG crosslinkages (10). In addition to having carboxypeptidase activity, PBP4 also functions as an endopeptidase.

An enzyme referred to as carboxypeptidase II exhibits L,D-carboxypeptidase activity, cleaving the peptide bond between *meso*-DAP and the terminal D-alanine (Figure 4, site 5) thereby creating a tripeptide side chain from the tetrapeptide side chains of the disaccharide-repeat unit (38). No gene encoding this enzyme has yet been identified.

Two distinct D,D-endopeptidases exist in *E. coli* , both of which cleave the D,D-alanine-DAP bond in PG (Figure 4, site 4). This bond is a major stabilizing force in the sacculus since it crosslinks two peptide side chains from adjacent repeat units, and it is also the target of β-lactam antibiotics. The endopeptidase activity of PBP4 is membrane-bound and

penicillin-sensitive, whereas the activity of the second endopeptidase is penicillin-insensitive, located in the periplasmic space and thought to be involved in septation (11).

In addition to the above enzymes, it has been proposed that two additional peptidases likely are present in *E. coli*. The existence of a D-glutamyl-*meso*-DAP peptidase which cleaves the bond between D-glutamic acid and *meso*-DAP would explain the isolation of DAP-D-alanine dipeptides from the periplasm (12). As well, the presence of L,D-cross links between two DAP residues in PG has led to the speculation that a specific L,D-endopeptidase exists (11). No additional supporting data has been reported to confirm the presence of these hypothetical enzymes.

In summary, the PG hydrolases are thought to have an essential role in the normal growth and turnover of the sacculus, since expansion of the cell requires that covalent bonds within the existing PG layer be cleaved to allow insertion of newly synthesized material and release of aged material. PG hydrolase activity has been implicated in a number of diverse cellular processes including cell elongation and division (11, 37), PG maturation (11, 48, 49), PG turnover and muropeptide recycling (11, 12, 50) and autolysis (11, 51), but the control mechanism(s) regulating PG hydrolase involvement remain(s) undetermined. Minor irregularities in hydrolase regulation could easily lead to changes in cell shape, whereas more significant deviations could lead to unbalanced growth and eventual lysis of the cell (10). Therefore, the regulation of PG hydrolases must allow continuous and controlled activity, providing sufficient hydrolysis to enable cell growth and division to occur but preventing autolysis which could result from

weakening the stress-bearing PG matrix. Cellular autolysis will be discussed in the following section.

The hydrolases must not only function coordinately, but they must act in concert with the synthetic enzymes involved in PG metabolism during elongation, aging and division. Since a large number of enzymes are involved in PG metabolism and cellular morphogenesis, maintenance of cell shape and integrity require delicately balanced and precisely coordinated action.

1.2.5 Cellular autolysis

Control of the lytic system of *E. coli* appears to be more efficient than that found in some Gram positive bacteria, such as *Bacillus subtilis* which lyse under non-optimal growth conditions, and *Streptococcus pneumoniae* which undergo spontaneous lysis upon entry into stationary growth phase (10). It has been suggested that partial culture lysis in the latter case serves to provide nutrients for growth of the surviving cells (11). In addition, some advantage may be conferred to the survivors by the opportunity for genetic transfer of DNA released by cell lysis (11). In general, normal *E. coli* cells do not lyse spontaneously at a high rate, and some kind of induction is required to cause autolysis (52). One of the main steps in the autolytic process is the degradation of PG by specific endogenous hydrolases (52). Of the PG hydrolases previously described in this paper, only the two endopeptidases and the two lytic transglycosylases may be considered strictly autolytic in *E. coli* (52).

An oversimplified but useful model of cell growth proposed by Weidel and Peltzer (7) describes the biosynthesis of PG as a tightly controlled balance between PG hydrolyzing and PG synthesizing enzymes. Inhibition of PG synthesis during growth causes depletion of precursors of the subsequent steps and leads to rapid arrest of the polymerization reactions. An unbalanced state is created in which synthesis is blocked but the hydrolases continue to cleave PG linkages unabated. The result is cell lysis. Under such circumstances, autolysis is not caused by the specific induction of PG hydrolases, but it results from a delay in or a lack of appropriate hydrolase control mechanisms (52). Consistent with this model, temperature sensitive (ts) mutants with defects in certain PG biosynthetic enzymes display a lysis phenotype when grown at their restrictive temperatures (26, 53-60).

Autolysis of *E. coli* can be induced under many conditions, including: (i) specific inhibition of PG synthesis, by deprivation of an essential component necessary for PG synthesis, for example DAP, or by treatment with certain antibiotics which can be used to block synthesis at a number of different steps, and (ii) exposure to certain non-specific chemical treatments. Although lysis results under both conditions mentioned, the nature of the autolytic process differs (11).

Autolysis triggered by specific inhibition of PG synthesis is apparently under topological restriction, shown by studies in which only enzyme located in the growth zone is deregulated and only biosynthetically new cell wall enzymes induce autolysis (11, 61). A wide variety of antibiotics inhibit PG synthesis, and regardless of the specific site of action

of the drugs, the mechanism of autolysis is apparently the same, *i.e.*, deregulation of the PG hydrolases from the synthetases. The rate of autolysis induced by antibiotics is strictly proportional to the rate of bacterial growth (62).

In contrast to autolysis caused by specific inhibition of PG synthesis, that triggered by chemical treatment, for example exposure of cells to EDTA, sucrose or NaCl, is less specific and involves interference with an inhibitory membrane environment. It has been suggested that some sort of delicate barrier exists between PG and its degradative hydrolases which prevents uncontrolled PG hydrolysis (63), and it has been shown that maintenance of this barrier depends on the presence of divalent cations such as magnesium, and on the preservation of the integrity of the cell envelope (63). Whether the barrier is due to an inhibitor or to topological inaccessibility, disruption of the barrier by alteration of the membrane environment leads to disturbances in autolytic control.

The mechanism involved in lysis by bacteriophage infection is not completely understood but induction of lysis involves specific changes in the cytoplasmic membrane so that either the host's autolytic enzymes or phage-encoded PG hydrolases gain access to the PG and initiate hydrolysis (10). For example, phage λ has its own lytic enzymes, a transglycosylase (product of the *R* gene) and an endopeptidase (product of the *Rz* gene), which are similar to the PG hydrolases of *E. coli*. These enzymes are jointly referred to as endolysin, and their lytic action depends totally on the presence of a third protein (product of the *S* gene) which causes lesions in the cytoplasmic membrane though which the endolysin can escape to the

periplasm. Cells infected with λS⁻ phage do not lyse but instead accumulate intracellular endolysin past the normal time of lysis (10). In these cells, mechanical disruption of the membrane, such as by addition of chloroform, allows the endolysin access to the PG and results in lysis.

Non-growing *E. coli* cells are resistant to lysis mediated by PG synthetic blocks (11), but not to chemical treatment with the lysis-causing agents previously described (63). Changes in the PG hydrolases and changes in the PG substrate to a less hydrolyzable form (11) are believed to contribute to this lysis resistance. PBP7 appears to be involved in synthesizing the autolysis-resistant PG characteristic of non-growing cells (31). Cells suspended in buffer are totally stable, even though active hydrolases are present (64). These findings support the proposal that the presence of an inhibitor or topological inaccessibility to the PG substrate is involved in preventing uncontrolled hydrolase action (10, 65).

Under normal growth conditions, rapid and sensitive regulatory mechanisms must exist which enable the hydrolases to perform their physiological functions without expressing their potentially suicidal action. These controls have not yet been fully elucidated, but evidence suggests that several distinct factors may be involved (11). Studies have indicated that the regulation of autolytic enzymes occurs mainly at the post-translational level since, except for the membrane-bound hydrolytic enzymes, activities of hydrolases increase exponentially in synchronously growing cultures, even when DNA synthesis is inhibited (66). Post-translational control may involve regulation of the activities of the enzymes, which could occur by restricted export to the periplasm or by topological restriction of enzyme

distribution (11) Alternatively, control may involve specific enzyme activation by substrate modification (31).

1.3 Bacteriophage Lambda and other Lambdoid Phages

1.3.1 Description of the lambdoid family of phages

Bacteriophage lambda (λ) is a member of a large family of similar temperate phages, referred to as the lambdoid family, which share similar genomic organization (67). Members of this family can exchange genetic information, resulting in the creation of new functional lambdoid phages. In fact, the continual interbreeding which contributes to the evolution and diversity of lambdoid phages has made it difficult to define the limits of the family and to determine the origin of the phages. In addition to phage λ, members of the family include *E. coli* phages ϕ80, P21, P434, and phage P22, whose normal host is *Salmonella typhimurium*. In this introduction, I will focus discussion on the lambdoid phages of *E. coli*, with an emphasis on bacteriophage λ.

Upon infection of the host bacterium, phage DNA is injected into the cell and a circular DNA intermediate is formed. At this point in the infection process, all members of the lambdoid family must commit to one of two alternate pathways of replication, either lysis or lysogenization of the host. Lytic growth involves the replication of the circular DNA, synthesis of bacteriophage gene products, packaging to form new phage particles, and release of the progeny virus though lysis of the host cell. Alternatively, during lysogenic growth, phage DNA is inserted into the chromosome of

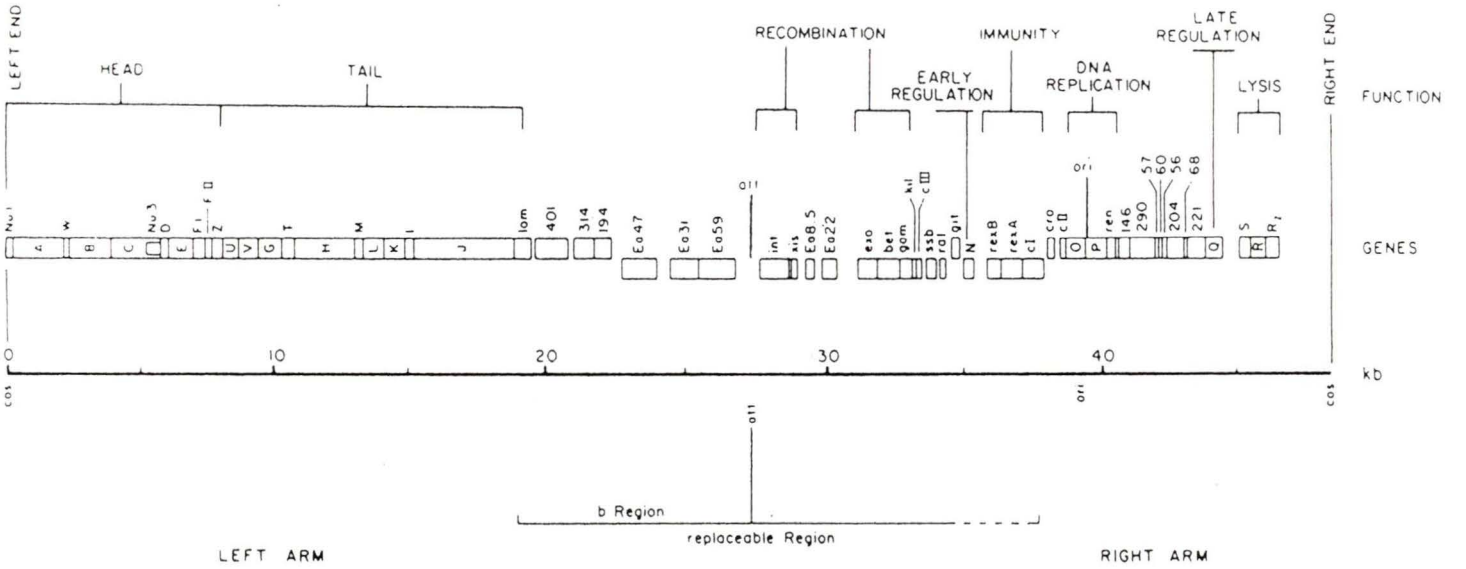
the host cell. The integrated phage DNA, referred to as a prophage, is subsequently replicated and transmitted to progeny bacteria along with the host chromosome (68). Comparison of the complex gene regulatory systems found in lambdoid phages reveals differences in regulatory specificity, but similarities in that all members have repressors, operators, and positive regulators that function in essentially the same manner, although the regulatory elements themselves are not necessarily interchangable among different phages.

All members of the family have a common genetic organization which allows genetic exchanges to take place. The genome is made up of functional units referred to as modules. As depicted in Figure 5, each module is composed of sets of genes encoding specific functions including immunity, recombination, replication, lysis, DNA integration, and phage structural components. Similar mechanisms are involved in the control of module expression in all family members. Although the nucleotide sequence can differ extensively in certain phages, the common genetic organization and similar mode of regulation permit exchange of whole genes, parts of genes, or groups of genes among different phages.

Exchanges between different phages or between phage and host may be accomplished though homologous recombination between regions flanking a gene or group of genes. For example, the attachment (*att*) sites of phage λ contain crossover points for reciprocal site-specific recombination (67). The integrase (*int*) protein of phage λ recognizes sequences in the *att* sites in both the bacterial and phage genomes and catalyzes a breaking and joining event that leads to insertion of viral DNA into the host

Figure 5. Modular arrangement of the phage lambda genome.

The genome of phage λ is organized into functional units called modules. Each module is composed of sets of genes encoding specific functions including: phage structural components (head and tail), DNA recombination, gene regulation, immunity, DNA replication, and host lysis. (Modified from references 68, 69 and 70)


LEFT END
HEAD
TAIL
RECOMBINATION
IMMUNITY
LATE REGULATION
EARLY REGULATION
DNA REPLICATION
LYSIS
RIGHT END
FUNCTION
GENES
kb
0
10
20
30
40
cos
cos
b Region
replaceable Region
LEFT ARM
RIGHT ARM

chromosome (68). It has been proposed that phage λ first acquired *att* and *int* by the insertion of a transposon into an ancestral virus (67). Since many of these transposon-like sequences exist in lambdoid phages, it is likely that λ evolution has included modules derived from various transposon insertions.

In addition to homologous recombinational events, illegitimate recombination, *i.e.*, recombination between non-homologous DNA sequences, may also partially account for the exchange of functional units leading to diversity in lambdoid phages. For example, functional phages may arise by illegitimate recombination between an infecting phage and a defective prophage in the host chromosome (67). As well, exchanges of non-homologous segments of DNA may be facilitated by the pattern of DNA homology interspersed with non-homology found in lambdoid phages, *i.e.*, exchange of regions of non-homology could occur via crossovers in the regions of homology. Interspersion of homologous DNA with non-homologous DNA is thought to have arisen by a combination of several factors. These include mutational divergence within modules, recombinational rearrangements between the mutated modules, recruitment of functionally equivalent modules from the host or from other viruses and replacement of λ modules with those newly aquired (67).

The origin of λ is uncertain but may involve a primordial lambdoid phage, as proposed by Campbell and Botstein (67). The suggestion that viral genomes may have originated at least in part from associations with their hosts is supported by similarities between certain lambdoid phage genes and host genes, such as chromosome replication origins (67). In

addition, the presence of cryptic λ-related DNA sequences in laboratory strains of *E. coli* and other enterobacterial species has been detected (71). The origins of these λ-like sequences are not known but they are generally thought to be incomplete lambdoid prophages. The cryptic sequences, which are comprised of modules, are able to recombine into the λ genome, functionally replacing defective viral modules. The cryptic λ-related DNA sequences will be discussed further in the following section.

In summary, the diversity observed among lambdoid phages including bacteriophage λ, amid a common modular organization supports the involvement of a common primordial lambdoid ancestor and/or continuing evolution of the phages though the recruitment, diversification, and exchange of functional segments (67).

1.3.2 λ-related cryptic prophages

The genomes of laboratory strains of *E. coli* harbor at least four sequences related but not identical to those of phage λ, which apparently are segments of lambdoid phages other than λ. The sequences have been detected by DNA hybridization with a λ DNA probe (71, 72). Some of the segments can recombine with an infecting λ phage to generate recombinants in which certain genes have been replaced by functionally homologous DNA. These endogenous incomplete prophages are referred to as Rac, Qsr', Qin and λcry (73). The sequences carried by the incomplete prophages are apparently stable and therefore may significantly affect the evolutionary potential of *E. coli* and of its phages (73).

How these sequences were created is unknown, but it is suspected that they represent defective integrated prophages. Defective prophages are incomplete phages, *i.e.*, lacking DNA sequences required to form a functional phage, which have recombined into the bacterial chromosome. They can be created from complete prophages by degenerate changes such as point mutations or deletions, aberrant excision and reinsertion, or complex interactions with insertion-sequence elements (67). Genes of these incomplete prophages are usually unexpressed, but can be induced by specific mutations or conditions, endowing the host cell with novel or supplementary functions (69, 73), and contributing to the bacterial phenotype.

Phage λ can integrate into the cryptic lambdoid prophages, and this has been observed when *E. coli* cells are lysogenized with a λ derivative deleted of its attachment site (74, 75). These integration events usually depend on homology between the DNA sequences in λ and the cryptic prophages (69).

Of the 4 known cryptic prophages, Rac has been the most thoroughly characterized. It is located at 29.6 to 30.1 minutes on the *E. coli* strain K-12 chromosome and has an estimated length of 26.5 kb (76). Rac is thought to be derived from a typical lambdoid prophage which became defective by deletion of the phage genes encoding lysis and capsid proteins. Rac contains a group of genes which can replace λ genes involved in the functions of integration, recombination, immunity and replication. Recombination between λ and homologous sequences in Rac results in the creation of a functional hybrid phage designated λ-reverse (73).

Qsr' is located at 12.5 minutes on the *E. coli* chromosome and carries λ-homologous sequences and genes that can replace the *Q-S-R* region of λ which is involved in host cell lysis. As mentioned previously, the *R* gene product is a transglycosylase which degrades PG by attacking the glycosidic linkages and the *Rz* gene may have endopeptidase activity involved in cleaving peptide crosslinks and/or bonds between PG and the outer membrane (77, 78); together, the *R* and *Rz* gene products constitute the enzymatic activity referred to as endolysin. The *S* gene encodes a hole-forming protein required for transport of endolysin across the cyoplasmic membrane to the periplasm. λ-mediated lysis of the host cell to release progeny phage particles requires the activities of all three of the lysis genes. The *Q* gene encodes an anti-termination protein involved in the transcriptional control of several genes including the *SRRz* lysis gene cluster (68).

Like Qsr', the Qin defective prophage carries λ-like sequences which can substitute for λ's *Q-S-R* region, however, the sequences in Qin differ from those in Qsr' and have different anti-termination specificities (79). Qin has been mapped at 34.2 to 34.6 minutes on the *E. coli* chromosome.

The most recently described incomplete prophage, λcryptic (λcry), is found in cells lysogenic for λ that have been induced with ultraviolet light. These cryptic λ lysogens carry most of the phage genes involved in DNA packaging, head and tail components and assembly, but lack the major promoters and the immunity, replication and recombination genes. λcry is located at 17 minutes on the *E. coli* chromosome.

As mentioned, it is not clear how or why the cryptic prophages have arisen, but two suggestions have been proposed. Strathern and Herskowitz (80) suggest that they serve no particular purpose and therefore may be regarded as "genetic debris" which resulted from previous bacteriophage infections and which will gradually be lost to the cell over time. Alternatively, Campbell and Bolstein (67) consider the sequences to be virus modules that have been co-opted by the host cell presumably for some advantageous purpose.

1.3.3 Bacteriophage λ as a DNA cloning vector

The grouping of λ DNA into discrete blocks of related genes has allowed construction of phage-derived cloning vectors containing deletions in large stretches of DNA non-essential to lytic growth. Approximately one third of the central region of λ DNA is dispensible for lytic growth (indicated in Figure 5 as "replaceable region"). λ-derived DNA cloning vectors commonly contain restriction sites that flank some or all of these dispensible genes. The major advantage of using λ-derived cloning vectors is that DNA can be inserted and packaged into phages *in vitro* (81). Another aspect of these vectors is that they represent single-copy elements, a disadvantage in terms of quantity of recovered recombinant DNA but an advantage in the cloning of genes which are unstable or lethal to the cell when expressed in multiple copies.

In addition to modified phage vectors, λ-derived plasmid vectors called phasmids have also been constructed for use in DNA cloning. These hybrids of phage and plasmid combine desirable properties of components

such as replicators, antibiotic resistance markers, unique restriction enzyme sites for cloning, and blue/white insert selection of recombinants. A cloning vector used to generate the *murH* and *lytG* clones referred to in this thesis is λSE6, a phasmid which was constructed by cloning the single-copy replicator (NR1) of plasmid pDPT427 into the phage λ1059 genome (82). This DNA molecule can be maintained lysogenically as an autonomous, single-copy number plasmid, or can be induced to replicate lytically under the control of the λ lytic cycle. The vector carries both ampicillin and kanamycin resistance markers, but cloning of DNA fragments into the vector results in the replacement of a 16 kb fragment containing the genes encoding ampicillin resistance and a protein required for lysogeny, the CI repressor. Consequently, genetic complementation assays must be performed in bacteria carrying a copy of the *c*I gene, such as λ lysogens. λSE6 has been used to clone genes in low-copy by complementation and its use in cloning the two loci discussed in this thesis will be addressed further in the following chapters.

1.4 The *murH* Family of Genes

Several distinct genetic loci that have been isolated in this laboratory appear to be functionally related based on their interactions with one another. To date, approximately nine genes in total may be considered members of the *murH* family of genes, based on suppression studies (83, 84, and M.-A. Noble, unpublished results). Figure 6 summarizes the loci involved and the specificity of their interactions with each other. The gene designations and approximate linkage map positions of the loci are as

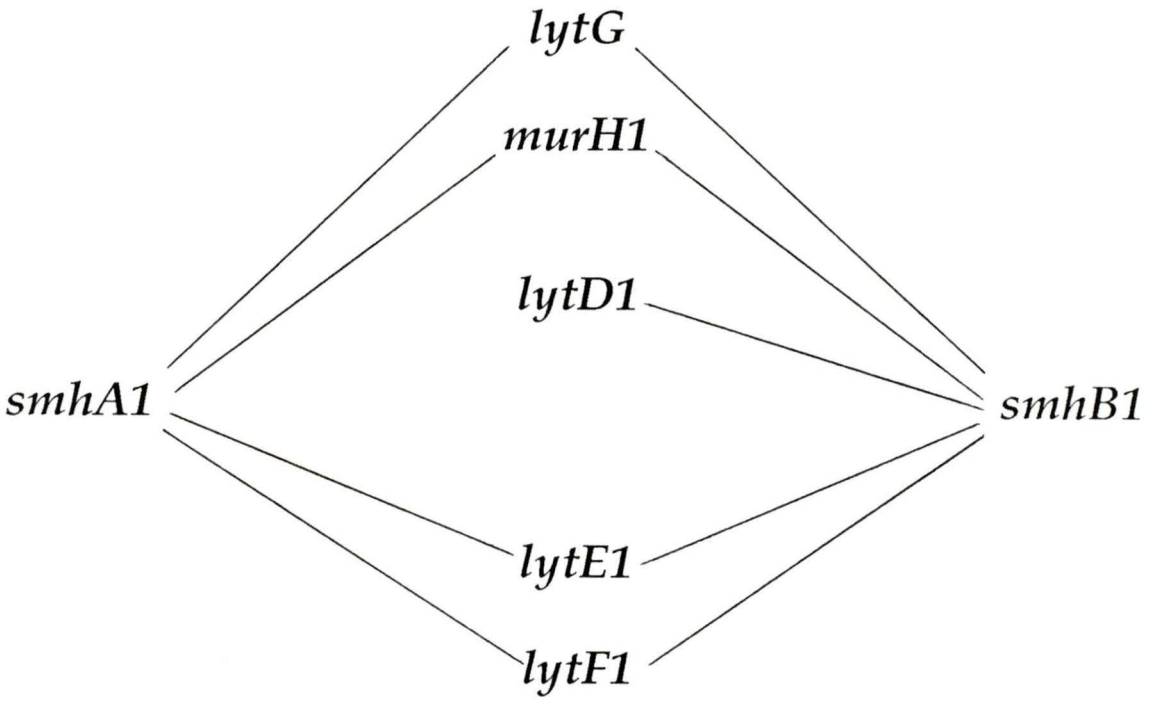


Figure 6. The *murH* family of genes

Proposed relationships between the members of the *murH* gene family based on suppression studies. Lines indicate either direct or indirect interactions between connected alleles. (Modified from reference 23).

follows: *murH1* (99.2 minutes), *smhA1* (24.5 minutes), *smhB1* (12.5 minutes), *lytD1* (12.7 minutes), *lytE1* (25 minutes), *lytF1* (62.4 minutes), and *lytG*(25 minutes). The isolation and characterization of two of these genes, *murH* and *lytG*, will be described in detail in this thesis. The isolation of the remaining genes and the proposed interactions between them will not be discussed further. It should be noted that the origin of the genes in this family and the functions of the products encoded by them are not yet known, although strains carrying a mutation in one of a number of these genes exhibit a ts lysis phenotype, *i.e.*, temperature-sensitive growth and lysis at the restrictive temperature (see Figure 10 as an example of this phenotype) characteristic of PG hydrolase-mediated autolysis. The possibility that at least some of these genes may be derived from cryptic λ-related sequences will be discussed.

1.5 Purpose of this Thesis

As discussed previously, many of the enzymes involved in PG biosynthesis have been identified and characterized, and the genes encoding them mapped on the *E. coli* chromosome. The purpose of my research was to contribute to the knowledge of the scheme of PG metabolism by attempting to clone and sequence two distinct but apparently functionally related genetic loci, mutations in which lead to phenotypes characteristic of PG hydrolase-mediated lysis, thus, indicating a potential involvement of the gene products in PG metabolism.

One locus, previously identified as *murH* and mapped to 99.2 minutes on the *E. coli* chromosome by D. Dai, encodes a gene product

originally thought to be involved in the last stage of PG synthesis (85), although subsequent work has rendered this unlikely. The designation of *murH* is a mnemonic for *mur*ein metabolism, locus *H*, in accordance with current *E. coli* genetic terminology; "murein" is synonymous with "PG". The *murH* gene was initially cloned in the single-copy number vector λSE6 (23), but subsequent efforts to subclone the gene in multicopy number vectors resulted in random deletions (presumably within *murH*) and loss of activity as determined by complementation assays (23). Thus, it appeared that *murH* was "toxic" to the cell in multicopy (23). My work has involved further attempts to subclone *murH* and is described in section 3.1. It should be noted that in this thesis "complementation" refers to the restoration of colony-forming ability at the restrictive temperature in a ts lysis mutant which is defective in the gene of interest. It should also be noted that the mechanism of this restoration of activity is not known, *i.e.*, suppression, rather than complementation, must also be considered a possible mechanism.

The other locus, *lytG*, was also initially thought to encode a gene product involved in PG metabolism, based on the ts autolytic phenotype of the *lytG* mutant strain which may involve PG hydrolase-mediated lysis. The *lytG* gene represents a newly identified locus which has not been previously described. This locus was designated *lytG* because of its similar ts lytic phenotype to an expanding group of *lyt* genes identified in this laboratory, some of which are included in the *murH* family of genes outlined in the previous section. The *lytG* locus was also cloned in the λSE6 single-copy

number vector (E. E. Ishiguro, unpublished results), and my attempts to subclone and sequence this locus are described in section 3.2.

As mentioned, the *murH* and *lytG* genes appear to be functionally related based on the finding that they share a common suppressor, *smhA*. A suppressor or second-site mutation is a mutation which occurs either within or outside an original mutant gene and which is able to correct the phenotypic defect of the original mutant (86). Suppressor mutations may be used to determine the functional interactions of a specific gene product with products of other genes, or to establish functional relationships between additional mutations in previously unidentified genes and the original mutant. Based on the common suppression of *lytG* and *murH* by *smhA*, the *lytG* mutation may be considered the most recent addition to the *murH* family of functionally related genes thought to be involved in PG hydrolase-mediated autolysis and currently being studied in this laboratory.

2 MATERIALS AND METHODS

2.1 Bacteria, Plasmids, and Bacteriophages

All bacteria used in this study were derivatives of *E. coli* strain K-12 . The bacterial strains, bacteriophages and plasmids used are listed at the beginning of each subsection in section 3.

2.2 Media and Culture Conditions

Bacteria were cultured in Tryptic Soy Broth (TSB, Difco Laboratories) or Luria Broth (LB, Gibco BRL). Solidified versions of these media, designated TSA and LA respectively, contained 1% agar. In some experiments, 2YT agar was used (1.6% Tryptone, 1.0% Yeast Extract, and 0.5% NaCl). Davis minimal agar (Difco Laboratories) containing appropriate supplements was used in genetic experiments involving auxotrophic markers.

Cultures in liquid media were incubated in gyrotory waterbath shakers at the indicated temperatures, and growth was monitored with a spectrophotometer at a wavelength of 600 nm. Unless otherwise stated, antibiotics were added at the following concentrations when required: tetracycline, 20 μg/ml; ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; spectinomycin, 50 μg/ml; chloramphenicol, 50 μg/ml. When required for blue/white selection, 5-bromo-4 chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added to a final concentration of 40 μg/ml in media used for multicopy number plasmids and 60 μg/ml in media used for single-copy

number plasmids. Addition of isopropyl-β-D-thiogalactopyranoside (IPTG) was at a concentration of .01 mM.

2.3 Maintenance of Bacterial Strains

For long term storage, all strains were prepared from an overnight culture grown at the appropriate temperature with antibiotic selection if applicable. Glycerol was added to a final concentration of 30% v/v, and the suspension was stored in 1.5 ml screw-capped plastic tubes at -70°C. Fresh cultures were prepared by innoculation of TSB (containing antibiotics if appropriate) with the frozen stock followed by overnight incubation at the appropriate temperature.

2.4 Determination of Temperature Sensitivity

The relative degree of temperature sensitivity exhibited by different mutant strains were quantified in terms of colony forming capability by a procedure called efficiency of plating (E.O.P.). Serial dilutions of cultures grown overnight in TSB at 30°C were plated in duplicate on TSA. One set of the plates was incubated at 30°C and the other at 42°C. Plate counts were determined after 18-24 h of incubation. Longer periods of incubation did not change the plate counts. The efficiency of colony formation was expressed as a ratio of the plate count in terms of colony-forming units (CFU) per ml obtained at 42°C to the plate count obtained at 30°C.

Broth lysis testing was used to determine the effect of temperature on growth in liquid medium. Bacteria were grown for 2-3 doublings in TSB at 30°C to a density of about 1×10^8 cells/ml. The culture was then divided

into two equal parts. One was maintained at 30°C as a control and the other was shifted to 42°C and observed for changes in turbidity.

2.5 General Recombinant DNA Techniques

Plasmid DNA was isolated by the alkaline lysis miniprep procedure (87), with the addition of a phenol/choloroform extraction step prior to ethanol precipitation, or by Magic Minipreps™ DNA Purfication System obtained from Promega Corporation. The following modifications for isolation of single or low-copy number plasmids were made to the Magic Minipreps™ protocol in order to concentrate the DNA: (i) 3.0 - 4.5 ml of overnight culture was concentrated by successive centifugations in a 1.5 ml microcentrifuge tube and removal of the supernatant prior to cell resuspension, and (ii) plasmid DNA was eluted in one-half the recommended volume of TE buffer.

DNA restriction endonuclease digestions, fill-in reactions of overhanging single strand DNA ends created from restriction digests, and ligations of either cohesive- or blunt-ended DNA were performed as described in Maniatis *et al.* (68).

Transformations were performed by electroporation (87) using a Gene Pulser® apparatus with Pulse Controller Unit (Bio-Rad Laboratories) and competent cells of the appropriate bacterial strain. Competent cells were prepared for electro-transformation by the following method based on the recommended procedure for high efficiency electro-transformation of *E. coli* in the Bio-Rad Laboratories Bacterial Electro-transformation and Pulse Controller Instruction Manual (version 1.0). A fresh overnight culture

of cells was diluted 1/100 in TSB and grown at the appropriate temperature with shaking to approximately A_{600} = 0.4 - 0.6. The culture was chilled on ice for 20 min and cetrifuged at 4000g for 15 min at 4°C. The pellet was resuspendedin an equal volume of cold sterile distilled H_2O, centrifuged as above, and resuspended in 1/2 the original volume of cold sterile distilled H_2O. The cells were again centrifuged as above and the resulting pellet was resuspended in 1/200 original volume of 10% glycerol. Competent cells were stored at -70°C for up to one month if not used immediately.

Agarose gel electrophoresis of DNA fragments was performed in 1% gels, except as indicated, following the procedure of Maniatis (68) using the TAE buffer system (0.04 M Tris-acetate; 0.002 M EDTA). Gels were stained by soaking in a solution of 0.5 µg/ml ethidium bromide followed by brief emersion in H_20 to destain. DNA was visualized using a UV light box. In preparative DNA procedures, Seaplaque Low Melt Agarose (FMC BioProducts, distributed by Handel Scientific Co. Ltd.) was used in making the gel and the appropriate band was excised from the gel using a razor blade. DNA was extracted from the agarose using a GENECLEAN® Kit (BIO 101 Inc., CA,USA). Purified DNA was stored at -20°C.

Restriction endonucleases, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA ligase, T4 DNA polymerase, and DNA molecular weight and size standards (λ DNA digested with *Hin*dIII, or λ DNA digested with HindIII and ϕX-174 DNA digested with HaeIII (DRIgest™ III) were purchased from Amersham, Pharmacia LKB Biotechnology, New England Biolabs, or Boehinger Mannheim.

Quantitation of purified plasmid DNA was calculated by spectrophotometric measurement (260 nm) of an appropriate dilution of DNA and calculation of the final DNA concentration based on A_{260} (1.0 units) = 50 μg/ml double-stranded DNA.

2.6 Cloning Methods

The fragment to be cloned and the vector were digested with the appropriate restriction enzyme in 5 μl or 10 μl reaction volumes. Agarose gel electrophoresis followed by ethidum bromide staining and visualization by UV light was used to determine that digestion went to completion. The enzymes were heat inactivated for 10-15 min at either 65°C or 85°C and placed on ice immediately. The DNA fragment of interest was purified by preparative gel electrophoresis. Vector DNA was added to insert DNA at a molar ratio of 1:2 or 1:3 and a ligation reaction was set up in a 10μl or 15 μl total volume. Cohesive-end ligations using T4 DNA ligase were incubated at 16°C overnight. Blunt-end ligations were incubated at RT overnight. Ligations were heat inactivated for 10 min at 70°C and placed on ice until used. To 50 μl of DH5α competent cells, 1-2 μl of the ligation mixture was added and the mixture was incubated on ice for 1 min, before the bacteria were transformed by electroporation. Transformation mixtures were plated on LA or 2YT agar containing IPTG, X-gal and appropriate antibiotics and incubated at 37°C. White colonies were picked for plasmid isolation and subsequent analysis by restriction digestion and agarose gel electrophoresis. Plasmids showing relevant restriction patterns were transformed into the appropriate mutant bacterial strain for complementation testing.

2.6.1 Subcloning *murH* activity from pDD41

All procedures involving the subcloning of the *murH* complementing activity from pDD41 were similar to that described above. The 11 kb *Bam*HI fragment containing *murH* complementing activity was isolated and purified from *Bam*HI restriction endonuclease digestion of pDD41 and ligated into the *Bam*HI restriction site of pUC18 (88). Ligation mixtures were transformed in DH5α competent cells by electroporation and white colonies were selected for plasmid purification on LB agar containing X-gal, IPTG and ampicillin. Similarly, the 11 kb *Bam*HI fragment was subcloned from pDD41 into the *Bam*HI site of pOF216 (89) and transformed, and white colonies were selected on LA containing X-gal, IPTG and spectinomycin.

2.6.2 Subcloning *lytG* activity from pUV20

All procedures involving the subcloning of the *lytG* complementing activity from pUV20 were similar to that described in section 2.6. The 3.0 kb or 8.2 kb *Bam*HI fragments were isolated and purified from *Bam*HI restriction endonuclease digestion of pUV20 and ligated into the *Bam*HI site of pUC19. Ligation mixtures were transformed in DH5α competent cells by electroporation and white colonies were selected for plasmid purification on LA containing X-gal, IPTG and ampicillin. Similarly, the 8.2 kb fragment was ligated into the *Bam*HI site of pCL1920 (90) and transformed. White colonies were selected on LB agar containing X-gal, IPTG and spectinomycin. The 8.2 kb fragment was also ligated into the *Bam*HI site of pWSK29 (91) and white colonies were selected on LA containing X-gal, IPTG and ampicillin.

2.7 Southern Blot Analysis

Southern blot analyses were performed with a non-radioactive labeling and detection kit obtained from Boehinger Mannheim. The DNA fragment to be used as a probe was purified by agarose gel electrophoresis. The probe was then labeled with the hapten digoxigenin (DIG). The DNA samples to be probed were digested with the specified restriction endonucleases and subjected to electrophoresis on a 1% agarose gel. The separated fragments were first visualized by staining with ethidium bromide and then transferred to a Zetaprobe™ membrane (Bio-Rad) using 10 X SSC buffer as described by Maniatis (68). The transferred DNA fragments on the membrane were denatured, fixed, dried and prehybridized for 1 h with 10 ml hybridization solution per 100 cm^2 membrane as described by Boehinger Mannheim (92). The membrane was then incubated with 2 ml of hybridization solution containing 1 μg of the DIG-labeled probe. The hybridization was carried out in a sealed plastic bag for 12-18 h at 68°C and the membrane was washed according to the Boehinger Mannheim protocol (92), then incubated with anti-DIG-alkaline phosphatase conjugate. DNA fragments which hybridized to the probe were detected using AMPPD® [3-(2'-Spiro-adamantane)-4-methoxy-4-(3''-phosphorloxy)-phenyl-1,2-dioxetane], a chemiluminescent substrate for alkaline phosphatase (Boehinger Mannheim), by exposure of the membranes to Kodak XK1 X-ray film.

2.8 Probing the *E. coli* Gene Mapping Membrane

A commercially prepared *E. coli* Gene Mapping Membrane was obtained from Takara Shuzo Co., Ltd. (Japan) though Bio/Can Scientific. This nylon membrane contained an ordered array of 476 immobilized phage λ clones. Each phage clone contained a different segment of the *E. coli* chromosome (strain W3110), and the entire collection of clones covered almost the entire genome with significant overlap. The seven gaps in the 100 minute genome, *i.e.*, areas not included in the phage clones, were found at the following approximate locations: 24 min; 36 min; 40.5 min; 72 min; 79.6 min; 90 min; and 96 min (93). Probing of the Gene Mapping Membrane was performed in the same manner as the Southern blot analysis using the non-radioactive DIG labeling and detection kit and the AMPPD® substrate for anti-DIG-alkaline phosphate conjugate from Boehinger Mannheim. Positive results were detected by plaque hybridization to one or more of the 476 overlapping clones immobilized on the membrane. Comparison of the positive clones with the Kohara restriction map of *E. coli* DNA (70, 93) revealed the chromosomal location of the DNA used as a probe.

2.9 DNA Sequencing

DNA sequencing was performed using the dideoxynucleotide chain terminating method of Sanger (94) in the presence of [^{35}S]dATP (10 mCi/ml) from Amersham Canada Ltd., or [^{35}S]dATP (12.5 mCi/ml) from Dupont. M13 forward (-20, 17 base) and reverse (-24, 16 base) sequencing primers (Pharmacia LKB Biotechnology) were used in the sequencing of DNA inserts cloned into the multiple cloning site of pUC19.

Construction of a 20 base synthetic primer of the following nucleotide composition: 5'-d[GACAGTTTGTGTTGTATACG]-3' (DNA Synthesis Lab, University of Calgary, Alta) was based on the nucleotide sequence from position 352 to 371 of the pKB41 subclone (see Figure 14) sequenced in the forward direction from the M13 universal primer. The DNA sequencing was carried out with a T7 DNA polymerase sequencing kit obtained from Pharmacia LKB Biotechnology. The accuracy of the manual sequencing results were verified by automated DNA sequencing performed in collaboration with M. Estable, Applied Biosystems Ltd., Toronto.

3 RESULTS

Introductory Comments

The Results are divided into two sections. Section 3.1 presents the background and the results related to the characterization of the *murH* locus, while section 3.2 presents the background and the results related to the characterization of the *lytG* locus.

3.1 Characterization of *murH*

3.1.1 Overview of chapter contents

Previous work by D. Dai (84, 85) is summarized in 3.1.2 to provide the necessary background for understanding the approach taken in generating the results of this section. The bacteria, plasmids and bacteriophages used are described in 3.1.3, and the results obtained in further characterizing the *murH* locus are presented in 3.1.4.

3.1.2 Background to the characterization of *murH*

Phenotypic properties of the *murH1* mutant

The *murH* mutant (VC484) exhibited temperature-induced autolysis when subjected to the restrictive temperature of 42°C. The ts lysis phenotype of the *murH1* mutant allele was shown to be indirectly associated with the inhibition of a late step in PG synthesis and PG hydrolase-mediated autolysis at the restrictive temperature. It was suggested that the *murH*

mutation alters a membrane-associated function which indirectly affects this aspect of PG synthesis (85).

Mapping of the *murH* locus

The *murH* locus in the VC484 *murH* mutant strain was mapped to 99.2 minutes on the *E. coli* chromosome using standard phage P1-mediated generalized transduction (85).

Suppression of *murH* by *smhA*

The extragenic suppressor *smhA* (mnemonic for suppressor of *mur*H, locus *A*) was isolated from spontaneously occurring temperature resistant derivatives of the *murH1* ts mutant plated at 42°C (84). The frequency of occurrence of the *smhA* suppressor mutation was 3×10^{-6}. The *smhA* allele apparently suppressed the lysis phenotype associated with *murHI* but the *smhA* mutant did not display a recognizable phenotype of its own and it was able to grow as well as wild type strains at temperatures of 30°C to 42°C. Mapping of the *smhA1* mutation (24.5 min) showed that it was a new genetic locus unlinked to the *murH1* locus (84).

Cloning the *murH* locus

A genomic library was prepared by ligating *Sau*3AI partial digests of *E. coli* chromosomal DNA from strain W311O into the *Bam*HI sites of the single-copy number vector λSE6 (23). The ligation mixture was packaged, amplified and transformed into the λ lysogen strain VC486 (*murH1*), and kanamycin resistant transformants were selected at 42°C. The clone

carrying *murH* complementing activity was designated pDD41, which carried a 13 kb insert of *E. coli* chromosomal DNA. An 11 kb fragment of this insert was released by digestion with *Bam*HI (23).

Attempts to subclone *murH* in multicopy

Attempts to subclone the 11 kb *Bam*HI fragment into the high-copy number vector pUC18 resulted in apparently random deletions of the fragment as indicated by a heterogeneity in size distribution of the inserts in recovered plasmids (23). Since the deletions were accompanied by a loss of *murH* complemetating activity, it was assumed that the deletions occured within the cloned *murH* locus. Similar observations were made when the 11kb *Bam*HI fragment was subcloned into other low-copy number vectors, thus it was concluded that the *murH* locus, or a closely linked marker, was unstable in multicopy number vectors (23).

3.1.3 Bacteria, bacteriophage and plasmids

For convenience, the bacterial strains, bacteriophage and plasmids used in this subsection are listed in Table 1.

Table 1. ***E coli*** **K-12 strains, bacteriophages and plasmids used in the characterization of** ***murH***

Strain	Relevant Genotype/Description	Reference/Source
DH5α	*thi hsd*R17 *rec*A1 *rel*A1 *end*A1 *gyr*A96 ϕ80d*lac*Z M15	BRL[a]
VC7	*thi-1 lysA23 rpsL109*	Laboratory collection
VC482	VC7 *zji-101*::Tn*10 murH1*	Laboratory collection
VC484	VC7 *murH1*	Laboratory collection
VC486	W3104 *zaa-100*::Tn*10 murH1*	Laboratory collection
W3104	λ lysogen	Laboratory collection
W3110	wild type	Laboratory collection
Bacteriophage	**Relevant Genotype/Description**	**Reference/Source**
λSE6	Kan^R Amp^R	82
Plasmid	**Relevant Genotype/Description**	**Reference/Source**
pDD41	Kan^R *murH*+	23
pOF216	Spc^R Str^R	89

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

3.1.4 Results

Further attempts to subclone the presumptive *murH* locus

In view of the apparent multicopy number toxicity problem encountered by D. Dai in previous attempts to subclone the *murH* locus from pDD41 (23), I decided to subclone the 11kb *Bam*HI fragment into the single-copy number vector pOF216. This vector was chosen based on the following three properties: i) a single-copy number origin of replication; ii) blue/white insert screening capability for selection of recombinant plasmids; and iii) a size (12.5 kb) which would allow screening of recombinant plasmids for complementation by electro-transformation. A representative example of the results of this subcloning is shown in Figure 7. Four different randomly selected pOF216 derivatives (A-D) were found to contain inserts ranging from approximately 7.5 kb to < 0.5 kb in size. Thus, the same subcloning problem as encountered with multicopy number vectors was observed in subcloning attempts using pOF216, *i.e.*, only deletion derivatives of the 11kb *Bam*HI insert were recovered from recombinant plasmids. Furthermore, when *murH* mutant strains were transformed with the purified pOF216 derivatives, the ability of the mutants to grow at 42°C was not restored. This indicated that the spontaneous deletions in the 11kb *Bam*HI fragment had apparently resulted in a loss of *murH* complementing activity. The basis for the apparent lethality of the *murH* locus was unclear and appeared to be more complex than multicopy "toxicity" as originally thought. Therefore, further subcloning attempts of *murH* were abandoned.

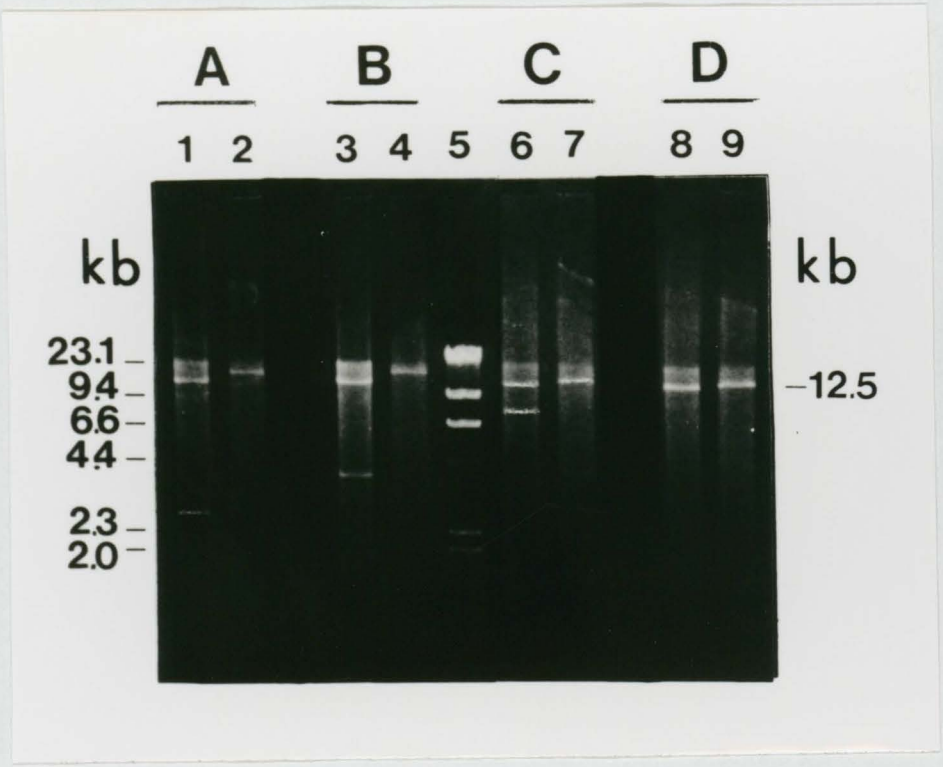


Figure 7. Electrophoretic analysis of four recombinant plasmids constructed from the subcloning of the 11 kb *Bam*HI fragment from pDD41 in pOF216

Four independent isolates (labelled A to D) were selected at random for *Bam*HI and *Eco*RI restriction analysis. *Bam*HI digests (lanes 1, 3, 6 and 8) show the 12.5 kb vector and the various sizes of insert DNA found in each of the four isolates. *Eco*RI digests (lanes 2, 4, 7 and 9) represent the total size of each of the recombinant plasmids, *i.e.*, size of vector plus insert. Lane 5 contains DNA size standards as indicated by the numbers at left in kb.

Probing an *E. coli* mapping membrane with the presumptive *murH* locus

The 11kb *Bam*HI fragment was purified from pDD41 and labeled with digoxigenin as described in the materials and methods. The probe was hybridized to a commercially prepared *E. coli* Gene Mapping Membrane. The results are shown in Figure 8. The probe hybridized to two different phage clones, identified as 3G6 and 25C105. The overlapping segment of DNA covered by these two clones encompassed an 11 kb *Bam*HI fragment located at 746 to 757 kb on the *E. coli* physical map, depicted in Figure 9. The presence on the physical map of a *Bam*HI fragment of the approximate size corresponding to the 11 kb *Bam*HI fragment containing *murH* activity strongly supported the mapping membrane result. This position corresponds to the 16 minute region of the 100 minute *E. coli* chromosome (70). No clones in the 99 minute region, *i.e.* ,the region to which the *murH* mutation was originally mapped (85), were detected.

Southern blot analysis of *E. coli*, phage λ and phage λSE6 genomes with the 11 kb fragment containing *murH* complementing activity

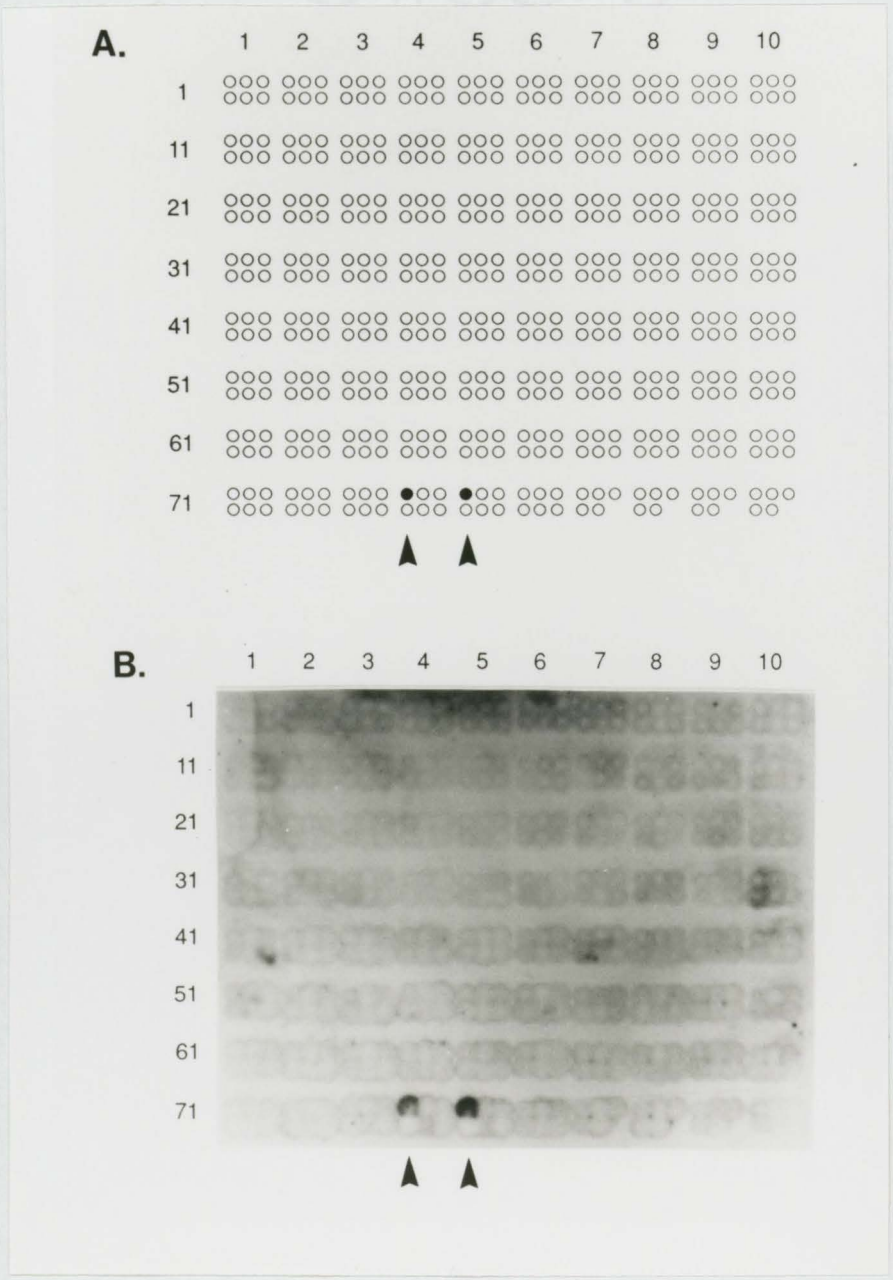
The same digoxigenin-labeled 11 kb *Bam*HI fragment that was used to probe the *E. coli* mapping membrane was used to probe restriction digests of *E. coli* DNA, phage λ DNA, and wild type phage λSE6. Figure 10 shows a single high MW band was detected with the probe on a *Bam*HI digest of *E. coli* chromosomal DNA. Strong hybridization of the probe to a 23 kb λ-*Hind*III fragment and a 6.6 kb λ-*Hind*III fragment was observed. The probe also bound to the vector component of *Bam*HI digested λSE6. The hybridization of the probe to *E. coli* chromosomal DNA was consistent with

the mapping membrane result. The source of the strong hybridization of the probe to phages λ and λSE6 DNA was undetermined, but indicated that the probe apparently contained a λ-related DNA sequence.

Figure 8. Hybridization of the 11 kb *Bam*HI fragment probe from pDD41 to an *E. coli* mapping membrane .

A. Template showing ordered array of 476 phage λ clones immobilized on the mapping membrane in 80 separate clusters. Each phage clone is represented by a circle and contains a different segment of the *E. coli* chromosome (strain W3110).

B. Hybridization of the 11 kb probe to phage λ clones in clusters 74 and 75 on the mapping membrane (indicated by arrow heads). These correspond to phage clones 3G6 and 25C105 respectively.


A.
1 2 3 4 5 6 7 8 9 10
1
11
21
31
41
51
61
71
B.
1 2 3 4 5 6 7 8 9 10
1
11
21
31
41
51
61
71

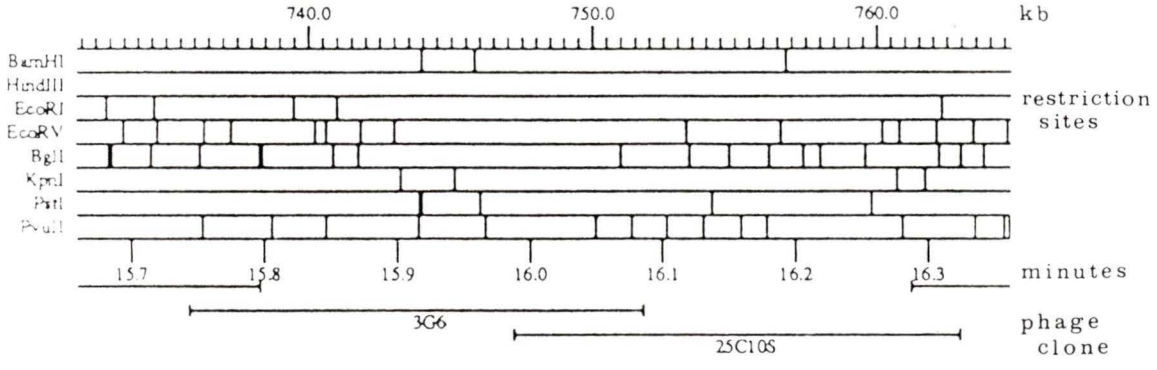


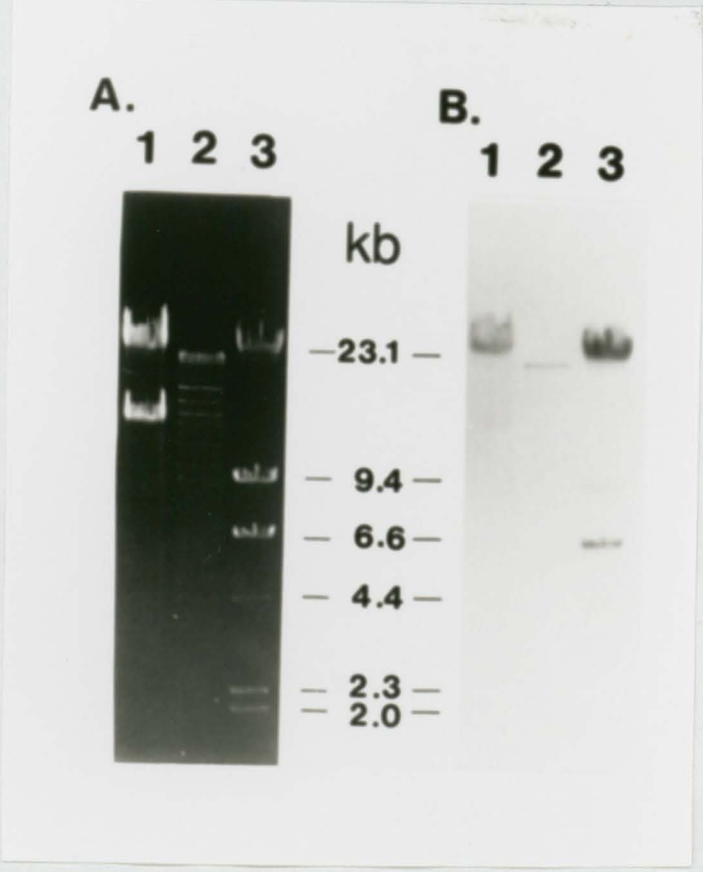
Figure 9. The 740-760 kb region of the *E. coli* physical map showing the location of phage λ clones 3G6 and 25C105.

Comparison of the positive phage clones 3G6 and 25C105 with the Kohara restriction map of *E. coli* DNA (73, 97) revealed the chromosomal location of the DNA used as a probe in Figure 8. The overlapping segment of DNA covered by these two clones encompassed an 11 kb *Bam*HI fragment located at 746 to 757 kb on the *E. coli* physical map. This position corresponds to the 16.0 to 16.2 minutes on the 100 minute *E. coli* chromosome. (Modified from reference 70).

Figure 10. Electrophoretic and Southern blot analysis of *E. coli* , and phages λ and λSE6 genomes probed with the 11 kb fragment containing *murH* complementing activity

A. Electrophoretic analysis of restriction digests of *E. coli* , and phages λ and λSE6 genomes. Lane 1: *Bam*HI restriction digest of wild type phage λSE6. Lane 2: *Bam*HI restriction digest of *E. coli* strain VC7 DNA. Lane 3: *Hin*dIII restriction digest of phage λ DNA. The numbers at the right indicate the sizes (kb) of DNA fragments in lane 3.

B. Southern blot analysis of the agarose gel shown in (**A**) after hybridization with the 11 kb *Bam*HI fragment containing *murH* activity. Lane 1: hybridization of the probe to the upper band of the λSE6 digest shown in (**A**). Lane 2: hybridization of the probe to a high Mr band of the *E. coli* chromosomal digest shown in (**A**). Lane 3: hybridization of the probe to both the 23 kb and the 6.6 kb λ-*Hind*III fragments shown in (**A**).


A.
1 2 3
kb
23.1
9.4
6.6
4.4
2.3
2.0
B.
1 2 3

3.2 Characterization of *lytG*

3.2.1 Overview of chapter contents

Important background information relevant to the results of this subsection is provided in 3.2.2, the bacteria, plasmids and bacteriophages used are listed in 3.2.3 , and results obtained in this thesis in further characterizing the *lytG* locus are presented in 3.2.4.

3.2.2 Background to the characterization of *lytG*

Cloning of *lytG*

In an effort to clone the *lytG* locus, an *E. coli* genomic library was constructed in λSE6 by E. Ishiguro and was subsequently screened for complementation at 42°C in the *lytG* mutant strain. A positive clone was isolated and designated pUV20. *Bam*H1 restriction digest of pUV20 released the cloned insert, resulting in two DNA fragments of 3.0 kb and 8.2 kb size in addition to the vector (E. Ishiguro, unpublished results).

Suppression of *lytG* by *smhA*

The *smhA* suppressor mutation isolated from spontaneous temperature resistant *murH* mutants was transferred to the *lytG* mutant strain. The *lytG smhA* mutant was able to grow at 42°C. Thus, the ts lysis phenotype conferred by the *lytG* mutation was apparently suppressed by the *smhA* allele (E. Ishiguro, unpublished results).

Genetic mapping of the *lytG* locus

The *lytG* gene was mapped to the 25 minute region of the *E. coli* chromosome using P1*vir*-mediated transduction of transposon insertions in the *lytG* mutant strain VC1005λ followed by selection of temperature-resistant (*i.e.*, *lytG*$^+$) drug-resistant transductants (E. Ishiguro, unpublished results).

3.2.3 Bacteria, bacteriophage and plasmids

For convenience, the bacterial strains, bacteriophage and plasmids used in this subsection are listed in Table 2.

Table 2. ***E coli*** **K-12 strains, bacteriophages and plasmids used in the characterization of** ***lytG***

Strain	Relevant Genotype/Description	Reference/Source
DH5α	*thi hsd*R17 *rec*A1 *rel*A1 *end*A1 *gyr*A96 ϕ80d*lac*Z M15	BRL[a]
VC7	*thi-1 lysA23 rpsL109*	Laboratory collection
VC1005λ	*lytG* λ lysogen	This study
W3104	λ lysogen	Laboratory collection
W3110	wild type	Laboratory collection
Bacteriophage	**Relevant Genotype/Description**	**Reference/Source**
λSE6	Kan^R Amp^R	82
Plasmid	**Relevant Genotype/Description**	**Reference/Source**
pUC19	Amp^R	88
pKB33	Amp^R *lytG*+ deletion derivative of 8.2 kb*Bam*HI fragment in pUC19	This study
pKB40	Amp^R	This study
pKB41	Amp^R*lytG*+ on 8.2 kb *Bam*HI fragment in pUC19	This study
pKB36	Amp^R pUC19 derivative	This study
pKB38	Amp^R pUC19 derivative	This study
pWSK29	Amp^R	91
pCL1920	Spc^R	90

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

3.2.4 Results

Phenotypic properties of the *lytG* mutant

The *lytG* mutant strain exhibited a temperature-dependent lysis phenotype, similar to that of the *murH* mutant strain, presumably caused by PG hydrolase mediated autolysis at the restrictive temperature. Figure 11 shows the effect of growth temperature on the *lytG* mutant strain, VC1005λ, grown in TSB. Spectrophotometric measurements (A_{600}) indicated that temperature-dependent autolysis is exhibited by strain VC1005λ when shifted from 30°C to 42°C.

Subcloning of *lytG* from pUV20

Figure 12 shows the agarose gel electrophoresis of plasmid constructs created in attempts to subclone *lytG* from pUV20. The 3.0 kb *Bam*H1 fragment isolated from pUV20 was successfully cloned into the *Bam*HI site of the multicopy number vector pUC19 (lane 5) and screened for *lytG* complementation in the *lytG* mutant strain at 42°C. No *lytG* complementing activity was present in this subclone, designated pKB40.

Attempts to subclone the 8.2 kb *Bam*H1 fragment into the pUC19 vector resulted in a spontaneous but apparently consistent deletion and possible rearrangement generating a subclone of total size 8.2 kb (*i.e.*, the size of the original 8.2 kb insert combined with the 2.7 kb vector was 8.2 kb in total) which retained the ability to restore growth at 42°C in the *lytG* mutant strain (lane 3). One of these subclones was retained and designated pKB33. Further attempts to subclone the *lytG* complementing activity from

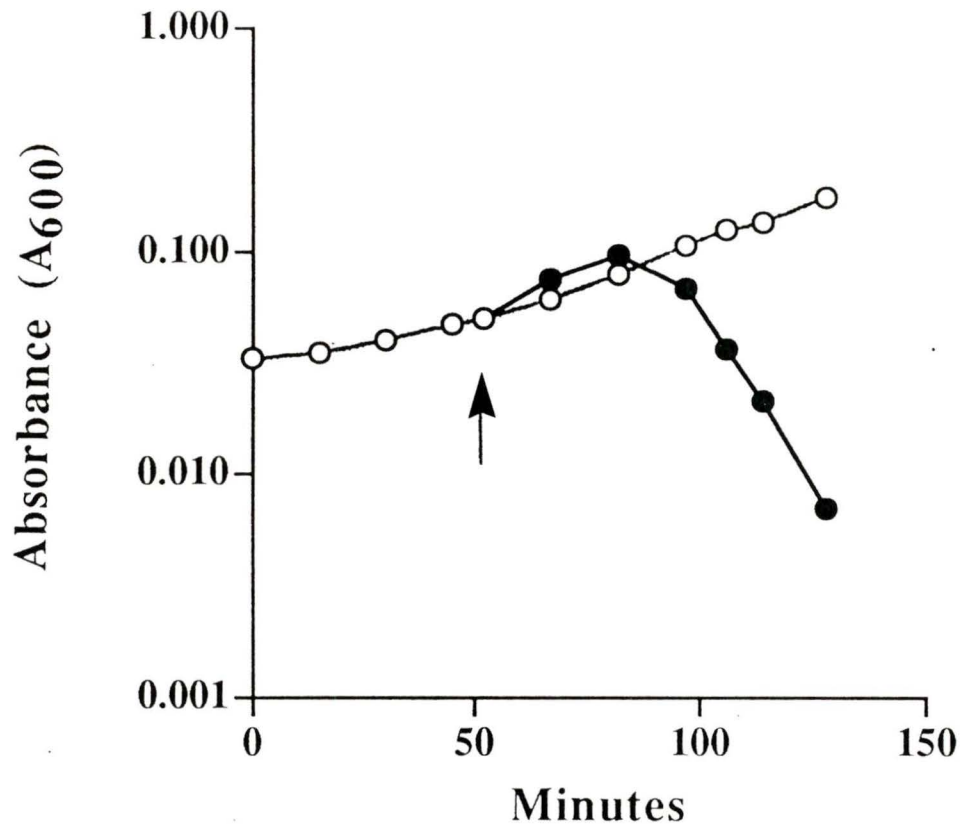


Figure 11. Temperature-dependent autolysis of strain VC1005λ (*lytG*).

A culture of VC1005λ growing at 30°C was either maintained at 30°C (–O–) or shifted to 42°C (–●–) at 50 minutes (arrow). Cell growth was measured at an absorbance of 600nm.

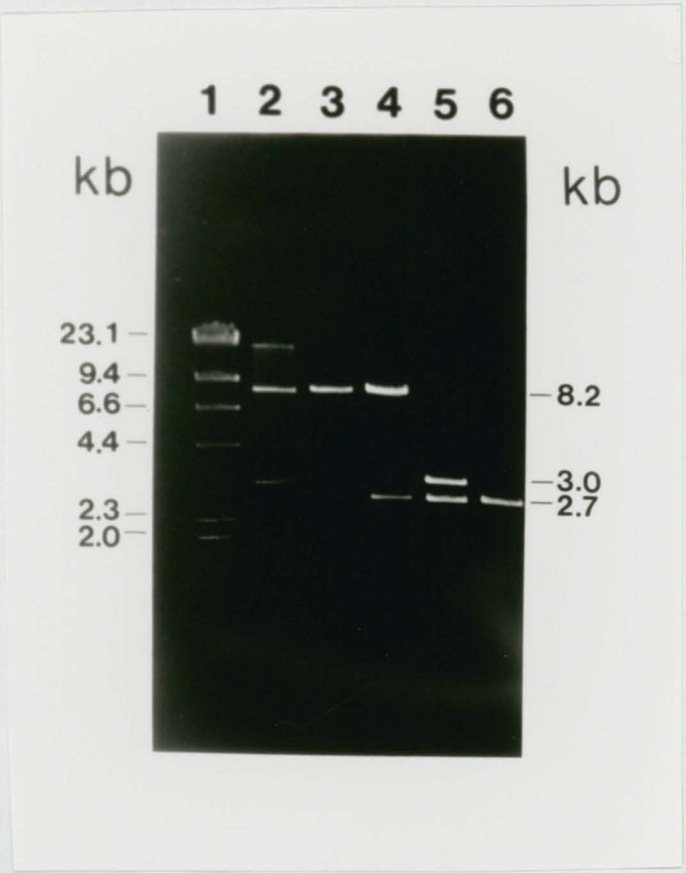


Figure 12 Electrophoretic analysis of the recombinant plasmids obtained from subcloning the 8.2 kb and 3.0 kb *Bam*HI fragments from pUV20 into pUC19.

Lane 1 : DNA size standards are indicated (kb) by the numbers at left. Lane 2: pUV20 digested with *Bam*HI. The upper band represents the λSE6 vector, the middle and lower bands are the 8.2 kb and 3.0 kb *Bam*HI fragments comprising the insert. Lane 3: recombinant plasmid pKB33 digested with *Bam*HI. Lane 4: recombinant plasmid pKB41 digested with *Bam*HI. The upper band is the 8.2 kb insert and the lower band is the 2.7 kb vector pUC19. Lane 5: Recombinant plasmid pKB40 digested with *Bam*HI. The upper band is the 3.0 kb insert and the lower band is the 2.7 kb vector pUC19. Lane 6: pUC19 vector digested with *Bam*HI showing a single 2.7 kb band. Sizes indicated by numbers at right are in kb.

pKB33 using various combinations of restriction enzymes and several different vectors of high, low or single-copy number, were unsuccessful. All resulted in derivatives with extensive deletions and no detectable *lytG* complementing activity (results not shown).

Only a single subclone was isolated from the subcloning attempts described above in which the 8.2 kb insert could subsequently be cut from the vector intact, and in which no obvious deletion or rearrangement of the vector were detected, based on restriction enzyme analysis (lane 4). This subclone exhibited only a partial ability to restore growth in the *lytG* mutant at the restrictive temperature. This was based on the observations that colonies exhibited irregular morphology and had difficulty growing at 42°C on solid media and broth cultures lysed when upshifted to 42°C. This subclone was designated pKB41.

Exhaustive attempts to subclone the 8.2 kb fragment into low-copy number (pWSK29, pCL1920) or single-copy number (pOF216) vectors resulted in deletions and possible rearrangements similar to those observed in multicopy number vectors, *i.e.*, in each case, the size of the resulting subclone was significantly smaller than the size of the original insert and vector combined (results not shown). In addition, temperature resistance was not restored when the *lytG* mutant was transformed with any of the above derivatives and selected at 42°C.

Probing the *E. coli* mapping membrane with *lytG*

The 8.2 kb *Bam*H1 fragment was purified from pUV20 and labeled with digoxigenin as described in the materials and methods. The probe was

hybridized to an *E. coli* Gene Mapping Membrane. No positive clones were detected on the membrane (results not shown).

Similarly, the 8.2 kb *Bam*H1 fragment was purified from pKB41 and labeled with digoxigenin. Again, no positive clones were detected on after hybridization to an *E. coli* Gene Mapping Membrane (results not shown).

Southern blot analysis of *E. coli* and phage λ genomes with the 8.2 kb fragment containing *lytG* complementing activity

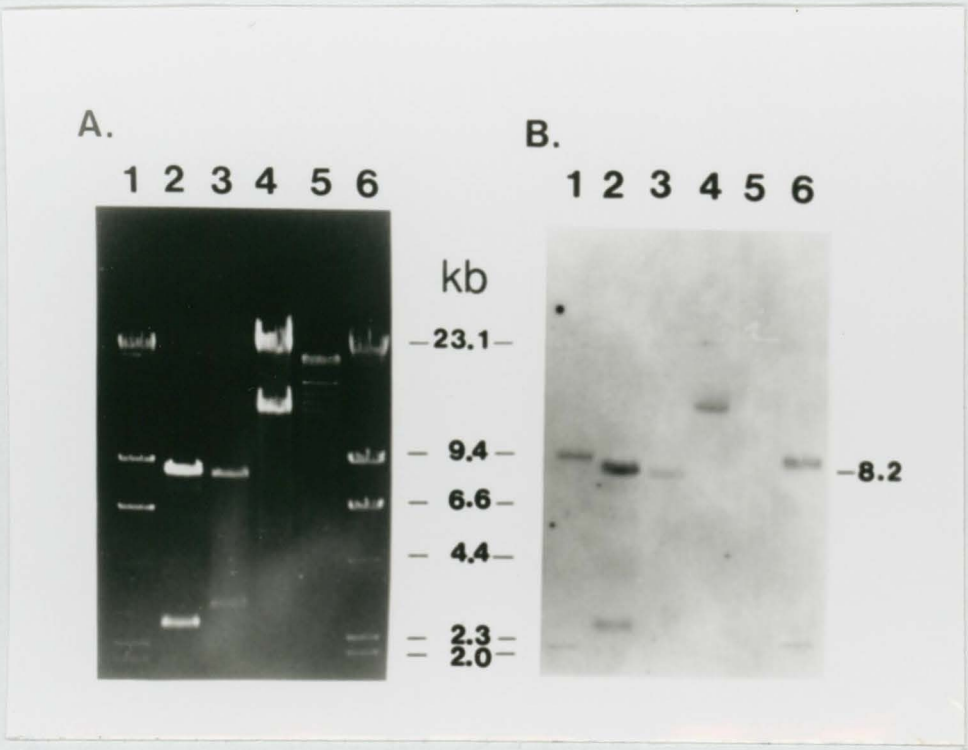
The digoxigenin-labeled 8.2 kb *Bam*H1 fragment derived from pKB41 that was used to probe the *E. coli* mapping membrane was also used to probe restriction digests of genomic *E. coli* DNA, phage λ DNA and phage λSE6 DNA. Figure 13 shows hybridization of the probe to the positive controls, the 8.2 kb *Bam*HI band of pKB41 (lane 2) and the 8.2 kb *Bam*HI band of pUV20 (lane 3). Hybridization to the 2.7 kb vector band of pKB41 (lane 2) indicated that the pUC19 vector was probably a component of the 8.2 kb band used as probe. This suggested that pKB41 was likely composed of a derivative similar to pKB33 (containing a similar deletion/rearrangement) recloned into the *Bam*HI site of pUC19.

No bands from the *E. coli* digest were detected with the probe (lane 5), consistent with the mapping membrane result. However, strong hybridization of the probe to a 9.4 kb λ-*Hind*III fragment and weaker hybridization to a 2.3 kb λ-*Hin*dIII fragment (lane 6) was observed. Hybridization of the probe to the lower 16 kb band of the λSE6 *Bam*HI digest, representing the "stuffer" or replacement region, (lane 4) was also observed.

Figure 13. Electrophoretic and Southern blot analysis of *E. coli* , and phages λ and λSE6 genomes probed with the 8.2 kb *Bam*HI fragment from pKB41

A. Electrophoretic analysis of restriction digests of pKB41, pUV20, *E. coli* chromosomal DNA, and phages λ and λSE6 genomes. Lanes 1 and 6: *Hin*dIII restriction digest of phage λ DNA showing six bands of sizes indicated by the numbers at right (kb). Lane 2: *Bam*HI digest of pKB41 showing 8.2 kb *Bam*HI band used to make the probe and 2.7 kb vector (pUC19) band. Lane 3: *Bam*HI digest of pUV20 showing upper λSE6 vector band, middle 8.2 kb *Bam*HI fragment and lower 3.0 kb *Bam*HI fragment. Lane 4: *Bam*HI restriction digest of wild type phage λSE6 showing two bands. The upper 33 kb band is the vector and the lower 16 kb band is the stuffer region which is replaced by a chromosomal insert when λSE6 is used in cloning. Lane 5: *Bam*HI restriction digest of *E. coli* strain VC7 DNA showing a range of several different sized bands.

B. Southern blot analysis of the agarose gel shown in (**A**) which was hybridized with the 8.2 kb *Bam*HI fragment from pKB41.
Lanes 1 and 6: positive hybridization of the probe to the 2.3 kb and 9.4 kb λ-*Hin*dIII fragment shown in (**A**). Lane 2: positive hybridization of the probe to the 8.2 kb and 2.7 kb bands of the pKB41 digest shown in (**A**). Lane 3: positive hybridization of the probe to the 8.2 kb *Bam*HI fragment from pUV20 shown in (**A**). Lane 4: positive hybridization of the probe to the lower band of the *Bam*HI digest of phage λSE6 shown in (**A**). Lane 5: no hybridization of the probe to the *E. coli* chromosomal *Bam*HI digest shown in (**A**).


A.
1 2 3 4 5 6
kb
23.1
9.4
6.6
4.4
2.3
2.0
B.
1 2 3 4 5 6
8.2

Restriction mapping of the *lytG* subclones

Attempts to construct an accurate restriction map of pKB33 were impeded by the extent and complexity of the deletion/rearrangement that had occurred in this clone. The resulting restriction map was speculative and has not been included here. Instead, a summary of the data is described below.

The restriction mapping data indicated that a significant portion of the pUC19 vector was missing from pKB33. It was estimated that the entire multiple cloning site, as well as undetermined amounts of DNA sequence on either side of this site, were deleted. Based on the restriction map of pUC19 (Figure 14), which is included here for refererence, it was determined that up to 689 base pairs to one side of the polylinker could potentially be deleted in the vector without disrupting the β-lactamase gene encoding ampicillin resistance. Similarly, up to 447 base pairs could potentially be lost from the other side of the vector without destroying the origin of replication. This total of 1136 base pairs (689 + 447) did not completely account for the approximate 2.7 kb missing DNA sequence in pKB33. Thus, it was suspected that the remaining sequence, if not more, was deleted from the 8.2 kb insert initially cloned into pUC19. Since it appeared that the entire multiple cloning site of the vector was deleted, the *Bam*HI site used in cloning the 8.2 kb insert, also must have been destroyed, yet a single *Bam*HI site was present in pKB33. This indicated one of the following possibilities: (i) one end of the original *Bam*HI cloning site in the vector was regenerated by recombination of the vector with the insert (indicating that this side of the vector would have been left intact), or

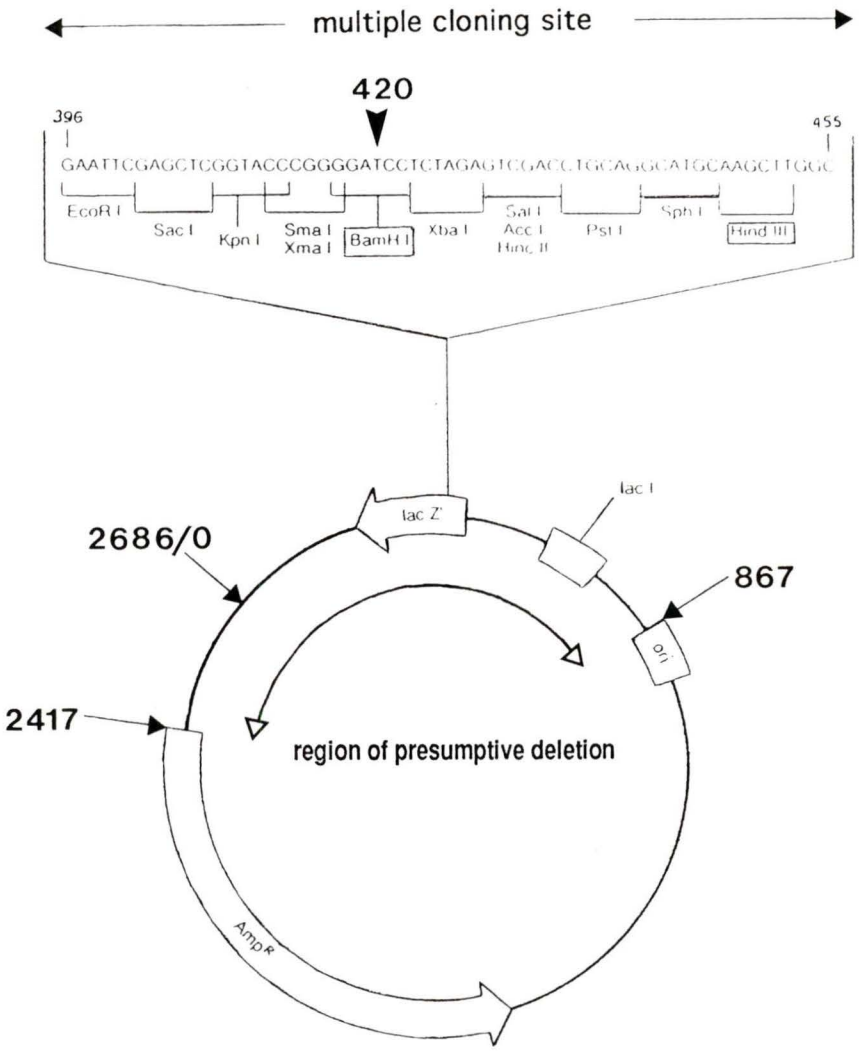


Figure 14. Restriction map of pUC19 (2686 bp)

The 8.2 kb *Bam*HI fragment containing *lytG* activity from pUV20 was ligated into the *Bam*HI site (indicated by the arrow head at 420 bp) of pUC19. The resulting derivative, pKB33, contained a deletion and possible rearrangement which was thought to include an undetermined amount of the vector, located within the 1136 bp region from 2417-2686 bp and 0-867 bp, indicated in center by open arrows (—▷). bp, base pair; Amp^R, ampicillin resistance; *lacZ'*, portion of the *E. coli lacZ* gene containing multiple cloning site; *ori*, origin of replication .

(ii) a new *Bam*HI site was fortuitously created in the insert, possibly by rearrangement of the insert DNA.

Restriction enzyme analysis of pKB41 was employed to aid in the construction of a restriction map of pKB33. Restriction mapping data showed that the vector, including the multiple cloning site, was intact in pKB41. This initially appeared to indicate that pKB41 was composed of the 8.2 kb *Bam*HI insert from pUV20 cloned into the *Bam*HI site of pUC19. However, based on the results of DNA hybridization studies (described above) and additional restriction enzyme analysis, it became apparent that the 8.2 kb insert of pKB41 contained DNA sequence similarity to pUC19. The most likely explanation for this finding is that pKB41 was composed of a *Bam*HI linearized derivative similar or identical to pKB33 which was cloned into the *Bam*HI site of pUC19. Thus, no intact 8.2 kb *Bam*HI fragment from pUV20 was successfully subcloned into any other vector.

DNA sequencing of the *lytG* subclones

Sequencing of pKB33 was attempted using M13 universal forward and reverse primers, but was unsuccessful presumably due to deletion of the sites for primer binding which would be located in the multiple cloning site of the vector.

Sequencing of pKB41 was initiated using the M13 universal forward and reverse primers, and the DNA sequence generated was used to construct an internal primer, located approximately 400 nucleotides from the cloning site of the vector, with the intention of 'walking' across the 8.2 kb insert. It was hoped that an internal primer constructed from the pKB41

sequence might also be useful in sequencing pKB33. Unfortunately, sequencing attempts using this internal primer were unsuccessful in both subclones. The reason for the lack of success was not determined. Multiple smeared bands were observed on the autoradiogram for pKB41 sequence using the internal primer, possibly due to multiple binding sites for the primer, while nothing was observed on the autoradiogram for pKB33, presumably due, again, to a deletion of the site for primer binding.

To obtain additional DNA sequence information, the M13 universal primers were used in sequencing portions of two more of the *lytG* subclones, designated pKB36 and pKB38. Neither of these subclones retained *lytG* restoring activity. Subclone pKB36 contained a 4.6 kb *Hin*dIII fragment which was derived from *Hin*dIII digestion of pKB33, and cloned into the *Hin*dIII site of pUC19. Multiple smeared bands, similar to those observed on the autoradiogram for the pKB41 sequence using the internal primer, were observed on the autoradiogram of the pKB36 DNA sequence using both the M13 forward and reverse primers. Subclone pKB38 contained a 0.6 kb *Hin*dIII fragment which was also derived from *Hin*dIII digestion of pKB33 and cloned into pUC19. DNA sequencing was successful using both M13 forward and reverse primers.

All DNA sequence data from pKB41 and pKB38 was analyzed using GeneWorks™ DNA analysis software (IntelliGenetics, Inc., CA., U.S.A.) and comparisons to known sequences were performed using the Basic Local Alignment Search Tool (BLAST) program (95). Figures 15 and 16 summarize the DNA sequence data obtained from sequencing portions of pKB41 and pKB38 respectively.

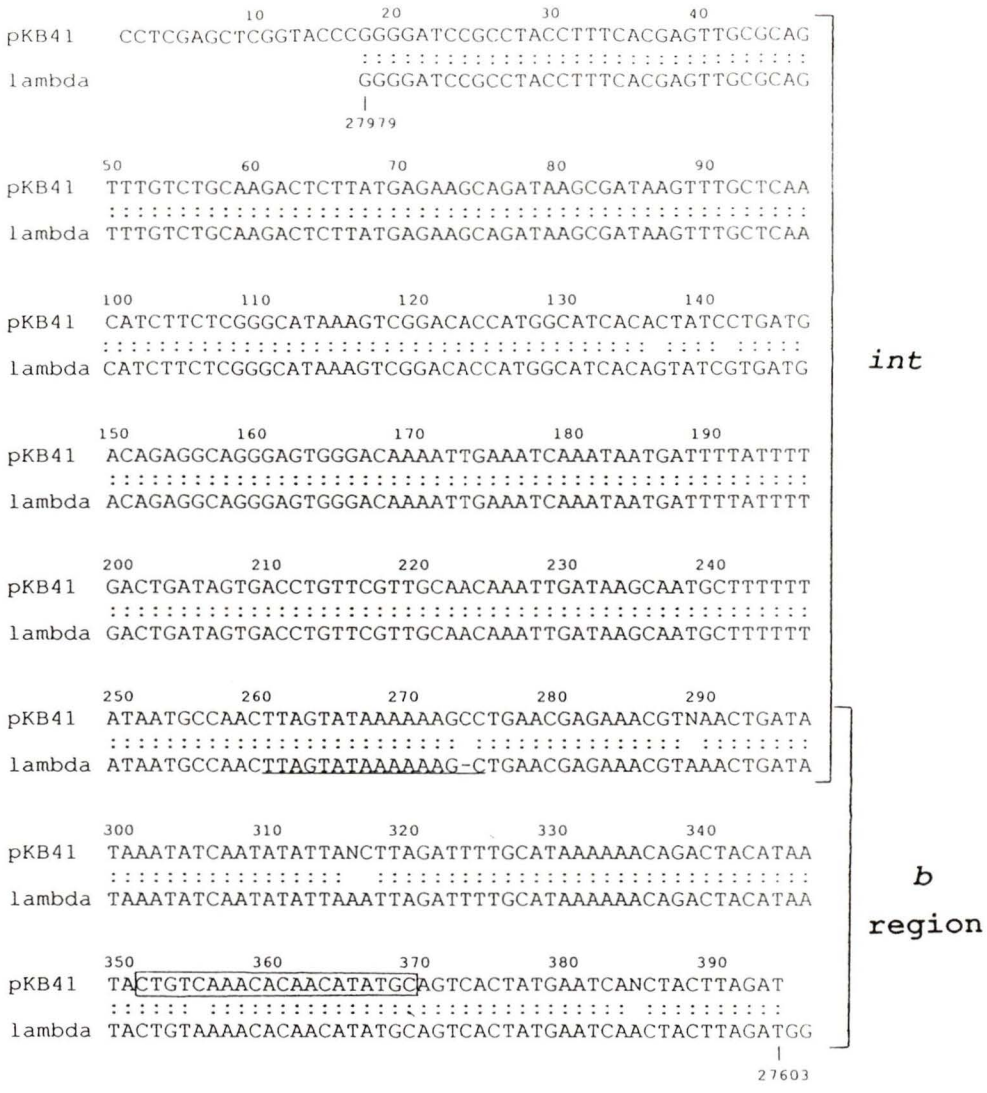


Figure 15. Partial DNA sequence of *lytG* subclone pKB41 and sequence comparison with phage λ DNA

400 nucleotides of the DNA sequence of pKB41 (top) aligned with nucleotides 27603 to 27979 of the phage λ genome (bottom). Regions of DNA sequence identity are indicated by dots (:). The λ*att* site is underlined. The sequence used to construct the 20 nucleotide DNA sequencing primer is boxed. A, Adenine; C, Cytosine; G, Guanine; T, Thymine.

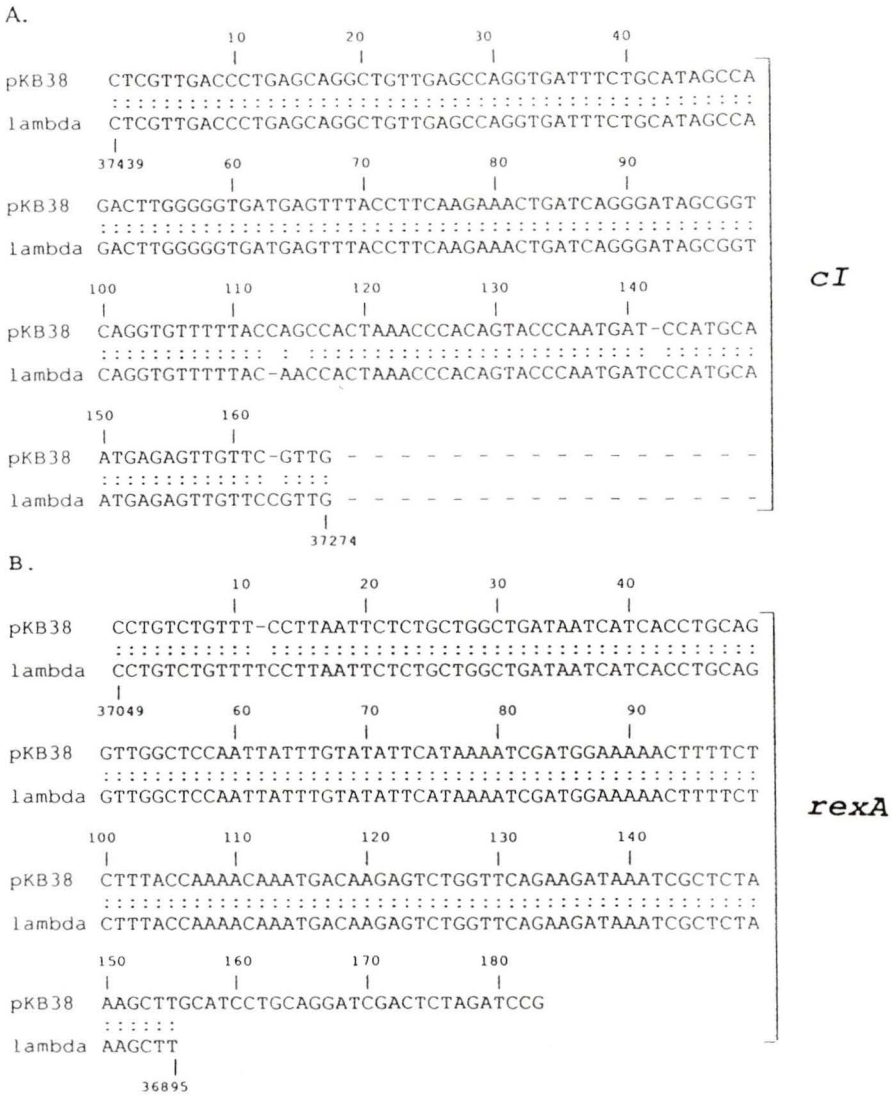


Figure 16. Partial DNA sequence of *lytG* subclone pKB38 and sequence comparison with phage λ DNA

A. 165 nucleotides of the DNA sequence of pKB38 (top) compared with nucleotides 37274 to 37439 of the phage λ genome (bottom).
B. 153 nucleotides of the DNA sequence of pKB38 (top) compared with nucleotides 36895 to 37020 of the phage λ genome (bottom).
The dashes (- -) indicate the 225 nucleotides separating these sequences. Regions of DNA sequence identity are indicated by dots (:). A, Adenine; C, Cytosine; G, Guanine; T, Thymine.

Similarity of *lytG* DNA sequence to phage λ

In total, over 1 kb of the 8.2 kb insert was successfully sequenced. As shown in Figures 15 and 16, over two-thirds of the nucleotide sequence obtained was identical to phage λ DNA sequence. The sequence identity flanked a region of λ of approximately 10 kb in size, encompassing the 27.6-37.4 kb region of the phage λ genome (refer to Figure 5). The remaining sequence, a continuous stretch of 360 nucleotides derived from sequencing pKB41 using the M13 reverse primer, was not similar to *E. coli* nor to λ DNA, but showed some identity to the DNA of pUC-derived cloning vectors (not shown), therefore it is assumed that this segment of the 8.2 kb fragment was at least partly composed of the pUC19 vector and was involved in the deletion/rearrangement described previously.

The DNA sequence derived from sequencing pKB41 using the M13 forward primer was identical, except for 8 nucleotides, to the 27603-27979 base region of phage λ DNA (see Figure 15). This DNA comprised the distal portion of the *int* gene, including the *att* site, and extended into the *b* region of λ (96).

The sequences derived from pKB38 were identical, except for five nucleotides, to the 36895-37049 base region (comprising a portion of the *rexA* gene) and the 37274-37439 base region (comprising a portion of the *cI* gene) of phage λ DNA (see Figure 16). Since these DNA sequences were located within a 544 nucleotide stretch of phage λ DNA, and the total size of pKB38 was approximately 0.6 kb, it seems reasonable to suggest that the remaining unsequenced DNA in this subclone is likely identical to the 225 remaining

nucleotides on the λ genome which separate these sequences, although this was not confirmed by DNA sequencing.

4 DISCUSSION

4.1 Characterization of *murH*

It was confirmed that some component of the 11 kb DNA fragment containing *murH* activity, derived from pDD41, was unstable when cloned into plasmid vectors of varying copy number. This conclusion is based on the observation that all plasmid derivatives that were isolated contained spontaneous deletions within the 11 kb fragment. D. Dai originally proposed that *murH* was "toxic" to the cell when expressed in multiple copies (23). The results of this study using a single-copy number vector were similar to those observed in the subcloning of the *murH* locus into multicopy number vectors. Based on these findings, I suggest that the instability of this fragment involves something other than (or in addition to) multicopy toxicity, as previously reported (23).

The question remains as to whether it is the *murH* gene itself, or some closely associated DNA sequence which contributes to the instability of this fragment (23). Considering the loss of *murH* restoring activity in the deletion derivatives, it is likely that the deletions encompassed at least portions of the *murH* gene. Spontaneous deletions as an apparent means of reducing the copy-number of toxic genes has been observed previously, for example in the *ompA* gene which encodes an outer membrane protein (97). Examples of other genes in *E. coli* which have been impossible to clone in multicopy number vectors, presumably due to toxic effects resulting from over-expression of the gene product, include *dacA*, encoding PBP5 (98), *pbpA*, encoding PBP2 (98), and *lpp*, encoding lipoprotein (99).

Although it originally appeared that the mutant *murH* allele conferred a defect in some aspect of the terminal step in PG synthesis (23), further work by D. Dai (85) indicated that the *murH* mutation did not directly elicit an inhibitory effect on PG synthesis. Thus, it was suggested that *murH* might be a pleiotropic mutation which caused, among other things, an alteration in membrane-associated function which indirectly affected the synthesis of PG (85). The function of the *murH* allele remains to be determined. The phenotype of the *murH* mutant strains suggests that autolysis at the restrictive temperature may be PG hydrolase-mediated, but the possibility exists that the ts lysis phenotype of the *murH* mutant is coincidently similar to, but independent of, that mediated by PG hydrolases, perhaps involving lysis by induction of a cryptic λ-related prophage.

The mutation in the *murH* mutant strain was genetically mapped to 99.2 minutes on the *E. coli* chromosome. Thus, it was surprising to find that the cloned fragment containing *murH* restoring activity mapped to the 16 minute region of the chromosome. This apparent discrepancy indicates that the *murH* chromosomal locus (99.2 minutes) was not cloned in pDD41 and therefore was not contained on the 11 kb fragment.

The mechanism involved in the restoration of the ability of the *murH* mutant to grow at the restrictive temperature is not known. The results herein suggest that the mechanism may involve suppression, rather than complementation. Complementation involves provision of a functional copy of the defective gene to restore gene activity in the mutant. In suppression, a mutational change in a second gene eliminates or suppresses a mutant phenotype. The suppressor-gene product either compensates for

the defect in the mutant or enables the altered gene to produce a functional product. In this case, it would appear that the 11 kb fragment cloned into pDD41 harbours a suppressor, located at 16 minutes on the *E. coli* chromosome, which is able to restore the ability of the *murH* mutant to grow at the restrictive temperature. To confirm this hypothesis, the dominance of *murH* would have to be determined. It is suspected that *murH* is a dominant allele and therefore can not be complemented by *murH*$^+$. If this is the case, it would not be possible to clone the *murH* allele using complementation, as no selection method would exist .

The reason that the 11 kb fragment was able to hybridize to two fragments (23 kb and 6.6 kb λ-*Hin*dIII fragments) representing two distinct regions (0-23 kb and 38-45 kb regions, respectively) of the phage λ chromosome, and similarly to the vector component of λSE6 which shares DNA segments of these regions (see Figure 10) is not clear, and cannot be answered based on the work herein. However, it is possible that the hybridization of the probe may be due to the existence of λ-related DNA at the 16 minute region of the chromosome, or the 16 minute region may fortuitously contain (an) *E. coli* gene(s) which share(s) homology with λ DNA. Furthermore, the *murH* suppressor may reside within this λ-related sequence.

In support of the former possibility, the cryptic prophage λ *cry*, which is located in the 17 minute area (73), is a possible candidate responsible for the hybridization of the probe to this region, since, as discussed in the introduction, this incomplete prophage contains DNA homologous to most

of the phage λ structural genes (*A* to *J*), which are found within the 0-23 kb region of λ DNA to which the probe bound.

Conversely, the cryptic prophage Qin, located at 34 minutes on the *E. coli* chromosome, contains DNA homologous to the lysis genes of phage λ (73), located within the 38-45 kb region of λ DNA to which the probe also bound, yet no hybridization of the probe was detected in the 34 minute region of *E. coli*. A possible explanation to account for this apparent discrepancy lies in the claim of Lichens-Park *et al.* (69) that Qin appears to be absent from the *E. coli* laboratory strain, W3110, that was used to construct the physical map of Kohara *et al.* (93). Thus, it is reasonable to assume that Qin was also absent from the commercially prepared *E. coli* Gene Mapping Membrane used in this work, and this would explain the lack of probe hybridization to the 34 minute region of the *E. coli* chromosome.

The possibility that the 16 minute region fortuitously contains an *E. coli* gene(s) which share(s) homology with λ DNA, is also worth considering, since other similarities between λ and host genes have been documented. For example, as previously mentioned, the origin of chromosomal replication of *E. coli* (*oriC*) has a very similar nucleotide sequence to that of several lambdoid phages (100), and some of the proteins which indirectly or directly act on these origins of replication are used by the phages as well as the host (67).

It is also possible that contaminating phage DNA may have been somehow cloned in the λSE6 vector along with the *E. coli* chromosomal DNA, accounting for the binding of the probe to phage λ DNA.

Until the DNA sequence of the 11 kb fragment is determined, or the gene product responsible for restoring the growth of the *murH* mutant at the restrictive temperature is obtained and characterized, the source of the instability and the restorative activity within the 11 kb fragment, the precise mechanism involved in correcting the mutant phenotype, and the involvement of a cryptic λ-related prophage will remain unclear.

4.2 Characterization of *lytG*

The phenotype of the *lytG* mutant strain (VC1005λ), like that of the *murH* mutants, was temperature sensitive, and cells lysed when grown at the restrictive temperature (Figure 11). As in the case of the *murH* , the precise function of the *lytG* locus is not yet known, but the lytic phenotype may be mediated by a PG hydrolase(s).

The *lytG* allele in strain VC1005λ (*lytG*) was previously mapped to the 25 minute region of the *E. coli* chromosome (E. Ishiguro, unpublished results), but mapping of the cloned DNA fragment believed to contain the *lytG* locus revealed that it did not hybridize to the chromosome at all. The reason for this discrepancy is not known but two possibilities will be discussed. Firstly, it is possible that the *lytG* locus falls into one of the 7 regions representing "gaps" in the mapping membrane (93), as indicated in the materials and methods, and one of these gaps occurs at approximately 24 minutes. On the other hand, if the DNA sequence of the presumptive *lytG* subclone used as a probe was part of a missing segment on the mapping membrane, probe hybridization to a chromosomal digest of *E. coli*

would have been be expected. Unfortunately, no hybridization of the probe to the *E. coli* digest was detected (see Figure 13).

The second, and probably more likely, possible explanation to account for the discrepancy in mapping parallels that of *murH*, *i.e.*, the chromosomal *lytG* locus was not originally cloned in pUV20. Instead, based on DNA hybridization and DNA sequencing results (discussed later), the insert was likely comprised of phage λ or phage λ-related DNA containing a suppressor which was able to restore *lytG* function to the mutant. It is not known if the insert in pUV20 was derived from a region of the *E. coli* genome containing λ-related DNA which happened to be absent from the mapping membrane, or whether the source of the insert was contaminating phage DNA in the vector preparation used for the construction of the genomic library.

The subcloning of the 8.2 kb fragment of DNA which contained the ability to restore growth to the *lytG* mutant, from pUV20, was attempted in order to localize the presumptive *lytG* locus on a smaller segment of DNA and into a more convenient plasmid vector for complementation testing, plasmid DNA purification, and DNA sequencing. All efforts to subclone the *lytG* activity from pUV20 into another vector resulted in either a loss of complementing ability and/or mutations in the form of deletions and possible rearrangements of the resulting derivatives. This finding appeared to be similar to, but apparently even more complicated than, the problems encountered in attempting to subclone *murH* from pDD41 into another vector. As with the instablity associated with *murH*, it was unclear whether the instability of the presumptive *lytG* locus was due to the *lytG* allele, or to

some closely associated DNA sequence, but it is possible that the mutations resulted in the reduction of some lethal effect of the overexpressed gene product.

Distinct from the problems encountered during the subcloning of *murH*, the subcloning of the *lytG* locus involved a deletion and possible rearrangement of the vector as well as the insert DNA. These apparently spontaneous changes to the insert and vector were remarkably consistent, given that all derivatives isolated from the subcloning of the 8.2 kb *Bam*HI fragment from pUV20 into pUC19 apparently resulted in the same construct as represented by pKB33. The single exception, pKB41, which initially seemed to represent the 8.2 kb insert successfully cloned into pUC19, on further analysis appeared to be the equivalent of linearized pKB33 recloned into the *Bam*HI site of the vector. The extent of the changes to the vector component of the *lytG* subclones pKB33 and pKB41 is difficult to explain, but could be due to a number of possibilities, which will be discussed later. A documented, and perhaps relevant, example of difficulties in attempting to clone other "uncloneable genes" is described below.

In the attempted cloning of the *t* gene of the bacteriophage K3 (closely related to phage T4) , which is believed to be involved in lysis, Riede (101) found that four clones which contained *t* complementing activity *in vivo* did not contain the whole T protein. Furthermore, despite apparently exhaustive efforts to clone and subclone different fragments in different directions on the pUC plasmids, a stable clone containing the whole *t* gene was not recovered. Instead, clones carrying deletions, duplications, or small insertions were isolated, and it was concluded that

even very small amounts of complete lysis protein of the phage might be lethal to the cell (101). The mystery, as expressed by Young (78), is that the incomplete clones, *i.e.*, missing N-terminal or C-terminal residues, are capable of "complementation". It is unclear how a fragment of *t* can be defective enough to be cloned under constitutive expression conditions but functional enough to perform its lethal function.

Positive hybridization to a 9.4 kb and a 2.3 kb *Hin*dIII fragment of phage λ DNA was detected with the presumptive *lytG* clone used as probe (see Figure 13), indicating DNA sequence similarity of the probe to phage λ. DNA sequencing of the presumptive *lytG* subclones was initiated to determine the source of the hybridization to phage λ DNA, as well as to attempt to determine the source of the *lytG* restoring activity. The latter objective was not successful, due to complications presumably resulting from the mutations in the subclones, thus the hypothesis that suppression is involved in correcting the phenotype of the *lytG* mutant was not confirmed. The former objective, *i.e.*, to find the reason for probe hybridization to phage λ DNA, was accomplished, and is described below.

Over 1 kb from three distinct regions of the 8.2 kb DNA fragment which carried *lytG* activity was sequenced, and over two-thirds of this sequence was identical to phage λ DNA. The regions of identity with phage λ were found within a 10 kb sequence, encompassing the 27.6-37.4 kb region of the phage λ genome (96). This is the same region of λ from which the 9.4 kb and 2.3 kb λ-*Hin*dIII fragments which hybridized to the probe are derived. This region contains genes involved in the functions of recombination, regulation and immunity (refer to Figure 5). It is worth

noting that this entire area is dispensible for lytic growth of phage λ and is also part of the "stuffer" region replaced with insert DNA in the λSE6 vector.

Sequence identity was found in three specific areas within this region. One area of almost 400 identical nucleotides encompassed the distal 250 nucleotides of the *int* gene, including the *att* site, and continued into the *b* region (96). The *int* gene product is the integrase protein which is required for recombination of phage λ into the bacterial chromosome at specific *att* sites on the phage and host chromosomes. Site-specific recombination leads either to phage insertion for lysogeny or to prophage excision after induction (102). The function of the *b* region is not known.

Another area of 165 identical nucleotides comprised part of the *rexA* (*r* exclusion) gene (96). The *rex* region encodes two coordinately expressed genes, *rexA* and *rexB* which are among the few genes that are expressed from the prophage. Expression of the *rex* genes causes the exclusion of many superinfecting phages. This inability to establish a productive infection in *rex*$^+$ λ lysogens occurs by an unknown mechanism which involves the inhibition of cellular metabolism (103, 104). One or both of the Rex proteins may be associated with the membrane (78, 102, 105)

The third area of sequence identity between a *lytG* subclone and phage λ DNA was found in a 153 nucleotide segment within the *cI* gene (96). This gene is located next to the *rexA* gene on the phage λ map (refer to Figure 5), and *cI* expression is under coordinate control with the expression of the *rex* genes from the prophage in a λ lysogen (105). The CI protein, also called λ repressor, is a regulatory element essential for lysogenic growth. CI is a DNA binding protein which blocks transcription of the genes required

for lytic growth thereby maintaining the lysogenic state of the cell. CI is also a positive regulator of its own synthesis (106), stimulating transcription of the *cI* gene unless very high concentrations of CI are present (107).

As the DNA sequences described above do not constitute complete genes, and it is not known what additional phage λ DNA sequence, if any, resides on the 8.2 kb fragment harbouring *lytG* activity, it is not possible to predict the involvement of any of the gene products in *lytG* suppression, but circumstancial evidence presented below suggests that the λ DNA residing on the *lytG* subclone may be involved in the unexplained deletions.

In an effort to determine whether the presence of λ DNA sequence in the *lytG* subclone was contributing to the deletion and/or rearrangement observed, I attempted to clone the 9.4 kb *Hin*dIII fragment of phage λ into the same pUC19 vector used to generate the *lytG* subclone. Purification of plasmid from the resulting colonies and subsequent restriction analysis revealed that all clones had undergone extensive deletions and presumably rearrangements resulting in derivatives which were ampicillin resistant yet were significantly smaller in size than the expected size of the vector and insert combined, an observation very similar to the derivatives resulting from attempts to subclone *lytG*. Although the full extent of the identity between the *lytG* subclone and phage λ DNA was not determined, it appears that whatever λ DNA is present on the *lytG* subclone may be involved in the deletion/rearrangement observed. It is not known whether the presence of the λ DNA alone can account for the instability of this fragment, or whether the combined presence of IS-like elements described below may also be involved.

The spontaneous, but apparently non-random, deletions observed in the presumptive *lytG* subclones may be representative of DNA containing transposon-like insertion sequence (IS) elements. IS elements are mobile segments of DNA that relocate at low frequency, though recombination which is not homology dependent. It should be noted that at least some of the IS elements have been shown to mediate duplications (108), as well as adjacent DNA deletions (73, 109), the latter of which may be relevant to the unexpected changes observed in the presumptive *lytG* subclones. Numerous IS and IS-like elements are apparently scattered thoughout the chromosome of *E. coli* (some are within the cryptic prophages), and found in many phage genomes as well (70). Therefore, the possibility exists that the DNA originally cloned into pUV20 may have contained an IS-like element at one end of the insert which contributed to the instability of this sequence. If so, an IS-like mediated deletion removing a portion of the insert as well as adjacent vector sequence, thereby deleting several of the cloning sites, might contribute to the unexpected changes observed in the subclones. Clearly the above suggestions regarding the complicated changes that are somehow induced in the subclones can only be considered very speculative .

Analysis of the limited DNA sequence of the 8.2 kb fragment containing *lytG* activity has contributed to the possibilities of why this DNA exhibits unconventional behavior, but does not explain how the λ DNA was incorporated into the pUV20 clone, whether the entire 8.2 kb fragment consists of λ DNA, what function (if any) the λ DNA is serving (for example, suppression of *lytG*), or where the *lytG* activity resides. One possible source of the λ DNA is the 16 kb stuffer region of the λSE6 vector. Another

possibility might be that it is DNA from contaminating phage. The potential involvement of a cryptic λ-related sequence, similar to the defective prophages described in the introduction, should also be considered.

Irrespective of where the phage λ DNA originated, the interesting question of whether it can suppress the *lytG* mutation remains. A pertinent example of phage λ DNA suppressing an *E. coli* mutant allele is found in another gene identified in this laboratory, and also part of the *murH* family of genes, *lytD*.

Like the *lytG* and *murH* mutants, the *lytD* mutant exhibited ts growth which was attributed to cellular autolysis at the restrictive temperature (111). Clones which restored *lytD* activity in the mutant strain were selected from a gene library cloned into λSE6, similar to the selection used to find clones which restored *lytG* and *murH* function. However, the "complementing" clones did not contain *E. coli* DNA; instead, they contained DNA derived from the *cI-cro* region of phage λ. It was shown that either the *cI* or the *cro* gene restored *lytD* activity (111). Like CI, Cro is a DNA binding protein involved in determining whether the lytic or lysogenic pathway is established in phage λ. Whereas CI is involved in establishing lysogeny by repressing the expression of genes required for lytic growth, Cro induces the lytic cycle by preventing the transcription of *cI*. Cro also negatively regulates its own synthesis. CI and Cro bind to similar sites to exert their antagonistic effects on gene expression, thus the fate of λ to grow in either one of the two alternative modes depends on the competition between CI and Cro for the binding sites involved.

Given the relationship between CI and Cro, and the observation that both genes suppressed the *lytD* mutation, it was suggested (111) that *lytD* suppression was dependent on the common DNA binding properties of CI and Cro. Furthermore, it was proposed that the *lytD* gene product may be a DNA binding protein with a similar specificity to that of CI and Cro, and LytD may regulate the expression of a gene(s) involved in a function which could directly or indirectly lead to cellular autolysis (111). The location of the *lytD* locus is 12.7 minutes, the same as that of the cryptic prophage Qsr' (69). The possibility that *lytD* might lie within the Qsr' module and therefore be involved in lambdoid gene regulation is interesting considering the lysis phenotype of the *lytD* mutant, and that the Qsr' module is composed of λ lysis gene-analogs and their positive regulator (111). Alternatively, it was suggested that LytD may regulate a non-lambdoid gene(s) involved in cellular function and may fortuitously possess structure-function features common to CI and Cro (111).

Like the case of *murH*, in order to confirm if, infact, the 8.2 kb fragment cloned into pUV20 encodes a *lytG* suppressor, the dominance of *lytG* would have to be determined. Again, as with *murH*, it is suspected that *lytG* is a dominant allele.

4.3 Concluding Remarks

It has been proposed that a functional relationship exists between *murH* and *lytG* based on the fact that the *smhA* mutation suppresses the ts lysis phenotype of the *murH* and *lytG* mutant strains (E.Ishiguro, unpublished results). Thus, if *murH* and *lytG* indeed are functionally

related, the similarities which have been found between these two distinct loci during their characterization might not necessarily be coincidental. For example, in light of the findings in the suppression studies described for *lytD* (111), the possibility that λ-related DNA may be responsible for the suppressor-like activity found within the *murH* and *lytG* clones might not be surprising. The source of the λ-related DNA, whether from a previously characterized cryptic prophage, from an as of yet unmapped defective phage located within the *E. coli* chromosome, or from some other source, is not known.

Based on the results obtained thus far, it is tempting to speculate that the involvement of λ-related DNA may be found in some or all of the mutant alleles comprising the *murH* family of genes. The conclusive determination of the source of the presumptive suppression in the *murH* and *lytG* clones, of the dominance of these alleles and of the basis for the instability of the DNA sequences within the clones, should help elucidate this complex set of observations for the *murH* and *lytG* genes.

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