THE MOLECULAR GENETICS OF CELL SHAPE IN ESCHERICHIA COLI

Thesis submitted for the degree of Doctor of Philosophy

at the University of Leicester

by

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TO MY MOTHER AND FATHER

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PUBLICATIONS

Some of this work has already been published:

Spratt, B.G., Boyd, A. and Stoker, N.G. (1980).
Defective and plaque-forming lambda transducing bacteriophage carrying penicillin-binding protein - cell shape genes : Genetic and physical mapping and identification of gene products from the *lip-dacA-rodA-pbpA-leuS* region of the *Escherichia coli* chromosome. J. Bacteriol. 143, 569-581.

Stoker, N.G., Fairweather, N.F. and Spratt, B.G. (1982).
Versatile low-copy-number plasmid vectors for cloning in *Escherichia coli*. Gene 18, 335-341.

"Thou comst in such a questionable shape ..."

Hamlet I(iv)

CONTENTS

Chapter 1

INTRODUCTION

The way in which Escherichia coli determines its rod-shape is still largely unknown. It is only as the details of the synthesis of the cell wall and its regulation are unravelled that this control will be clarified. The work in this thesis involves the preliminary study of a small cluster of genes involved in cell-shape determination.

In the introduction, I shall briefly discuss the structure of the E.coli cell wall and the biosynthesis of the peptidoglycan layer; the penicillin-binding proteins, and their functions in the late stages of peptidoglycan synthesis; cell-shape mutants, and how peptidoglycan synthesis varies throughout the cell cycle. Finally, I will discuss the aims of this study.

I. THE BACTERIAL CELL WALL

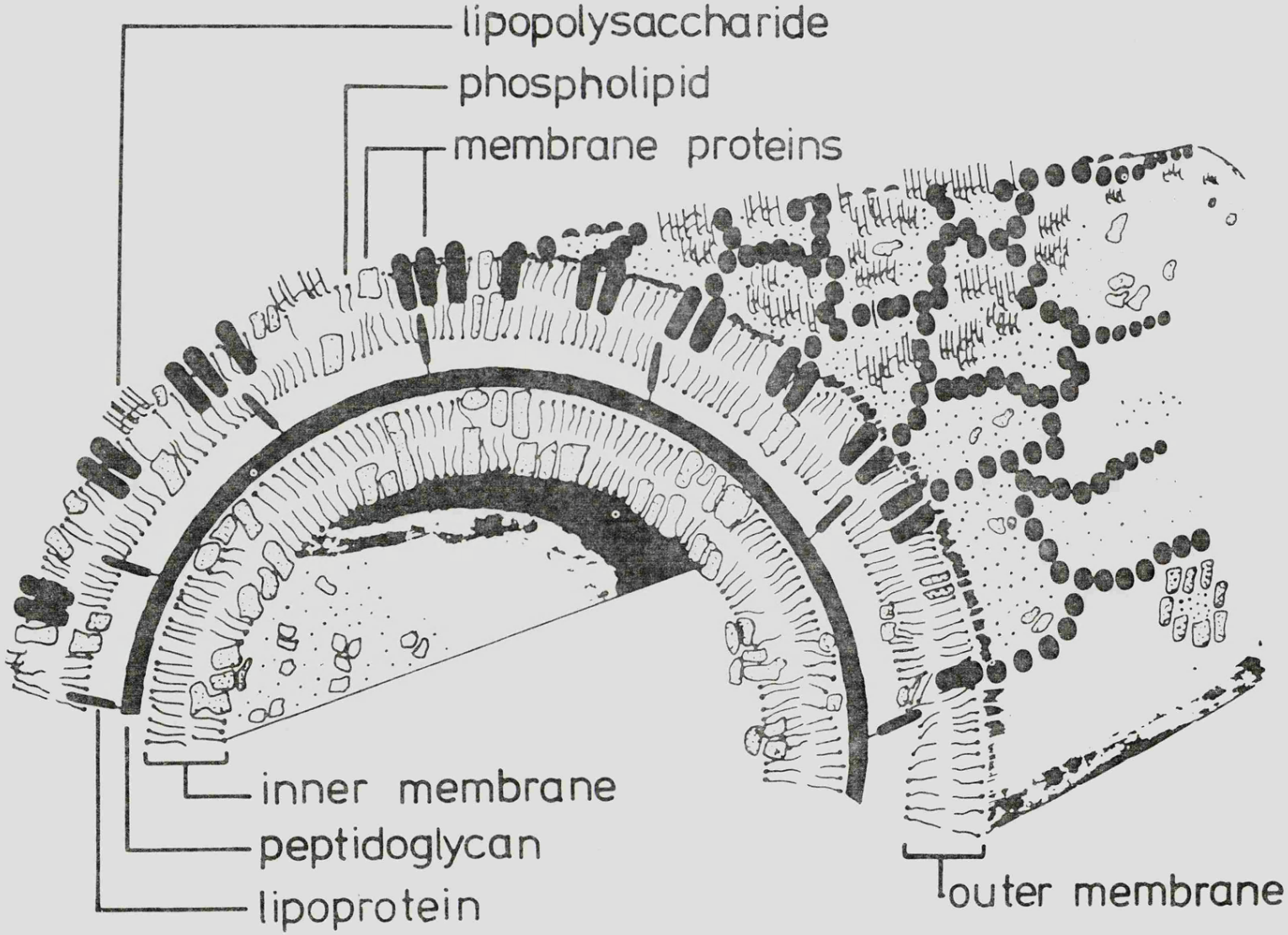
I(a) Introduction

The cell wall of gram-negative bacteria is a complex structure made up of three layers (see Figure 1.1). There is a cytoplasmic or inner membrane, which corresponds to the eukaryotic plasma membrane. This is surrounded by the outer membrane, and between these two membrane layers there is a layer of peptidoglycan, which acts as the cell's "skeleton". Comprehensive reviews of the structure and biosynthesis of the cell wall have been written by Ghuysen (1977), Daneo-Moore and Shockman (1977), Tipper and Wright (1979), Wright and Tipper (1979), Inouye (1979), Mirelman (1979, 1981) and Rogers et al. (1980).

Figure 1.1

Schematic representation of the cell wall of E.coli.

Modified from an original provided by Professor I.B. Holland.


lipopolysaccharide
phospholipid
membrane proteins
inner membrane
peptidoglycan
lipoprotein
outer membrane

I(b) The Inner Membrane

The inner membrane is a typical biological membrane consisting of a phospholipid bilayer with associated proteins forming a fluid mosaic (Singer and Nicholson, 1972). It is the main permeability barrier of the cell, and is the site of many cellular functions such as energy metabolism, the biosynthesis of lipids, peptidoglycan and lipopolysaccharide, and the active transport of many different nutrients. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) shows the inner membrane to contain a large number of different proteins, contrasting with the simpler profile shown by the outer membrane.

I(c) The Outer Membrane

The outer membrane contains more protein per unit mass than the inner membrane, consisting of large amounts of a rather small number of proteins. While it is a bilayer, the two leaflets are not symmetrical. The inner leaflet is made up of phospholipids and proteins, but the outer leaflet also contains a large amount of lipopolysaccharide (LPS).

This membrane acts as a barrier to the proteins in the periplasmic space, which include hydrolytic enzymes and specific amino acid and sugar transport binding proteins. The outer surface also acts as a protective barrier against the cell's environment. Gram negative bacteria are much more resistant than gram positive bacteria to certain dyes, chemicals, enzymes and antibiotics. This is particularly important for the Enterobacteriaceae, which come into contact with bile salts. The LPS is instrumental in preventing many compounds from entering the cell, and is also known as endotoxin, the major toxin of pathogenic enteric bacteria.

While it acts as an effective barrier on both sides, the outer membrane is permeable to the nutrients and ions required for growth. It

provides specific and non-specific channels through which these can diffuse. The exclusion limit of the membrane varies between 600 and 1000 daltons, depending on the molecular dimensions of the solute. The nutrients may then be actively transported across the inner membrane.

There are three major proteins or groups of proteins in the outer membrane. The porins, proteins Ia and Ib, which are coded for by the ompF and ompC genes respectively, form passive diffusion pores. Protein II*, which is coded for by the ompA gene, may have a structural role. Finally, the most abundant protein in the cell is lipoprotein, which has a molecular weight of about 7 Kd, and makes up 5-7% of the total cell protein. It consists of 58 amino acids with a glycerylcysteine at the amino-terminus, to which two fatty acids are attached by ester linkages, and another by an amide linkage. Approximately one-third of the lipoprotein molecules are covalently bound to the peptidoglycan (see section I(e)). The function of the lipoprotein is obscure, but seems to have a role in maintaining the integrity of the outer membrane.

There are 10 to 20 "minor" proteins in the outer membrane, which may under certain growth conditions become "major" proteins. Some of these proteins are involved in the uptake of nutrients under specific growth conditions.

I(d) The Peptidoglycan

The structure of peptidoglycan varies among different bacterial species. Here, only E.coli will be discussed. The peptidoglycan has been aptly described as a "bag-shaped molecule" or sacculus. It consists of linear strands of alternating residues of N-acetylglucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc). Pentapeptide side chains are attached

to each muramic acid residue, and covalent bonds formed between side chains from different glycan strands produce a net-like structure (see Figure 1.2). The peptidoglycan sacculus so formed is a rigid layer which gives the cell its mechanical strength and is crucial in the maintenance of the shape of the cell. Bacteria lacking an intact peptidoglycan layer lose their shape and form spheroplasts which lyse in growth medium of normal osmotic strength. E.coli peptidoglycan is remarkably stable, being turned over at a rate of less than 2% per generation.

I(e) The Relationships Between The Peptidoglycan And The Outer Membrane

The outer membrane is closely associated with the peptidoglycan. The only membrane constituent covalently attached to the sacculus is lipoprotein. One-third of the molecules are joined by a peptide bond formed between the C-terminal lysine of lipoprotein and meso-diaminopimelic acid residues in the peptidoglycan, the two D-alanine residues of the pentapeptide sidechains being cleaved off (see Figure 1.3). 10% of peptide sidechains are bound to lipoprotein in this way, although this may double in stationary phase cells (Wensink et al., 1982).

Rosenbusch (1974) observed that when peptidoglycan was isolated by incubation of cell envelopes in SDS at 60°C, proteins were arranged in a lattice structure with hexagonal symmetry on the outer face. These proteins were dissociated by treatment in SDS at 100°C, although lipoprotein remained covalently bound. The protein which formed the lattice he called "matrix" protein, and was shown to consist of proteins 1a and 1b (Lugtenberg et al., 1977). Yamada and Mizushima (1978) showed that purified 1a or 1b together with lipopolysaccharide will form a lattice structure if added to purified peptidoglycan. However, it is clear that this ordered array is formed by interaction with lipoprotein, rather than

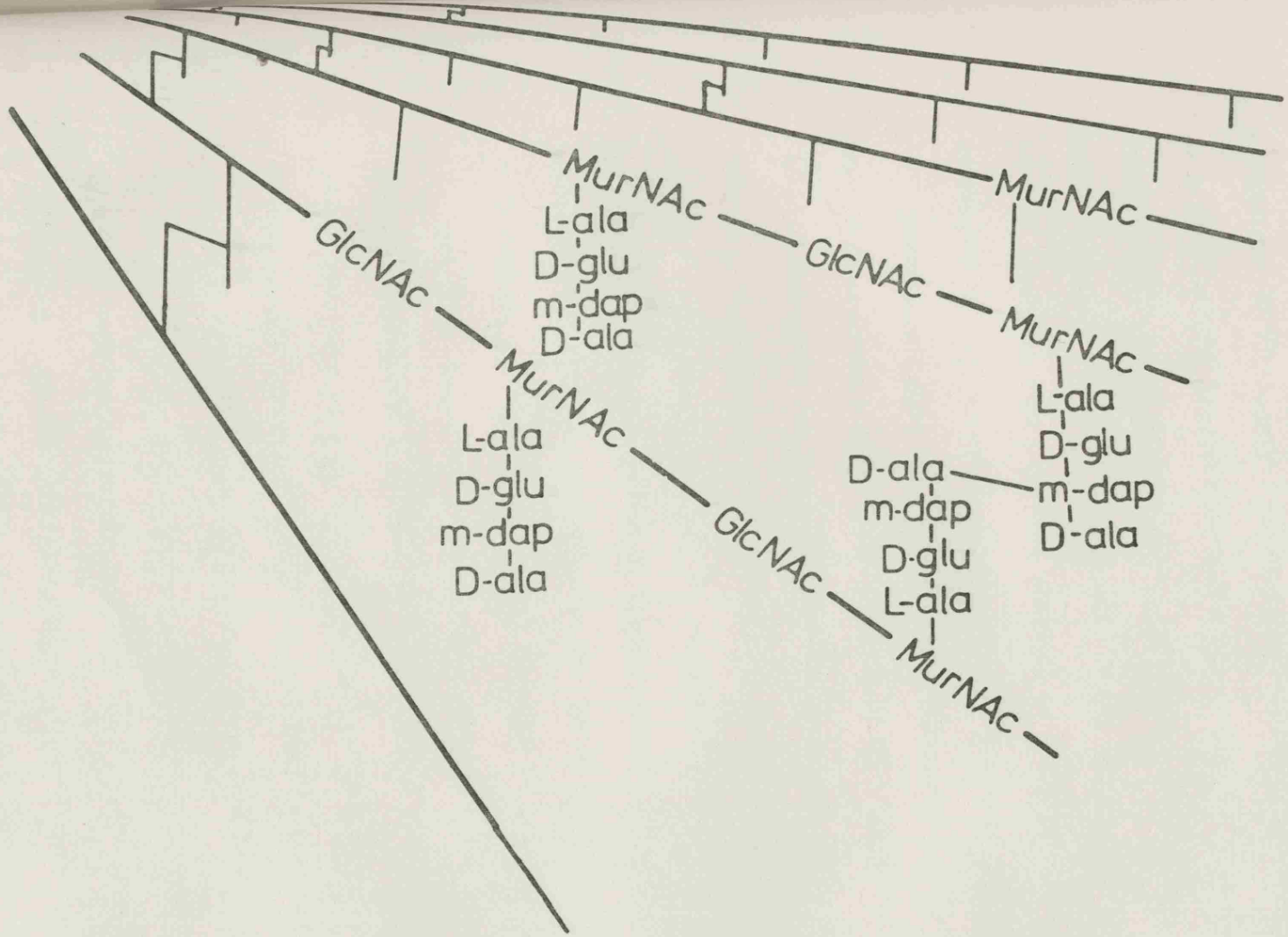

MurNAc
L-ala
D-glu
m-dap
D-ala
GlcNAc
MurNAc
GlcNAc
MurNAc
L-ala
D-glu
m-dap
D-ala
GlcNAc
MurNAc
L-ala
D-glu
m-dap
D-ala
GlcNAc
MurNAc

Figure 1.2

The structure of part of the peptidoglycan of E.coli.

MurNAc, N-acetyl muramic acid; GlcNAc, N-acetyl glucosamine; L-ala, L-alanine; D-glu, D-glutamate; m-dap, meso-diaminopimelate; D-ala, D-alanine.

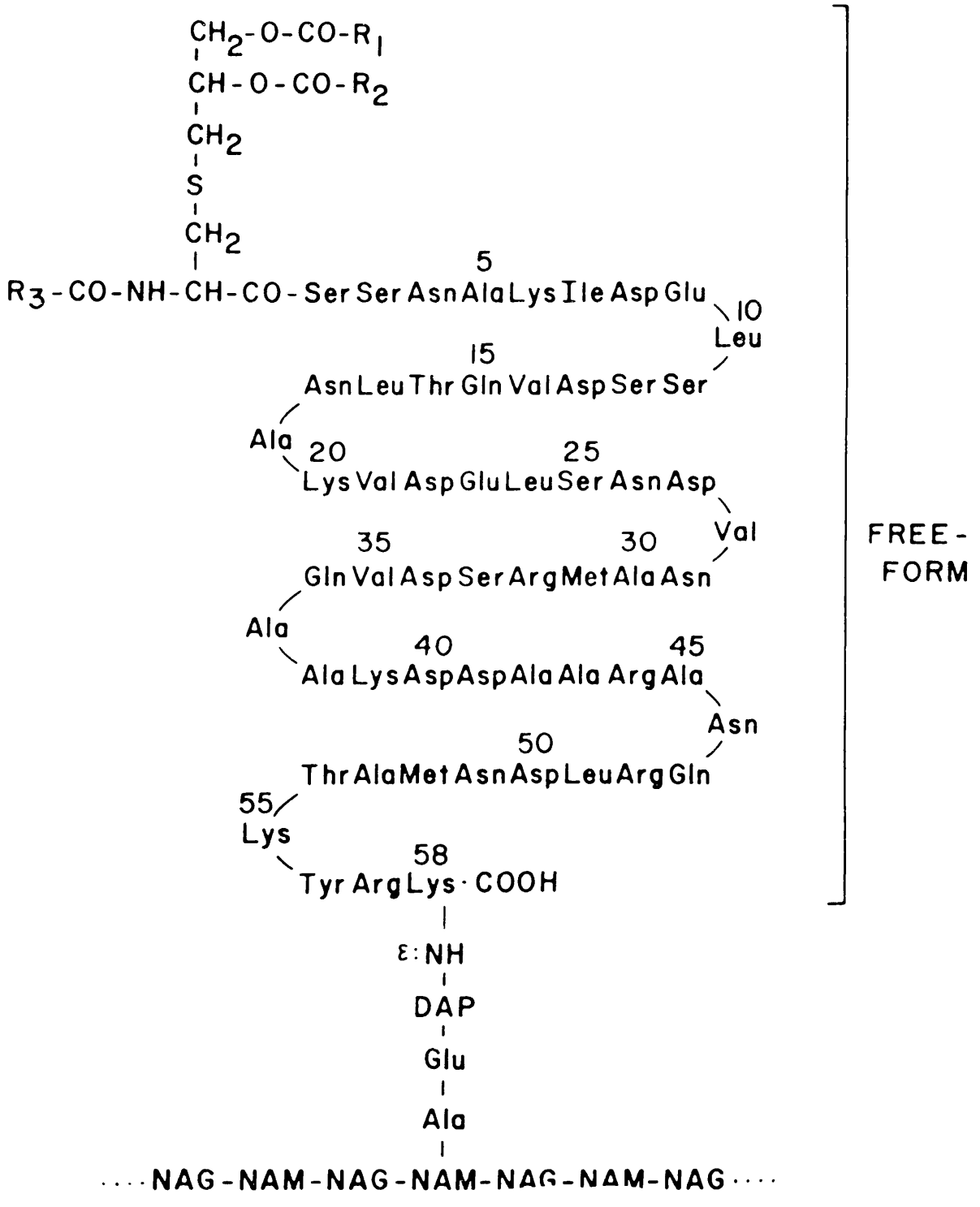
Taken from Spratt (1978b).

Figure 1.3

The structure of lipoprotein, showing the linkage with peptidoglycan.

NAM, N-acetyl muramic acid; NAG, N-acetyl glucosamine; Ala, L-alanine; Glu, D-glutamate; DAP, diaminopimelic acid. R_1, R_2 and R_3 represent hydrocarbon chains of fatty acids.

Taken from Inouye, 1979.


CH2-O-CO-R1
CH-O-CO-R2
CH2
S
CH2
R3-CO-NH-CH-CO-Ser Ser Asn Ala Lys Ile Asp Glu Leu Asn Leu Thr Gln Val Asp Ser Ser Ala Lys Val Asp Glu Leu Ser Asn Asp Val Gln Val Asp Ser Arg Met Ala Asn Ala Ala Lys Asp Asp Ala Ala Arg Ala Asn Thr Ala Met Asn Asp Leu Arg Gln Lys Tyr Arg Lys · COOH
5
10
15
20
25
30
35
40
45
50
55
58
FREE-FORM
ε:NH
DAP
Glu
Ala
....NAG-NAM-NAG-NAM-NAG-NAM-NAG....

with the peptidoglycan, since no such lattice is formed when peptidoglycan lacking bound lipoprotein is used.

Studies with bifunctional crosslinking reagents (Endermann et al., 1978) show that protein II* is closely associated with the peptidoglycan, while proteins 1a and 1b cannot be crosslinked to the sacculus in this manner. Sonntag et al. (1978) showed that a lpp ompA mutant is spherical, and has an outer membrane almost totally detached from the sacculus, supporting the idea that lipoprotein and protein II* are the most important outer membrane proteins interacting with the peptidoglycan.

II. THE BIOSYNTHESIS OF PEPTIDOGLYCAN

II(a) Introduction

Peptidoglycan biosynthesis may for convenience be considered in three stages (see Figure 1.4).

1. The synthesis of precursor molecules in the cytoplasm.
2. The transfer of these precursors to a lipid carrier in the cytoplasmic membrane, forming the basic peptidoglycan subunit.
3. The translocation across the membrane to the outer surface, followed by polymerization of the peptidoglycan and crosslinking.

The synthesis of UDP-MurNAc-pentapeptide was initially studied in gram positive cocci such as Staphylococcus aureus and Streptococcus faecalis, and initial studies on the membrane-associated step were also performed in S.aureus. Investigations into polymerization and crosslinking of the peptidoglycan have been made using in particular E.coli, B.subtilis and B.stearothermophilus and Streptomyces species. Although widely separated taxonomically, the organisms looked at have similar mechanisms of peptidoglycan synthesis. This work has been reviewed by Tipper and Wright (1979) and Rogers et al. (1980). Only peptidoglycan synthesis in E.coli will be discussed here.

Figure 1.4

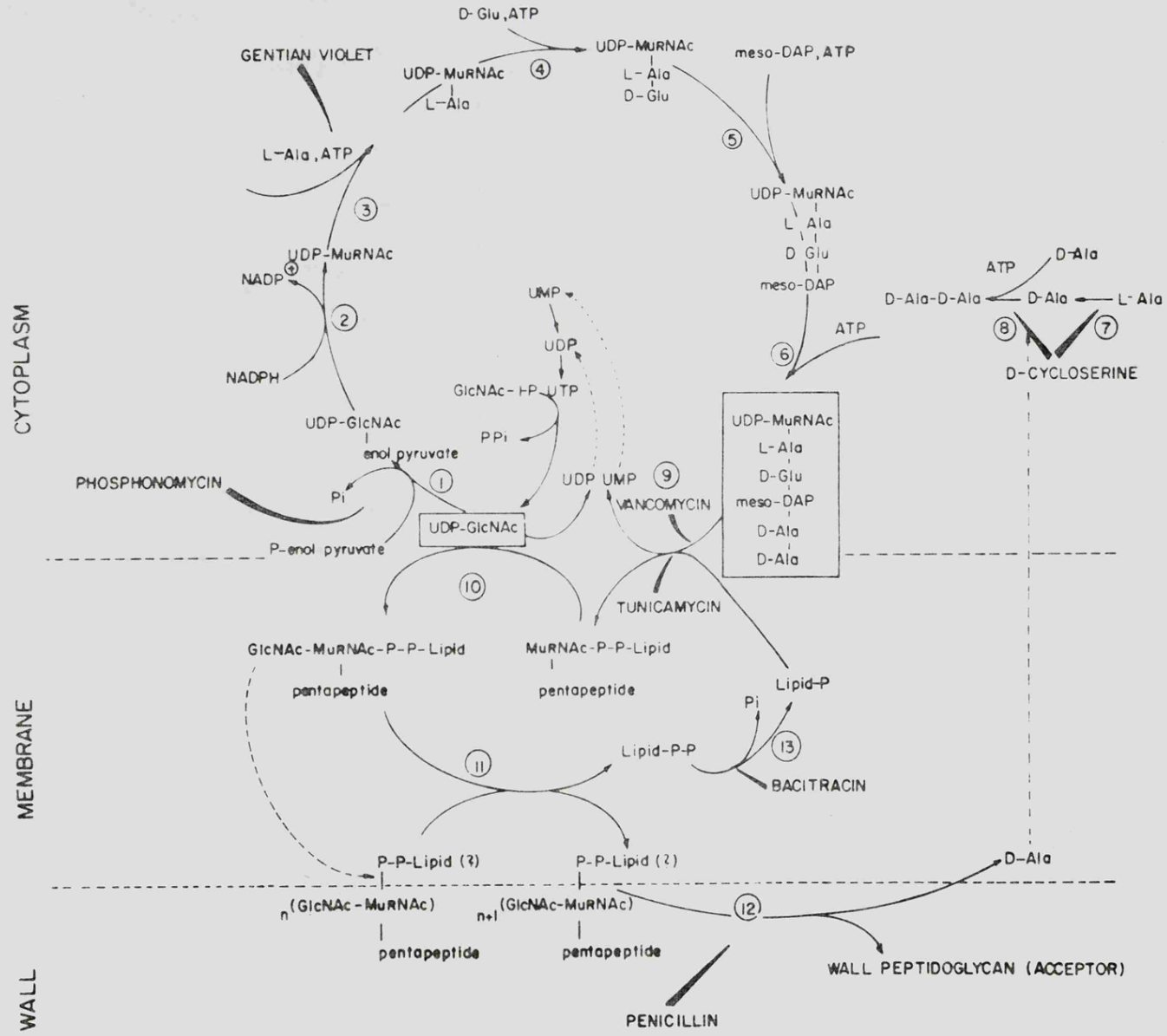
Biosynthesis of peptidoglycan in E.coli.

The three stages of synthesis that occur in the cytoplasm, in the membrane, and outside the membrane are separated by dashed lines. The sites at which various antibiotics act are also indicated.

Mutants have been isolated which have defects at stages:

1. (phosphoenolpyruvate : UDP-GlcNAc transferase; murA)
2. (UDP-N-acetylglucosamine-enolpyruvate reductase; murB)
3. (L-alanine ligase; murC)
5. (m-diaminopimelate ligase; murE)
6. (D-alanyl-D-alanine ligase; murF)
7. (alanine racemase; alr)
8. (D-alanyl-D-alanine synthetase; ddl)

Taken from Mirelman (1980).


CYTOPLASM
MEMBRANE
WALL
D-Glu, ATP
UDP-MurNAc
L-Ala
D-Glu
meso-DAP, ATP
GENTIAN VIOLET
L-Ala, ATP
NADP
NADPH
UDP-GlcNAc
enol pyruvate
PHOSPHONOMYCIN
Pi
P-enol pyruvate
UMP
UDP
GlcNAc-1-P-UTP
PPi
UDP UMP
VANCOMYCIN
meso-DAP
ATP
D-Ala-D-Ala
D-Ala
L-Ala
D-CYCLOSERINE
TUNICAMYCIN
GlcNAc-MurNAc-P-P-Lipid
MurNAc-P-P-Lipid
pentapeptide
Lipid-P
Lipid-P-P
BACITRACIN
P-P-Lipid (?)
n(GlcNAc-MurNAc)
n+1(GlcNAc-MurNAc)
WALL PEPTIDOGLYCAN (ACCEPTOR)
PENICILLIN

II(b) Synthesis Of Precursors

The low molecular weight precursors of peptidoglycan are the two nucleotide sugars, UDP-N-acetylglucosamine (UDP-GlcNAc) and UDP-N-acetylmuramyl-L-Ala-D-isoGlu-meso-DAP-D-Ala-D-Ala (UDP-MurNAc-pentapeptide). These are synthesized by soluble cytoplasmic enzymes.

The first step is the synthesis of UDP-GlcNAc. This is converted by phosphoenolpyruvate (PEP) : UDP-GlcNAc transferase and UDP-GlcNAc-3-enolpyruvate reductase to UDP-MurNAc. The amino acids are now added sequentially by specific enzymes. An exception is D-Ala-D-Ala, which is preformed and added on as a dipeptide.

A number of the genes coding for these enzymes have been identified. murC, murE and murF code for the L-alanine, m-DAP and D-ala-D-ala ligases respectively. ddl codes for D-ala-D-ala synthetase. These four genes map at about 1.5' on the E.coli chromosome, within a larger cluster of genes involved in peptidoglycan synthesis and cell division (called the mra cluster; see Figure 1.5). murC and ddl appear to be transcribed from the same promoter (Lutkenhaus and Wu, 1980), and other transcriptional units may exist. At least two other genes with an unidentified role in peptidoglycan synthesis also map within this cluster.

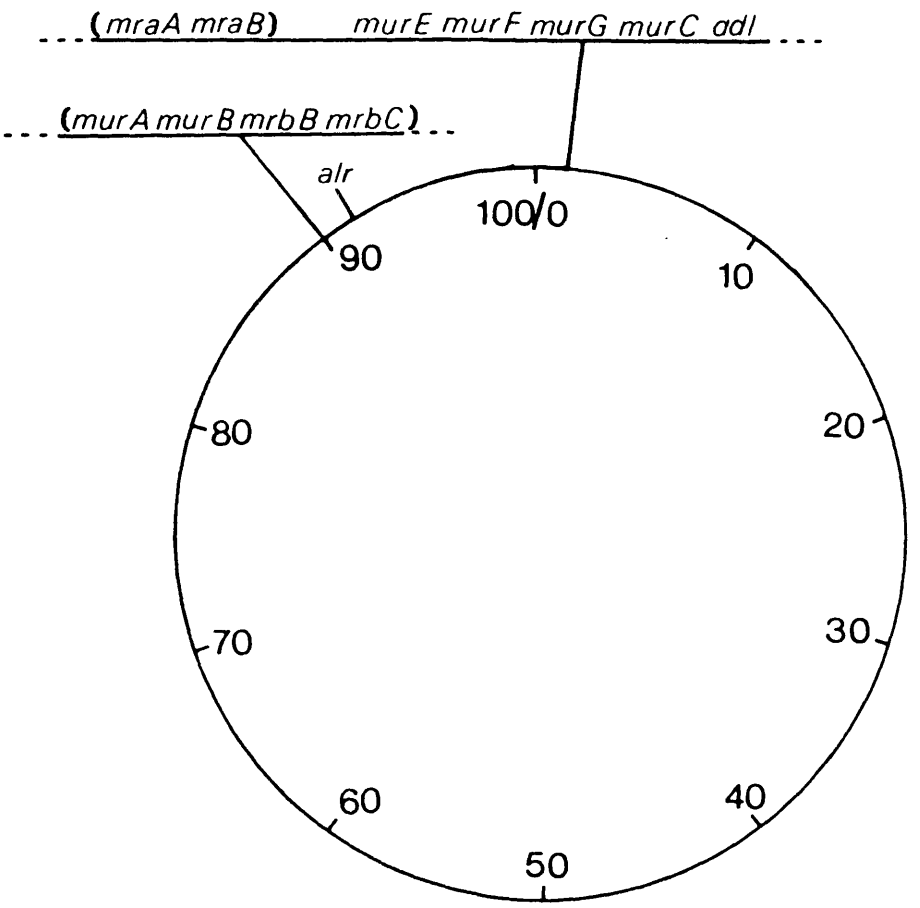
A second cluster of genes involved in peptidoglycan synthesis (mrb) lies at 90' on the chromosome. murA codes for PEP : UDP-GlcNAc transferase (Wu and Venkateswaran, 1974) and murB codes for UDP-GlcNAc-enolpyruvate reductase (Matsuzawa et al., 1969). Other related genes also lie within this cluster.

The only gene involved in the early stages of peptidoglycan synthesis which does not map within these two clusters is alr, the

Figure 1.5

Map of the E.coli chromosome showing the locations of genes involved in the early stages of peptidoglycan synthesis.

The map shows genes murG, mraA, mraB, mrbB and mrbC which have an unidentified role in peptidoglycan synthesis. The brackets indicate that the precise location or order of the genes is not known.


(mraA mraB)
murE murF murG murC adl
(murA murB mrbB mrbC)
alr
100/0
10
20
30
40
50
60
70
80
90

structural gene for alanine racemase. However, it has been suggested that its main function is the conversion of L-alanine to pyruvate via D-alanine (Wijsman, 1972), and it is therefore not surprising that it does not map with the other peptidoglycan synthesis genes.

II(c) Transfer To The Membrane

The UDP-MurNAc-pentapeptide is now transferred to an undecaprenyl phosphate carrier in the cytoplasmic membrane. GlcNAc is transglycosylated from UDP-GlcNAc to the undecaprenyl-P-P-MurNAc-pentapeptide, to form an activated disaccharide-peptide, which is the basic subunit of peptidoglycan synthesis.

II(d) Polymerization And Cross-linking

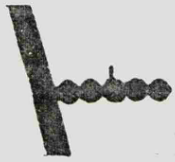
The precursor subunit is transferred to the periplasmic side of the cytoplasmic membrane, where it is attached to the growing glycan chain to form nascent uncross-linked peptidoglycan (Figure 1.6). The average length of glycan strains in E.coli is about 50 disaccharides. Finally, the nascent peptidoglycan is cross-linked to the preexisting sacculus. This cross-linking gives the peptidoglycan its strength. Covalent bands are formed between the penultimate D-alanine residue in one peptide side chain and the free amino group of the DAP in a second peptide, with the loss of the terminal D-alanine in the first side chain. This transpeptidation reaction allows the synthesis of peptide bonds outside the cytoplasmic membrane in the absence of energy donors such as ATP.

Relatively little is known about these final stages in peptidoglycan synthesis. Also, little is known about how and where lipoprotein is attached. The cell sacculus is an insoluble macromolecule, insoluble even in hot SDS. Nascent peptidoglycan becomes insoluble the moment the first covalent bond is formed with the sacculus. Other fractions of

Figure 1.6

Cross-linking of nascent peptidoglycan to the cell wall.

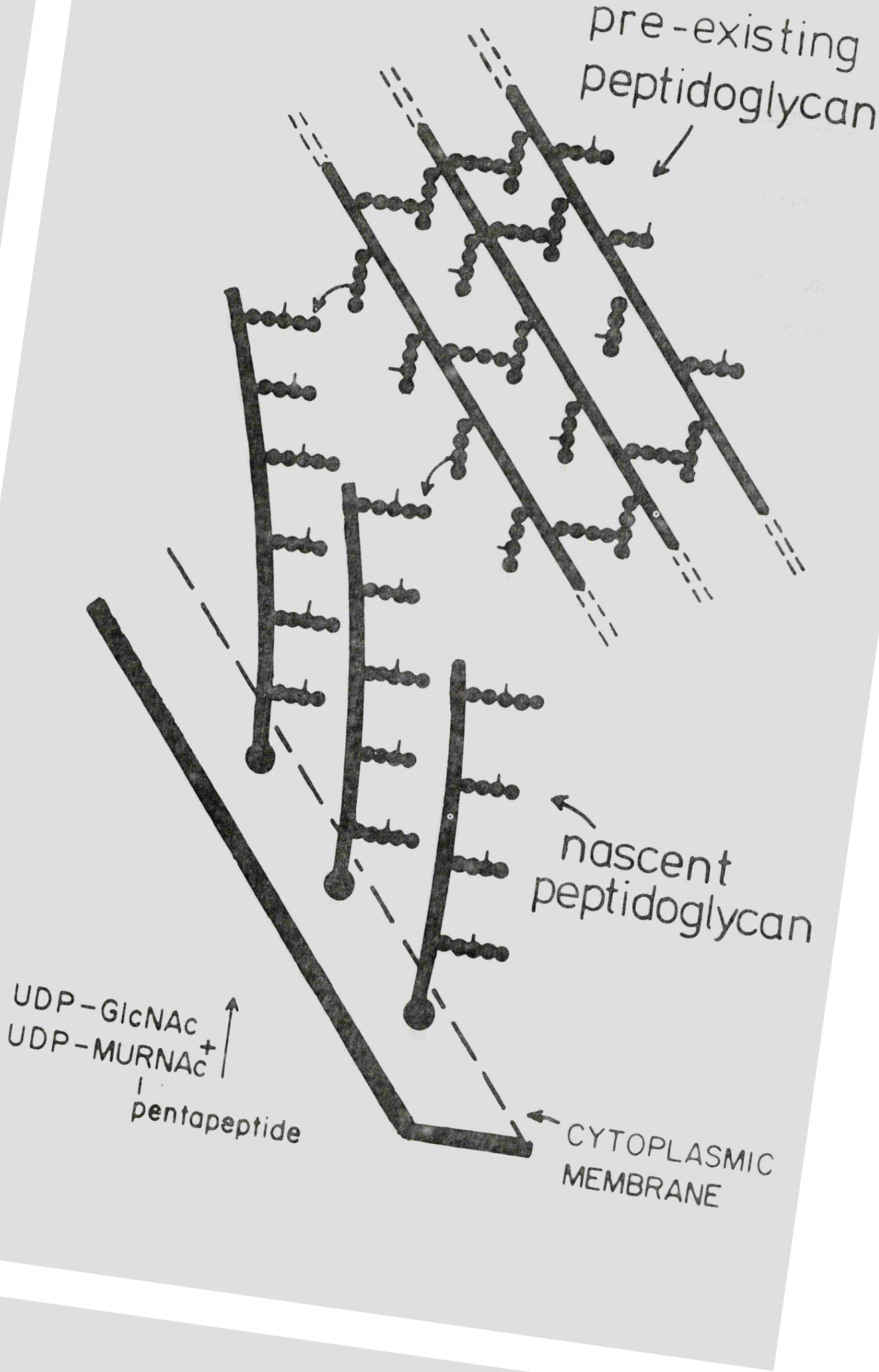
UDP-GlcNAc and UDP-MurNAc-pentapeptide are transferred via a membrane-bound carrier to the outer surface of the cytoplasmic membrane. The nascent peptidoglycan forms cross-links with the peptidoglycan in the sacculus with the loss of the terminal D-ala residue.



= GlcNAc

MurNAc-L-ala-D-glu-m-dap-D-ala-D-ala

Taken from Spratt (1978b).


pre-existing
peptidoglycan
nascent
peptidoglycan
UDP-GlcNAc
+
UDP-MURNAc
|
pentapeptide
CYTOPLASMIC
MEMBRANE

peptidoglycan have been described (Mirelman et al., 1976; Mett et al., 1980), which probably represent late intermediates in peptidoglycan synthesis.

Mett et al. (1980) labelled peptidoglycan, disrupted the cells using a French pressure cell, and separated the supernatant from the envelopes. They identified a soluble macromolecular peptidoglycan fraction which was loosely crosslinked in the supernatant (19% of pentapeptides cross-linked compared to 27%), with a high proportion of pentapeptides (20%; sacculus 1-4%) and some bound lipoprotein (4.6%; sacculus 8-10%). Mirelman described a TCA-insoluble, SDS-soluble fraction, which was approximately 22% crosslinked, with 10% pentapeptide side chains.

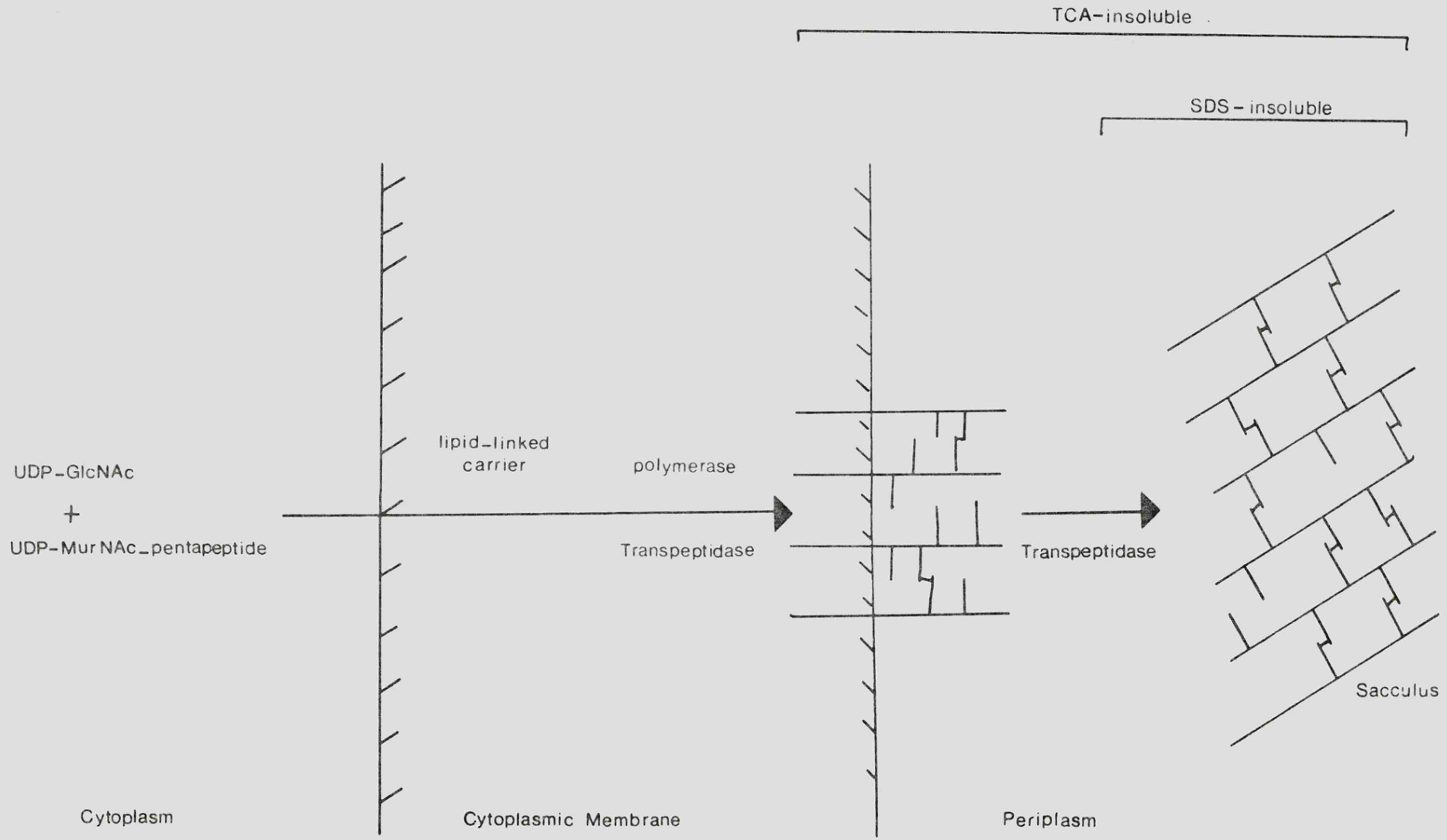
Mett et al. concluded that lipid-disaccharide-pentapeptides (or oligosaccharides) are polymerized into a series of loosely cross-linked, higher molecular weight peptidoglycan strands. These became gradually more cross-linked and more tightly associated with the envelope, such that on mechanical disruption of the cells, the loosely cross-linked peptidoglycan is still in the cell-soluble fraction (presumably the periplasm). Intermediates with higher cross-linking can be solubilized from the cell envelope only with detergent. Finally they became covalently joined to the sacculus (Figure 1.7). The cross-linking of lipoprotein to the peptidoglycan follows a similar pattern.

The work described, however, does not begin to answer how the assembly of peptidoglycan is regulated in different regions of the cell, at different points in the cell cycle.

Figure 1.7

Diagram illustrating the different fractions of peptidoglycan.

The sacculus is insoluble when boiled in 4% SDS. The partially cross-linked peptidoglycan which is not covalently linked to the sacculus is soluble in boiling SDS, but is precipitated in 5% trichloroacetic acid.


TCA-insoluble
SDS - insoluble
UDP-GlcNAc
+
UDP-Mur NAc_pentapeptide
lipid-linked carrier
polymerase
Transpeptidase
Transpeptidase
Sacculus
Cytoplasm
Cytoplasmic Membrane
Periplasm

III. THE MECHANISM OF ACTION OF β-LACTAM ANTIBIOTICS

III(a) The Structural Analogue Hypothesis

The crosslinking of the peptidoglycan is the reaction inhibited by the β-lactam antibiotics (see Figure 1.8). All reactions upto and including the formation of long uncrosslinked peptidoglycan chains are insensitive to these antibiotics.

The first clue as to the mechanism of action of β-lactam antibiotics was provided by Wise and Park (1965) who measured the concentration of alanine residues in cell walls prepared from Staphylococcus aureus grown in the presence and absence of sub-lethal concentrations of penicillin. They demonstrated that the walls from cells grown in penicillin contained more alanine than those grown in its absence. This was confirmed independently by Tipper and Strominger (1965) who showed directly the presence of an increased number of uncrosslinked pentapeptide monomer units. It therefore seemed that penicillin inhibits the final transpeptidation reaction in peptidoglycan synthesis.

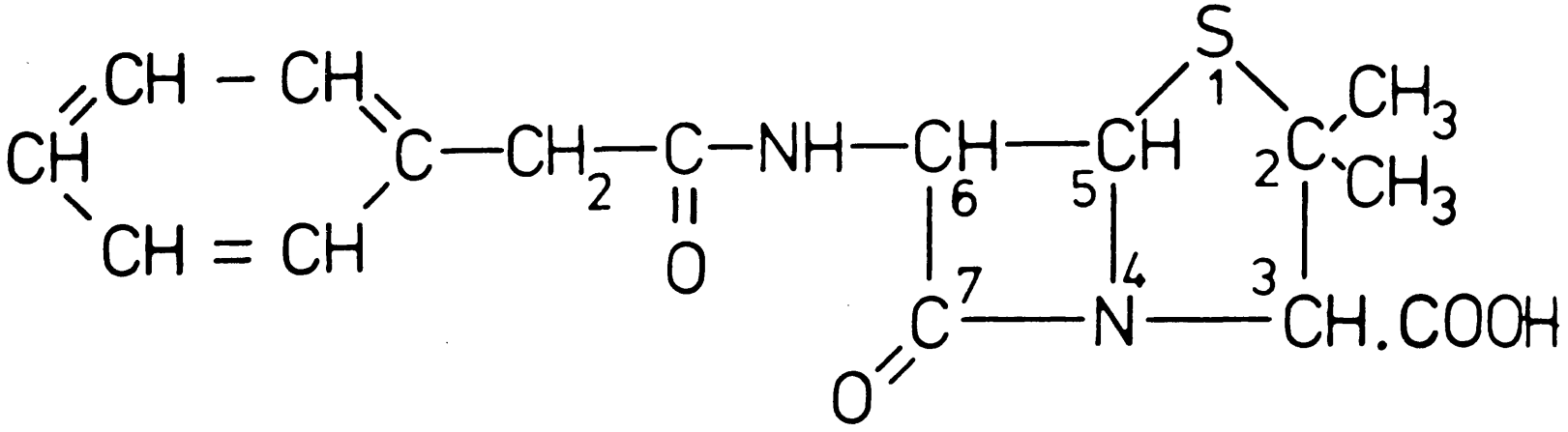
Tipper and Strominger hypothesized that penicillin was a structural analogue of acyl-D-alanyl-D-alanine (see Figure 1.9). They proposed that transpeptidation normally proceeds via an acyl-D-alanine-enzyme intermediate with the release of the terminal D-alanine residue. The acylated enzyme would then react with a free amino group in a neighbouring side chain to form the peptide crosslink, with the release of the enzyme. In the presence of penicillin, the enzyme binds the antibiotic, causing the cleavage of the highly-strained β-lactam ring and resulting in an inactive penicilloyl-enzyme complex. The interaction between penillin and the enzyme occurs in two stages:

Figure 1.8

The chemical structures of (A) benzylpenicillin and (B) the amidinopenicillanic acid, mecillinam.

Taken from Spratt (1978b).

(A)



(B)

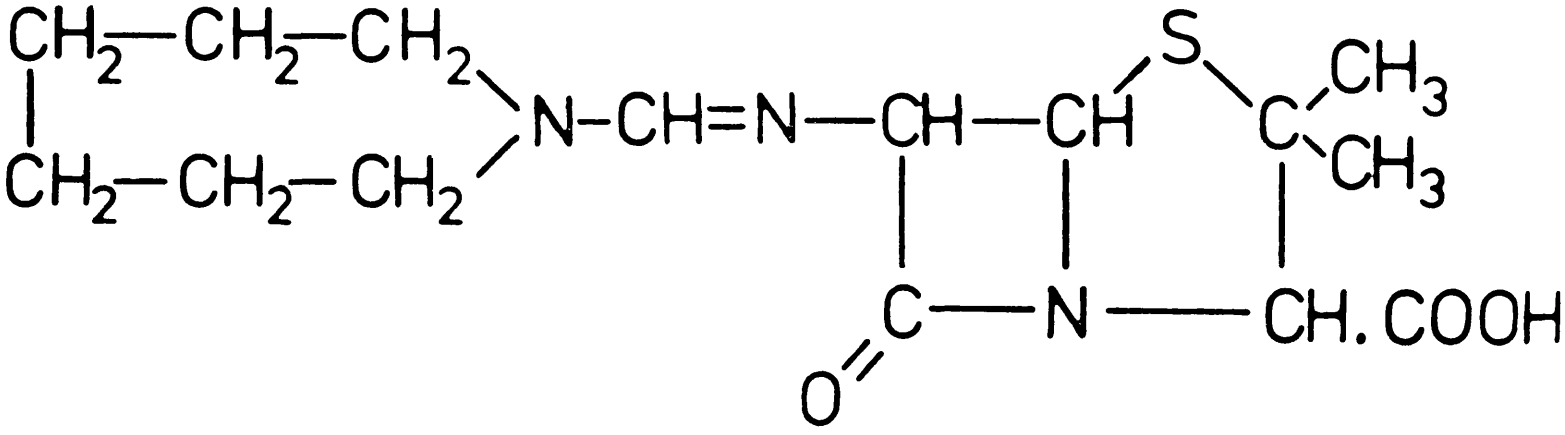
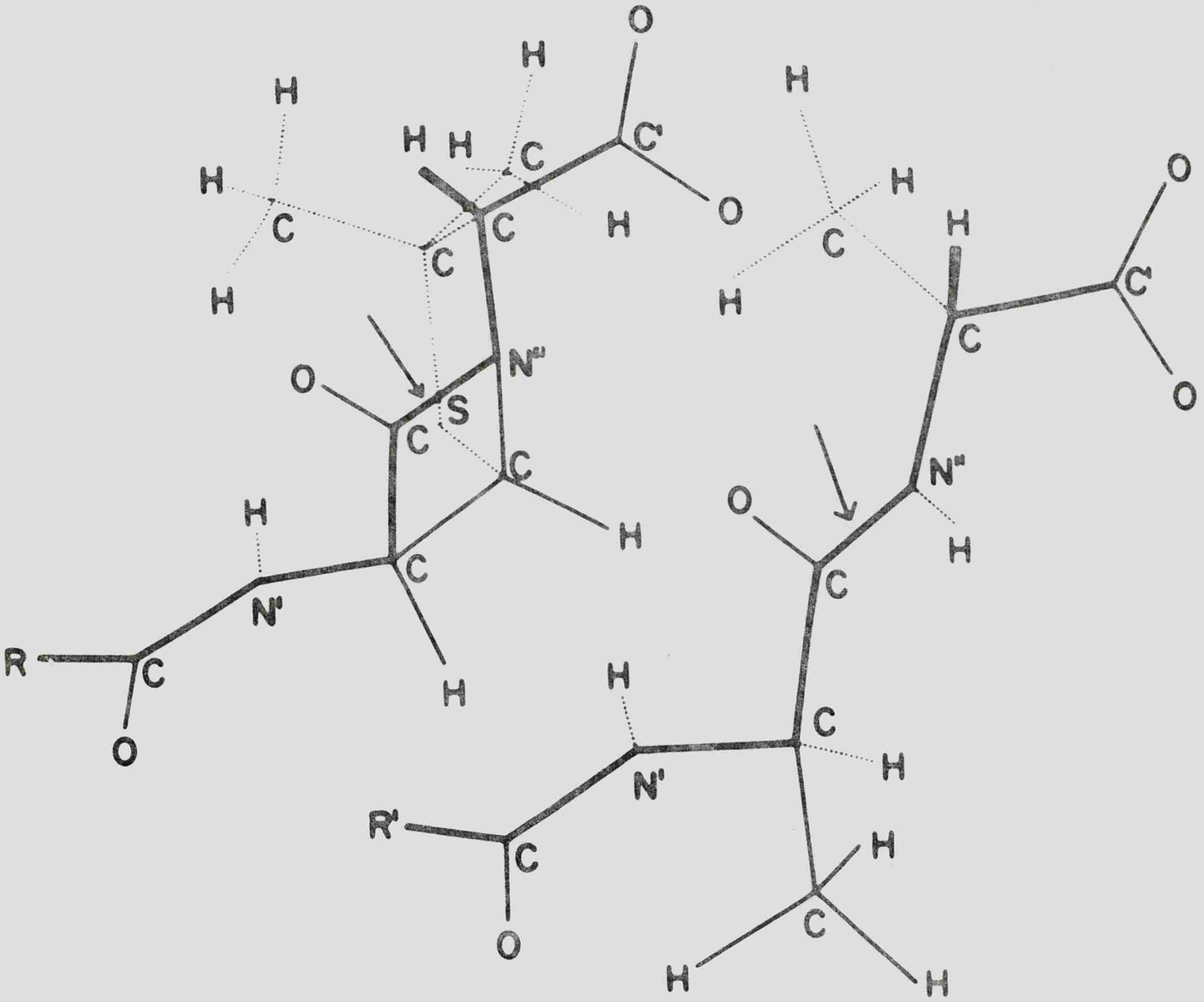


Figure 1.9

Molecular models of a penicillin (left) and the terminal -D-ala-D-ala of the pentapeptide sidechain of the substrate for the transpeptidase (right). The arrows mark the analogous CO-N bond which is cleaved in the reaction of the transpeptidase with substrate or with penicillin.

Taken from Blumberg and Strominger (1974).



R
R'
N'
N''
S
C'

$$E + P \rightleftharpoons EP \longrightarrow EP^* \longrightarrow E + X + Y$$

Penicillin (P) reacts with enzyme (E) through a rapid equilibrium process to form the inactive complex (EP). The penicillin then becomes covalently bound to the enzyme, forming the modified complex (EP*). This can be enzymatically broken down to release active enzyme and fragmentation products of the penicillin (X and Y). The rate of this breakdown varies considerably depending on the enzyme and the β-lactam involved, and is in many cases extremely slow.

While this theory is very attractive, and has in fact been established for some enzymes (see Section III(c)), other hypotheses have been put forward. In particular, Ghuysen and his colleagues proposed an allosteric mode of action, where penicillin binds to a site on the enzyme, other than the active site, so producing a conformational change to the enzyme's active site (see Ghuysen et al., 1979).

III(b) Penicillin-Sensitive Enzymes

Izaki et al. (1966) showed that transpeptidation was not the only penicillin-sensitive enzyme (PSE) activity. The mechanism of action of β-lactam antibiotics is therefore more complex than originally thought. Three PSE activities have been identified - transpeptidase, D-alanine carboxypeptidase and endopeptidase (see Figure 1.10).

(i) Transpeptidase

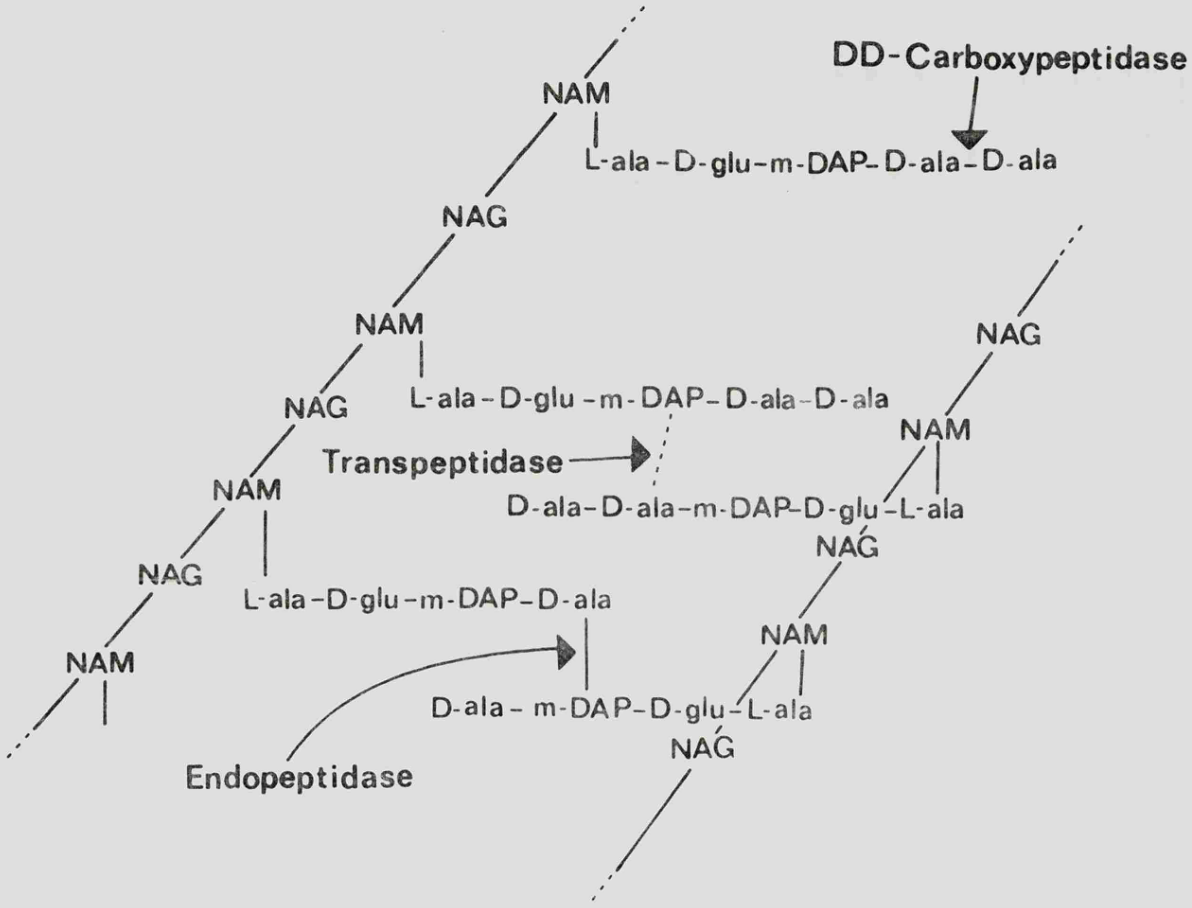
As described above, the transpeptidase catalyses the formation of a cross-link between the penultimate D-alanine residue on one peptide side-chain (donor), and the free amino group in the meso-diaminopimelic acid residue on a second peptide sidechain (acceptor), with the concomitant release of the terminal D-alanine in the donor peptide. Transpeptidation

Figure 1.10

Diagram illustrating the action of transpeptidase, DD-carboxy-peptidase and endopeptidase.

The dotted line indicates the creation of a bond between the D-alanine and m-diaminopimelic acid residues.

NAM, N-acetyl muramic acid; NAG, N-acetyl glucosamine, L-ala, L-alanine; D-glu, D-glutamate; m-DAP, meso-diaminopimelic acid; D-ala, D-alanine.


DD-Carboxypeptidase
NAM
L-ala-D-glu-m-DAP-D-ala-D-ala
NAG
NAM
NAG
L-ala-D-glu-m-DAP-D-ala-D-ala
Transpeptidase
NAM
D-ala-D-ala-m-DAP-D-glu-L-ala
NAG
NAG
L-ala-D-glu-m-DAP-D-ala
NAM
NAM
D-ala-m-DAP-D-glu-L-ala
NAG
Endopeptidase

in vitro was first demonstrated using *E.coli* membrane preparations, obtained by sonication of the cells followed by a series of differential centrifugations (Izaki *et al.*, 1966). More recently, ether-treated cells have been used (Mirelman *et al.*, 1976), which are permeable to exogenous nucleotide precursors, and the enzymes presumably retain close association to the cell wall.

Several groups have purified PSEs from various species to homogeneity, and have demonstrated model transpeptidase activity, using either natural or unnatural substrates (see Table 1.1). These model activities may or may not be of relevance *in vivo*.

(ii) D-alanine Carboxypeptidase

Chemical analysis of the peptidoglycan showed that D-alanine residues not involved in cross-link formation were absent from the peptidoglycan. This suggested that terminal D-alanine residues might be removed by an enzyme or enzymes with D-alanine carboxypeptidase activity. These enzymes were first described in *E.coli* by Weidel and Pelzer (1964) and were found to be penicillin-sensitive by Izaki *et al.* (1966).

Izaki and Strominger (1968) identified two D-alanine carboxypeptidase activities in an *E.coli* B strain. DD-carboxypeptidase (carboxypeptidase I) cleaved the terminal D-alanine residue from UDP-MurNAc-pentapeptide (see Table 1.1). This reaction was highly penicillin-sensitive. L-D carboxypeptidase (carboxypeptidase II) cleaved the L-D linkage between D-alanine and m-diaminopimelic acid in UDP-MurNAc-tetrapeptide, and was not inhibited by β-lactam antibiotics.

The DD-carboxypeptidase activity was purified to homogeneity by Tamura *et al.* (1976). It was subdivided into two membrane-bound

Table 1.1.

Methods of measuring enzyme activities in E.coli

Enzyme activity	Substrates	Reaction products
D,D carboxypeptidase	UDP-MurAc → L-Ala → D-Glu-OH (L) → DAP → D-Ala → D-Ala H (D) OH + H_2O	UDP-MurAc → L-Ala → D-Glu-OH (L) → DAP → D-Ala + D-Ala H (D) OH
Unnatural model transpeptidase	UDP-MurAc → L-Ala-D-Glu-OH (L) → DAP → D-Ala → D-Ala H (D) OH + gly(or D-ala*)	UDP-MurAc → L-Ala → D-Glu-OH (L) → DAP → D-Ala → gly(or D-Ala*) H (D) OH + D-Ala
Natural model transpeptidase (with concomitant hydrolysis of donor)	L-Ala → D-Glu-OH (L) → DAP → D-Ala → D-Ala H (D) OH + H_2O as both donor and acceptor	L-Ala → D-Glu-OH (L) → DAP → D-Ala → D-Ala L-Ala → D-Glu-OH (L) → DAP → D-Ala → (D) OH H (D) OH + L-Ala → D-Glu-OH (L) → DAP → D-Ala + D-Ala H (D) OH
Endopeptidase	—GlcNAc-MurAc— L-Ala D-Glu-OH (L) → DAP → D-Ala → (D) OH H (D) OH —GlcNAc-MurAc— L-Ala D-Glu-OH (L) → DAP → D-Ala	—GlcNAc-MurAc— L-Ala D-Glu-OH (L) → DAP → D-Ala H (D) OH

Taken from Rogers et al. (1980)

activities, carboxypeptidases IA and IB, and a soluble form, IC. It appears that carboxypeptidases IB and IC are determined by the same enzyme, as a mutation in the dacB gene abolishes both activities (see Section III(g)). Carboxypeptidase IA differs from these activities, however, in that it is inhibited by p-chloromercuribenzoate, indicating the presence of a sulphydryl group important to enzymatic function, while carboxypeptidase IB is not. Furthermore, carboxypeptidase IA shows significant unnatural model transpeptidase activity, while carboxypeptidases IB and IC do not.

E.coli peptidoglycan contains both tri- and tetrapeptide side chains, so both carboxypeptidases I and II function in vivo. Carboxypeptidase II appears to be periplasmic, and a role for this enzyme in the regulation of the binding of lipoprotein to peptidoglycan has been postulated (see section IV(e)), although there is no real evidence for this.

(iii) Endopeptidase

The endopeptidase removes cross-links by cleavage of the D-ala-mDAP bond synthesized by transpeptidases (see Table 1.1). Two endopeptidase activities have been identified, only one of which is penicillin-sensitive (Tomioka and Matsuhashi, 1978). The endopeptidase substrate formally resembles that of the D-alanine carboxypeptidase, and this is supported by the fact that many carboxypeptidases also possess endopeptidase activity. The major penicillin-sensitive DD-endopeptidase is identical to carboxypeptidase IB (see section III(g)). Carboxypeptidase IA shows no endopeptidase activity.

III(c) Evidence For The Structural Analogue Hypothesis

The DD-carboxypeptidases are the most abundant and the easiest to purify of the PSEs, and they have been the subject of the most detailed study of the interaction of penicillin and substrate with PSEs. They have been purified from several different organisms, including E.coli, B.subtilis, B.stearothermophilus and several Actinomycetes. Some of the purified enzymes also have model transpeptidase and endopeptidase activity.

The DD-carboxypeptidases from Streptomyces R61 (Frere et al., 1976), B.subtilis and B.stearothermophilus (Yocum et al., 1979) were incubated with radioactively labelled penicillin and denatured. After protease digestion, radioactive peptides were isolated and sequenced. In each case, the penicillin was covalently bound to an L-serine residue. Yocum et al. (1979) then repeated this using the radioactively labelled substrate analogue [^{14}C]-diacetyl-L-lys-D-ala-D-lactate, trapping the intermediate substrate-enzyme complex by rapid denaturation. They showed that in both bacilli carboxypeptidases, the substrate bound to the same serine (residue 36) to which penicillin bound.

This evidence very strongly supports the structural analogue hypothesis for the inhibition by penicillin of these DD-carboxypeptidases (see Section III(a)). However, there is as yet no such evidence regarding the major transpeptidases. Furthermore, while there is considerable amino acid sequence homology between the major DD-carboxypeptidases from different organisms, these seem to show little detectable homology with transpeptidases (Waxman et al., 1982; Maruyama et al., 1982; B.G. Spratt and J.K. Broome-Smith, unpublished results). Therefore, other theories such as the action of penicillin as an allosteric inhibitor cannot be ruled out regarding these high molecular weight PBPs. Studies of the

site of binding of penicillin to these PBPs should soon resolve this issue.

III(d) Direction Of Transpeptidation

Transpeptidation is a directional process. A donor peptide provides a terminal D-ala-D-ala, while a recipient peptide provides an amino group. A nascent peptidoglycan strand can therefore attach itself to the pre-existing wall as a donor or recipient. It can also attach itself to another nascent strand. Many bacteria, including E.coli, possess active DD-carboxypeptidases, which remove the terminal D-alanine residue from pentapeptide sidechains. This means that a high proportion of the free side chains in the old peptidoglycan would be tetrapeptides which can only act as acceptors in transpeptidation reactions, with nascent peptidoglycan supplying the donor peptides. Bacteria which have been shown to synthesize peptidoglycan in this manner are Bacillus licheniformis (Ward and Perkins, 1974), Bacillus megaterium (Giles and Reynolds, 1979) and Pseudomonas aeruginosa (Mirelman and Nuchamowitz, 1979). As in E.coli, these bacteria possess penicillin-sensitive transpeptidases, and inhibition of these transpeptidases by penicillin leads to death of the cell.

An exception to this is Gaffkya homari, which catalyses transpeptidation in the opposite orientation (Hammes and Kandler, 1976). Approximately 50% of the pentapeptides in nascent peptidoglycan have one or both D-alanine residues removed by the sequential action of DD- and DL-carboxypeptidases. The pentapeptides remaining in the mature peptidoglycan then act as donors, while the tri- and tetrapeptides in the nascent peptidoglycan act as acceptors in a transpeptidation reaction which is insensitive to inhibition by β-lactam antibiotics. The killing target of

β-lactams in this organism is the DD-carboxypeptidase, since inhibition of this enzyme prevents the formation of tetrapeptides (Hammes, 1976). Presumably, pentapeptides are poor substrates as acceptors in this transpeptidation. The DD-carboxypeptidase may also be the killing target of β-lactam antibiotics in Streptococcus faecalis (Coyette et al., 1978).

III(e) Penicillin-binding Proteins

Early studies on the binding of penicillin showed the existence of a small number of high-affinity binding sites (see review by Blumberg and Strominger, 1974). These sites were identified as cytoplasmic membrane proteins, and were assumed to correspond to the penicillin-sensitive enzymes. Penicillin binds covalently to these sites, so penicillin-binding proteins (PBPs) can be easily detected by incubating membranes with [^{14}C] benzylpenicillin followed by separation of the proteins by SDS-PAGE and fluorography. All organisms tested have multiple PBPs. E.coli has seven PBPs (Spratt, 1977c; Figure 1.11), and most gram negative bacteria have similar, though not identical, proteins. Gram positive organisms generally possess three to five PBPs, and do not seem to correspond closely to the characteristic pattern of PBPs found among gram negative bacteria.

The PBPs of E.coli have been studied using a variety of techniques. Different β-lactam antibiotics bind the individual PBPs with different affinities. This can be detected by measuring the competition between a particular β-lactam and [^{14}C] benzylpenicillin for each PBP. Correlation of these data with the morphological effects of different antibiotics allows conclusions to be drawn about the effect of inhibition of the different PBPs. A second approach is the purification of individual PBPs and the identification of enzyme activities. Thirdly, mutants have been isolated which have an altered or non-functional PBP.

Figure 1.11

Detection of the PBPs of *E.coli* by scintillation autoradiography.

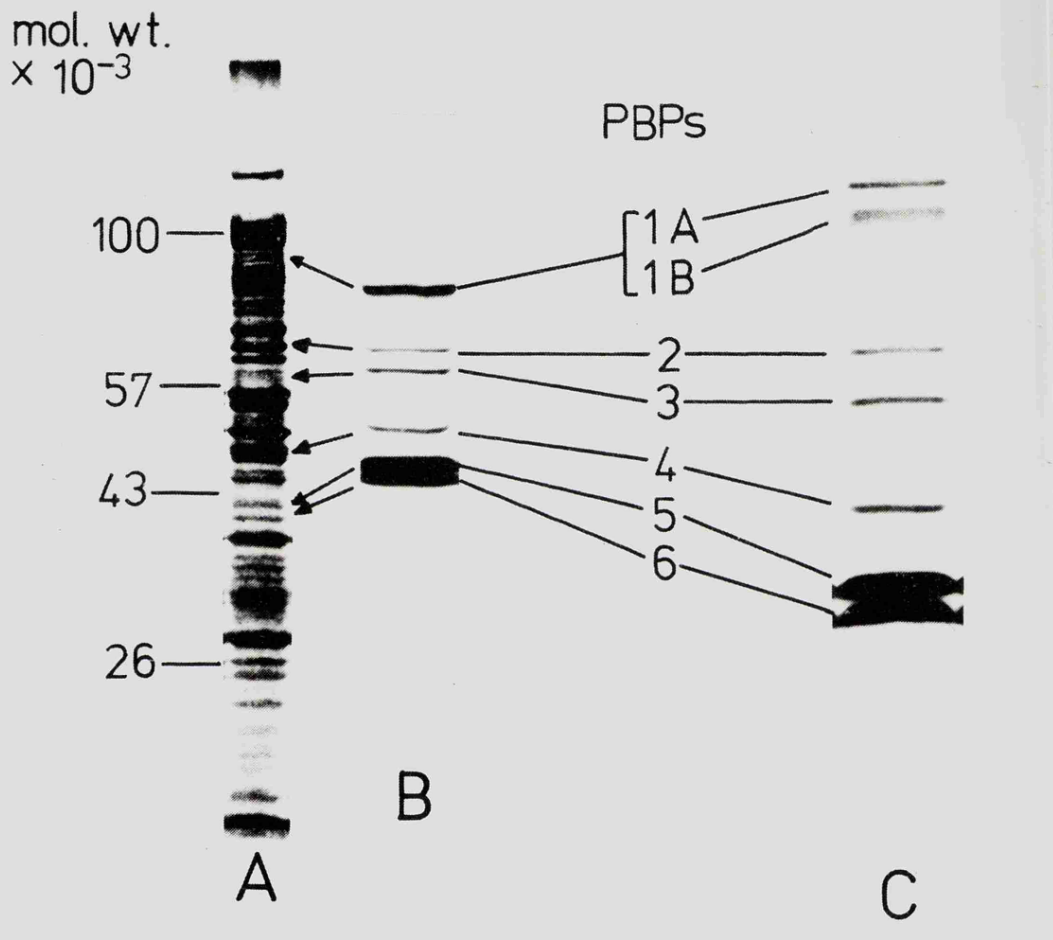
[^{14}C]benzylpenicillin was bound to cell envelopes and the cytoplasmic membrane proteins were fractionated on a SDS-polyacrylamide gel.

(A) The gel was stained to show the complex pattern of proteins in the membrane.

(B) A similar gel was autoradiographed to detect PBPs. Arrows mark the position of the PBPs on the stained gel.

(C) An autoradiograph showing PBPs fractionated in gel system using a different acrylamide:bis-acrylamide ratio, which resolves PBP1 into 2 components.

Taken from Spratt (1978b).


mol. wt.
× 10⁻³
100
57
43
26
PBPs
1A
1B
2
3
4
5
6
A
B
C

Fourthly, the structural genes for the PBPs have been cloned, allowing more sophisticated analytical and mutagenic techniques to be employed.

III(f) Identification Of The Killing Targets Of β-lactams

Since penicillin binds to seven proteins in E.coli, it is of interest to know which of these proteins is the killing target of the antibiotic. Growth of E.coli in the presence of a high concentration of penicillin causes the cells to lyse. However, penicillin can also cause cells to grow as long filaments, as filaments with bulges, or as spheres (see Figure 1.12). Spratt (1975) examined the effects of different concentrations of various β-lactams on the growth of E.coli. For most of the antibiotics (e.g. ampicillin and cephalexin), low concentrations caused cells to grow as filaments, leading eventually to lysis. At higher concentrations, cell elongation is inhibited, leading to more rapid lysis. With some β-lactams, bulges appear in the middle of filamenting cells. High concentrations of virtually all β-lactams rapidly inhibit cell elongation, leading to cell lysis and spheroplast production. The 6β-amidinopenicillanic acid, mecillinam (see Figure 1.8), is unique in causing cells to grow as spheres over at least a thousand-fold concentration range.

These experiments suggested that cell lysis (inhibition of elongation) was caused by binding of penicillin to PBP IB, that filamentation (inhibition of division) was caused by binding to PBP3, and that growth of cells as spheres was caused by binding to PBP2. Indeed, mecillinam binds specifically to PBP2.

These findings were confirmed by the isolation of bacteria carrying mutant PBPs. A mutant, SP63, was isolated which divided normally at 30°C, but formed filaments at 42°C (Spratt, 1975). [^{14}C] benzylpenicillin bound to PBP3 in this mutant at 30°C, but not at 42°C. The thermo-

Figure 1.12

Electron micrographs of E.coli grown in the presence of β-lactam antibiotics.

(A) control cells

(B) a filament produced by a low concentration of penicillin

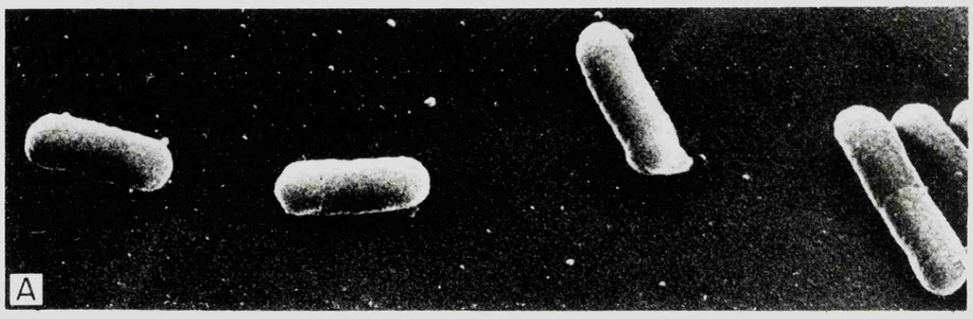
(C) a lysed cell extruding a spheroplast, produced by a high concentration of penicillin

(D) spherical cells produced by mecillinam

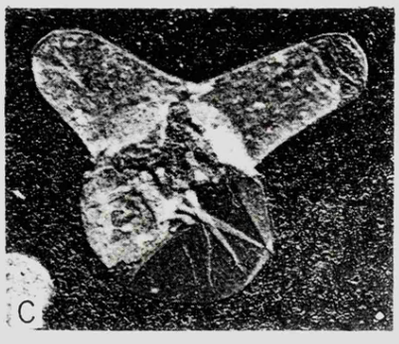
(A), (B) and (D) are scanning electron micrographs x 11,000.

(C) is an electron micrograph of a negatively stained preparation x 17,000.

Taken from Spratt (1978b).


A


B


C

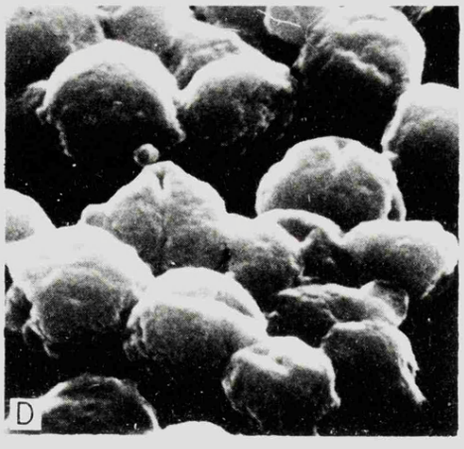

D

Table 1.2 Properties of *E.coli* PBPs

PBP	MW	Molecules Per Cell	Effect Of Inactivation	Function
1A	92,000	170	Rapid cell lysis occurs if both PBP1A and PBP1B are inactivated	Elongation
1B	90,000	60		
2	66,000	20	Formation of spherical cells	Cell shape
3	60,000	50	Formation of filamentous cells	Cell division
4	49,000	110	No obvious growth defect	?
5	42,000	1,800	No obvious growth defect	?
6	40,000	570	No obvious growth defect	?

labile PBP3 was found to be the cause of the morphological effects thus establishing the role of the protein in cell division. The structural gene, pbpB (also called sep and ftsI), was shown to map at about 1.8 minutes, close to the mra gene cluster involved in early peptidoglycan synthesis (Suzuki et al., 1978).

A mutant, SP137, was isolated which grew as a normal rod at 30°C, but which became spherical at 42°C (Spratt, 1977b). Binding of penicillin to PBP2 was correspondingly temperature-sensitive showing that PBP2 is involved in the maintenance of the rod-shape of the cell. The structural gene, pbpA, maps at 14.4 minutes (Suzuki et al., 1978).

A temperature-sensitive mutant which had a thermolabile PBP IB was found to lyse at the restrictive temperature (Tamaki et al., 1977). Other mutants with a defective PBP IB were viable, but the introduction of a temperature-sensitive mutation in PBP IA caused the cells to lyse at high temperature (Suzuki et al., 1978). It was concluded that PBP IB is the major cell transpeptidase, but that PBP IA can in some circumstances take over from PBP IB (Matsuhashi et al., 1978). Mutants lacking PBP IA alone were quite healthy (Spratt et al., 1977), and they showed a slower rate of lysis in the presence of several β-lactams. Mutants lacking both PBPs IA and IB are not viable. Mutations in dacB, dacA and dacC, the structural genes for PBPs4, 5 and 6 respectively, are not lethal (see section III(g)). These results are summarized in Table 1.2.

III(g) Enzyme Activities Catalysed By PBPs

As described in the last section, PBP IB was postulated as the major cell transpeptidase. Membrane preparations from mutants lacking either PBP1A or PBP1B (Suzuki et al., 1978) were examined. Mutants defective in PBP IB were capable of synthesizing

lipid-linked intermediates, but no peptidoglycan. Mutants lacking PBP IA showed only a slight decrease in peptidoglycan synthesis as compared with wild-type cells. This suggests that PBP IB is more than a transpeptidase, or membranes from PBP IB mutants would catalyse synthesis of uncross-linked peptidoglycan strands. Purified PBP IB has been found to catalyse synthesis of crosslinked peptidoglycan from undecaprenyl-P-P-disaccharide-pentapeptide (Nakagawa et al., 1979; Suzuki et al., 1980), so it is a peptidoglycan transglycosylase as well as a transpeptidase, although only the transpeptidation is penicillin-sensitive. The same dual activity was found with purified PBP IA (Ishino et al., 1980).

PBP2 has been purified (Curtis and Strominger, 1981) but did not catalyse any of the model enzyme systems tried. Recently, Ishino et al. (1982) have demonstrated a mecillinam-sensitive transpeptidase activity in membranes that contained approximately 50-fold overproduction of PBP2 and in which the other PBPs had been inactivated. PBP2 is therefore thought to be a transpeptidase. It is not clear whether any trans-glycosylase activity is associated with the protein.

Ishino and Matsuhashi (1979) reported that membranes from cells lacking PBP IB, but with a ten-fold overproduction of PBP3 catalysed a cephalexin-sensitive transpeptidation reaction. They later confirmed that purified PBP3 catalysed both transglycosylase and transpeptidase activities (Ishino and Matsuhashi, 1981).

Therefore, all the high molecular weight PBPs appear to be trans-peptidases, and most or all are also transglycosylases. It must be pointed out, however, that these conclusions are often based on very low activities in vitro.

Table 1.3 Enzyme activities catalysed by *E.coli* PBPs

PBP	Enzyme Activity Catalysed	Comments
1A	Transpeptidase/transglycosylase	These are the major transpeptidases and transglycosylases in the cell.
1B	Transpeptidase/transglycosylase	
2	Transpeptidase/? transglycosylase	
3	Transpeptidase/transglycosylase	
4	DD-carboxypeptidase/DD-endopeptidase	May act *in vivo* as a secondary transpeptidase (see Chapter 8).
5	DD-carboxypeptidase/model transpeptidase activity	These are the major DD-carboxypeptidase of the cell. They may be used to regulate the degree of cross-linking in the peptidoglycan.
6	DD-carboxypeptidase/model transpeptidase activity	

PBPs4, 5 and 6, which together constitute 89% of the PBP complement of the cell (Spratt, 1977c), all have DD-carboxypeptidase activity. PBP4 corresponds to carboxypeptidase IB, and also has DD-endopeptidase activity (Tamura et al., 1976; Iwaya and Strominger, 1977). Recent evidence suggests that this enzyme functions mainly as a transpeptidase in vivo, catalysing a secondary cross-linking of the peptidoglycan (see section IV(g)). However, mutants lacking PBP4 grow normally, except for a reduced rate of lysis in the presence of ampicillin (Iwaya and Strominger, 1977).

PBP5 and 6 together correspond to carboxypeptidase IA (Matsuhashi et al., 1979; Amanuma and Strominger, 1980). They both possess very weak β-lactamase activity, but differ in the rate of deacylation of the penicilloyl-enzyme complex. The half-lives of the penicilloyl-enzyme at 30°C are 5 minutes and 19 minutes respectively. They have both been shown to catalyse model transpeptidase activity in vitro, but the relevance of this in vivo is not clear. Mutants in which either of the genes coding for PBPs 5 and 6 (dacA and dacC respectively) has been deleted have been constructed and seem to grow without serious abnormalities (Spratt, 1980a; Broome-Smith and Spratt, 1982). dacA mutants show supersensitivity to β-lactam antibiotics, (Tamaki et al., 1978), but the reason for this is not clear. Until mutants lacking PBPs 4, 5 and 6 have been constructed, carboxypeptidase activity cannot be considered non-essential. There is evidence that carboxypeptidases may be involved in the cell cycle (see section IV(e)). The enzyme activities shown by PBPs are summarized in Table 1.3.

III(h) Cloning Of Genes Involved In Late Peptidoglycan Synthesis And Cell-Shape Determination

The cloning of genes involved in peptidoglycan synthesis should be of great benefit, firstly for the purification of the relevant proteins, or their overproduction in membrane preparations, in order to allow identification of activities catalysed by these enzymes, and secondly to allow more sophisticated analysis of the genes and their regulation.

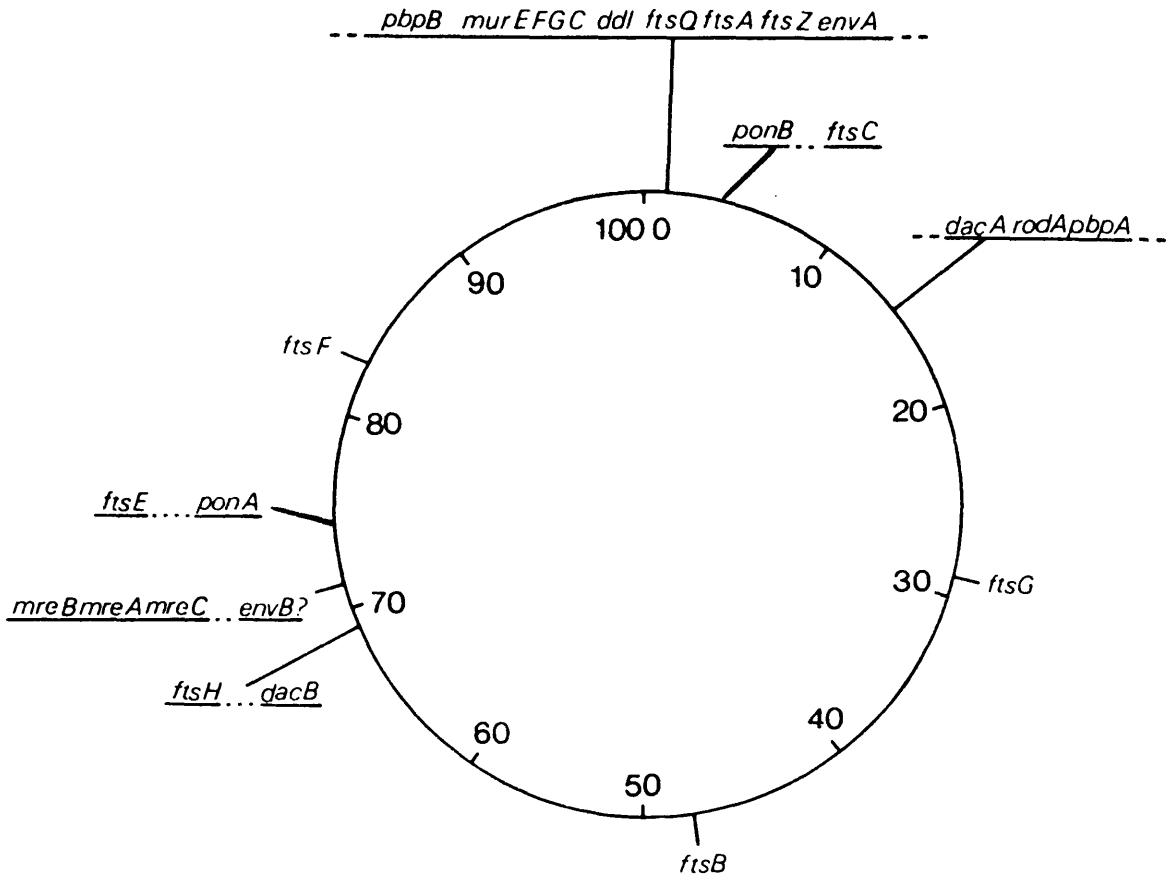
All seven PBP structural genes have been cloned. Takeda et al. (1981) screened the Carbon-Clarke collection and found plasmids carrying separately ponA (encoding PBP IA), ponB (PBP IB), pbpB (PBP3) and dacB (PBP4). PBPs IA, IB and 3 were overproduced 5- to 15-fold by these plasmids. PBP4 was not overproduced, but the reason for this is not known. It may be due to regulation of its expression, or if overproduction of the protein is deleterious to the cell, a mutation (e.g. in the promoter) may have been acquired. A plasmid was also isolated which corrected a temperature-sensitive PBP3 mutation, but which did not overproduce PBP3, and showed no homology with pbpB. They suggested that this plasmid carried a gene (which they called sui) which at high levels (due to gene dosage) can correct the pbpB$^-$ mutation. pbpB has also been cloned in a λ vector (Irwin et al., 1979).

No clone carrying pbpA (PBP2), dacA (PBP5) or dacC (PBP6) was found in the Carbon-Clarke plasmid collection. dacC was cloned onto a multicopy plasmid by Spratt(1980b), and pbpA and dacA were cloned together onto a λ transducing phage (Spratt et al., 1980). The phage, λdlip5, also carries the cell shape gene rodA. This cluster of genes (pbpA-rodA-dacA) will be examined in more detail in this thesis. The locations of the PBP genes on the E.coli chromosome are shown in Figure 1.13.

Figure 1.13

Map of the E.coli chromosome showing the locations of genes encoding penicillin-binding proteins and other genes which affect cell-shape.

dacC, the structural gene for PBP6 has not been mapped. The dotted lines separating ponA and ftsE, ponB and ftsC, and dacB and ftsE indicate that the genes have been mapped to approximately the same region, but are not otherwise known to be closely linked. envB may be identical to mreB (see text).


pbpB murEFGC ddl ftsQ ftsA ftsZ envA
ponB ftsC
dacA rodA pbpA
100 0
10
20
30
ftsG
40
50
ftsB
60
70
mreB mreA mreC envB?
ftsH dacB
80
ftsE ponA
ftsF
90

These clones have already been used to purify PBPs IA and IB, and to identify minor enzyme activities catalysed by PBPs 2 and 3 (see section III(g)). *dacA* (Broome-Smith and Spratt, unpublished work) and *pbpB* (Maruyama *et al.*, 1982) have been sequenced, and the sequencing of the other genes is in progress.

Matsuhashi *et al.* (1982) have reported the cloning of another cluster of genes, the *mre* cluster, onto a λ transducing phage. They have identified three closely-linked genes - *mreA*, which may be involved in regulation of expression of PBPs IA and 2, *mreB* (probably identical to *envB* see section IV(c)), involved in cell shape determination, and *mreC*, which together with an *mreB*$^-$ mutation confers resistance to mecillinam. The order of genes is reported to be *fabE-mreB-mreA-mreC*. No gene products have been identified yet.

The *fts* genes are directly or indirectly involved in cell division, and therefore influence cell shape (see section IV(c)). Nishimura *et al.* (1977) screened the Carbon-Clarke collection for plasmids which corrected *fts* mutations. Plasmids carrying separately *ftsB*, *ftsE* and *ftsM* were found. *ftsA*, *ftsQ* and *ftsZ* have been cloned together onto a λ transducing phage (Lutkenhaus and Donachie, 1979; Lutkenhaus *et al.*, 1980; Begg *et al.*, 1980). The *ftsA* and *ftsZ* gene products have been identified (Lutkenhaus and Donachie, 1979; Lutkenhaus and Wu, 1980). The map locations of the *mre* cluster and these *fts* genes are shown in Figure 1.13).

IV. CELL SHAPE

IV(a) Components Involved In Cell Shape Maintenance

That peptidoglycan is important in maintaining the rod-shape of E.coli is unquestioned. Degradation of the peptidoglycan by lysozyme leads to the formation of spheroplasts. However, the role played by other components of the cell wall is less clear. Cells lacking both lipoprotein and protein II* from the outer membrane grow as osmotically-stable spherical cells (Sonntag et al., 1978), although the loss of only one of these proteins does not have this morphological effect. Thus, there appears to be a role for the outer membrane in cell shape maintenance. Indeed, under certain conditions, purified outer membranes retain the rod-shape of the cell (Henning et al., 1973). Proteins Ia and Ib will form a lattice structure on peptidoglycan (see Section I(e)). These proteins were therefore thought to be involved in cell-shape maintenance. The claims were shown to be wrong, since triple mutants lacking lipoprotein, and proteins Ia and Ib show no gross defect (Sonntag et al., 1978).

It is perhaps naive to speak of whether peptidoglycan alone is responsible for cell-shape maintenance, since the cell wall is such a complex, integrated structure. As for cell-shape determination, this must reflect the specificities of the enzymes which synthesize the cell wall in general, and the peptidoglycan in particular.

IV(b) The Site Of Insertion Of Nascent Peptidoglycan

Elucidation of the functions of specific enzymes in cell-shape determination requires an understanding of how nascent peptidoglycan is incorporated into the sacculus. Is it incorporated at specific sites (growth zones), or in a diffuse manner over much of the cell surface? Work on Streptococcus faecalis has shown that growth takes place at a

central zone in this organism (see Daneo-Moore and Shockman, 1977). It might therefore be expected that rod-shaped bacteria grow with a central growth zone.

The results for E.coli obtained by various groups, using various methods, are often unclear and are frequently contradictory. This is a reflection of the enormous technical problems encountered. One technique used to study peptidoglycan synthesis in E.coli is to pulse-label the peptidoglycan with [^{3}H]DAP (which is specifically incorporated into this layer), followed by autoradiography of the sacculi. The results obtained by Ryter et al. (1973), Schwarz et al. (1975) and Koppes et al. (1978) using this approach are fairly consistent. The silver grains in the autoradiographic film were analysed statistically. They found the highest probability of finding silver grains at the centre of the cell in all size groups, but in the longer cells and dividing cells, lateral regions of incorporation were also detected.

If new peptidoglycan is preferentially inserted at the cell centre, it might be expected that it is displaced from there in a conserved manner towards the poles, as in S.faecalis. There is no evidence for this. On the contrary, Ryter et al. (1973) reported that after a quarter of a generation, newly-incorporated peptidoglycan had become randomly distributed over the entire cell surface. However, Braun and Wolff (1975) criticized this work on the grounds that Ryter et al. did not use sufficient non-radioactive DAP for an effective chase. Braun and Wolff found that using identical conditions, [^{3}H]DAP continued to be incorporated unimpaired during the chase for one generation, due to the large pool of [^{3}H]DAP in the cytoplasm. They concluded that peptidoglycan precursors were incorporated over most of the cell surface (though

preferentially at the cell centre), and that in the work of Ryter et al. a quarter of a generation was required for silver grains to be created. The concentration of radioactivity at the septum, they suggested, was due to enhanced synthesis when the septum was formed. Schwarz et al. (1975) reported that the number of grains in the central zone was reduced by 50% if similar autoradiographs of temperature-sensitive division mutants were prepared immediately after transfer to the restrictive temperature, suggesting that much of the localized incorporation is division-specific. After sustained growth at this temperature, there was little evidence of localized incorporation.

As regards the lateral growth zones which have been described, it is difficult to distinguish between diffuse growth, many narrow secondary growth zones, and secondary growth zones not precisely positioned from cell to cell, leading to a blurring of the result (A.L. Koch, unpublished observations). In order to distinguish between these possibilities, high specific activities of [^{3}H]DAP are required followed by longer autoradiography in order to allow sufficient silver grains to be visible over the whole cells, in addition to the intense equatorial zone grains. Nanninga et al. (1982) concluded that it is not possible to distinguish clearly between diffuse growth and zonal growth. They suggested that there is an incorporation gradient, where the highest probability of incorporation lies in the actual or prospective division site.

A second approach to studying the site of incorporation of nascent peptidoglycan is to examine the autolytic sites in cells. The rationale for this approach assumes that the growth of the peptidoglycan layer is a balance between the action of synthetic and hydrolytic enzymes. Inhibition of synthetic enzymes (e.g. by β-lactam antibiotics) should

disturb the balance, leading to net hydrolysis of the sacculus. At low concentrations, penicillin and ampicillin inhibit division but not elongation of the cell. Characteristic filaments are produced with bulges at the centre (Schwarz et al., 1969). At higher concentrations, elongation is inhibited, and a small "sphere" is detectable alongside the rod as lysis begins. Ultimately, the cell wall splits, producing a spheroplast. This method of studying peptidoglycan synthesis is not quantitative, as the autoradiographic studies are. It has been used to locate the major zone of incorporation.

Donachie and Begg (1970) and Staugaard et al. (1976) observed that these penicillin-sensitive sites tended to cluster at the poles of small cells, and at the centre of larger, dividing cells. However, while Donachie and Begg reported that intermediate sized cells showed a gradual displacement of the sphere from the pole to the centre with increasing length (hence the concept of the "unit cell"), Staugaard et al. found no well-defined pattern. There were some experimental differences in these studies; they used different β-lactam antibiotics, and Donachie and Begg used light microscopy, while Staugaard et al. used electron microscopy - but the results are difficult to reconcile.

Mathys and Van Gool (1979) used cephaloridine, a β-lactam antibiotic which inhibits cell elongation even at low concentrations. Their results showed different patterns of autolytic sites depending on the growth rate of the bacteria. At fast growth rates, their results agreed with Staugaard et al. (1976). However, at slower growth rates, the sensitivity of the cells altered. At very slow growth rates, the sensitivity decreased sharply at all cell lengths, and no spheres could be detected in the smallest or largest cells. Those spheres which were

detected showed no clear preference for any site. They concluded that the pattern of surface growth may alter depending on the requirements of the cell, which are in turn determined by the growth rate. It is clear that these experiments on the autolytic sites in E.coli are not easy to interpret.

In summary, the pattern of insertion of nascent peptidoglycan into the sacculus is still uncertain. By no means all new peptidoglycan is inserted at a central growth zone. However, there appears to be more peptidoglycan synthesis at the centre of the cell than can be accounted for by septum formation. How this centrally-incorporated material which is not required for septum formation is redistributed is difficult to determine, as there seems to be some incorporation all over the cell surface, complicating the results in pulse-chase experiments. There is no one accepted view, and more data is required. In particular, autoradiographic studies using a higher specific activity precursor would be useful, as well as more information on physical parameters, such as osmotic pressure and the flexibility of the envelope.

IV(c) Cell-Shape Mutants

E.coli can be considered as a cylinder, with hemispherical poles (see Donachie, 1981). It might be imagined that the shape is determined by two enzymatic processes - one which synthesizes the cylindrical side walls, and one which synthesizes the poles. This view is supported by the existence of the two types of mutants - filaments and spherical cells - which one would predict if one of these shape-determining processes were inhibited. Study of such mutants, therefore, and the identification of the gene products which have been altered, will lead to a greater understanding of the mechanisms by which cells determine their shape.

(i) Filamenting mutants

Filamentation of cells is induced by many agents. Some cause DNA damage, and so induce the many responses collectively known as "SOS" responses, one of which is the inhibition of cell division. Others, such as penicillin, cycloserine and vancomycin affect peptidoglycan synthesis. Numerous genes involved in cell division have been identified by the isolation of temperature-sensitive filamenting (fts) mutants, which divide normally at 30°C, but filament at 42°C (see review by Helmstetter et al., 1979).

As described earlier (Sections III(f) and III(g)), inactivation of PBP3 leads to filamentation. This is apparently due to failure of the cell to complete septation, rather than initiate it (Olijhoek et al., 1982). Transpeptidase and transglycosylase activities have been identified in the purified enzyme (Ishino and Matsuhashi, 1981). Therefore these functions are specifically required at division. There is also a suggestion that cephalexin, which preferentially binds to PBP3, inhibits an event about 15 minutes before division (James et al., 1975). The ftsA and ftsZ products have been identified (see Section III(h)). No enzymatic functions have been assigned, but physiological studies have identified a short period of about 10 minutes just before the normal time of division, during which synthesis of the ftsA gene product must take place if septation is to follow (Donachie et al., 1979). Further analysis of these and other filament-causing mutations will be of great interest.

(ii) Spherical mutants

A variety of mutants have been described which grow as spherical cells, in contrast to the normal rod-shaped morphology. Some of these mutations are conditional.

Wild-type strains of Arthrobacter species grow as rod-shaped or spherical cells according to their nutritional environment (Ensign and Wolfe, 1964). Biochemical analysis of the peptidoglycan in rods and spheres suggested that there were shorter glycan chains in spheres than in rods (Krulwich et al., 1967a). This may be due to the greater activity in the spheres of an autolytic enzyme. A difference was also claimed in the amino acid composition of the interpeptide bridges (Krulwich et al., 1967b). Little more has been discovered about this process in Arthrobacter, due in part to its poorly defined genetic system.

Round mutants of E.coli carry mutations which map in two regions of the chromosome. pbpA (the structural gene for PBP2) maps at 14'. Both constitutive (Spratt, 1975) and temperature-sensitive (Henning et al., 1972; Spratt, 1977b; Iwaya et al., 1978) mutations in this gene have been isolated. rodA mutants have a very similar phenotype, except that they possess normal PBP2, and are very closely linked to pbpA (Matsuzawa et al., 1973; Spratt et al., 1980). envB mutants which are also spherical map at 70.5' (Adler et al., 1968; Normark, 1969). Also, a lpp ompA double mutant grows as spheres, although each mutation in isolation has no effect on morphology (Sonntag et al., 1978). A pH-conditional mutant has been isolated in Klebsiella pneumoniae (Satta and Fontana, 1974). The mutant grows as rods at pH5.8 and as spheres at pH7.0. The gene does not appear to be the homologue of pbpA or envB.

Cells defective in adenyl cyclase (*cya*$^-$) or in cAMP receptor protein (*crp*$^-$) have also been described as round (Yamasaki *et al.*, 1976; Kumar, 1976). Addition of exogenous cAMP to *cya*$^-$ mutants causes them to revert to the normal rod-shape. However, these cells are not true spheres. Scott and Harwood (1981) examined the dimensions of the *cya* mutants, and concluded that they are in fact short rods, resulting from the slow growth rate. This is supported by the observation that whereas spherical cells form giant spheres in the presence of nalidixic acid, cAMP-deficient cells form filaments, showing that they retain the ability to make side walls. The *cya* and *crp* mutations have pleiotropic effects, and the slow growth rate is not surprising. In the presence of mecillinam, they do form proper spheres.

Physiological, structural and biochemical alterations have been looked for in spherical mutants, and in cells made spherical by the action of mecillinam, which appears to inhibit PBP2 exclusively (see Section III(f)), Henning *et al.* (1972), Fontana *et al.* (1979) and Goodell and Schwarz (1975) have demonstrated that the isolated sacculi from these cells are spherical, showing the shape to be an intrinsic feature of the peptidoglycan. Analysis of *pbpA*^{ts} mutants (Henning *et al.*, 1972; Botta and Buffa, 1981) and of mecillinam-treated cells (Park and Burman, 1973; Matsuhashi *et al.*, 1974; Braun and Wolff, 1975) have shown a 50% decrease in the rate of peptidoglycan synthesis, but no effect on transpeptidase, carboxypeptidase or endopeptidase activity was detected. The extent of cross-linking was normal, and no difference could be detected in the structure of the peptidoglycan, the length of the glycan chains or the thickness of the sacculi. Recently, however, it has been found that spherical cells differ from rod-shaped cells in the kinetics of

incorporation of peptidoglycan into the sacculus (Markiewicz et al., 1982). The implications of this will be discussed in Chapter 8.

Spherical cells are generally extremely sensitive to the effects of detergents such as deoxycholate and SDS, and to a number of antibiotics (Matsuzawa et al., 1973; Sonntag et al., 1978). This effect is frequently produced by factors which cause increased permeability of the outer membrane (Leive, 1974). Matsuzawa et al. (1973) found that the rodA mutant they were working with had increased resistance to certain antibiotics. They, Adler et al. (1968) and Normark (1969) reported an increased resistance to UV-irradiation. Westling-Häggström and Normark (1975) suggested that this is due the fact that the spherical cells are larger than normal cells, and have a higher cellular content of DNA.

Several studies have looked at the ionic requirements of spherical cells. The pbpA^{ts} mutant isolated by Henning et al. (1972) and the lpp ompA mutant (Sonntag et al., 1978) were susceptible to lysis, but were stabilized by the addition of Mg^{2+}. Spherical sacculi isolated from the K.pneumoniae mutant (Fontana et al., 1979) became amorphous when EDTA was added. This suggests that Mg^{2+} is essential for the rigidity of spherical sacculi. Rayman and McLeod (1975) reported that low concentrations of Mg^{2+} stabilized the rod-shaped peptidoglycan layer from a marine pseudomonad. However, Matsuhashi et al. (1974) found that the Mg^{2+} concentration had no effect on the growth or morphology of mecillinam-treated cells. Tybring and Melchior (1975) showed that mecillinam-treated cells were protected from lysis by the addition of NaCl or KCl, but Henning et al. (1972) reported that high concentrations of NaCl had no such protective effect on their pbpA^{ts} mutant.

A *pbpA*^{ts} mutant (Botta and Buffa, 1981) and the *lpp ompA* mutant (Sonntag *et al.*, 1978) were shown to have an outer membrane detached from the peptidoglycan layer. This contrasts with the results of Braun and Wolff (1975) who reported that mecillinam-treated cells contained approximately twice as much lipoprotein bound to the peptidoglycan as normal cells. There have also been reports of increased vesicle formation (Henning *et al.*, 1972) and of wrinkling of the cell surface (Iwaya *et al.*, 1978), suggesting an imbalance between peptidoglycan and membrane synthesis.

A property of many of these spherical mutants is an increased rate of autolysis. This was found in spherical *A.crystallopoietes* (Krulwich *et al.*, 1967a) and in *pbpA*^{ts} mutants (Henning *et al.*, 1972; Botta and Buffa, 1981). This may be due to the increased concentration or activation of one of the cell's autolytic enzymes.

In conditional mutants, or in mecillinam-treated cells, the transition from rod-shaped to spherical cells occurs fairly rapidly and without loss of viability (Iwaya *et al.*, 1978; Botta and Buffa, 1981). However, the transition back to rod-shaped cells is not so smooth, and some lysis occurs. Goodell and Schwarz (1975) examined the rod-sphere-rod transition of mecillinam-treated cells in Methocel medium, a viscous liquid in which bacteria remain stationary and do not lose their original orientation. They found that when spheres became rods they generally keep their original polarity. A small proportion (7%) of cells became Y-shaped, but the authors did not report whether these eventually became normal rods. The rod-sphere-rod morphogenesis took several generations to complete, but single cells could in principle do it without dividing. However,

protein synthesis was required for the sphere-rod transition. The retention of polarity by these cells contrasted with spheroplasts. A diaminopimelic acid auxotroph was deprived of the amino acid, causing spheroplast formation. When diaminopimelic acid was added back, most of the spheroplasts could not revert to rods, and synthesized spherical sacculi. A few occasionnally formed rod-shaped outgrowths, and rods were often produced from these. However, the outgrowths occurred randomly, and bore no relation to the cell's original polarity.

Many different features of spherical mutants have been described. They may not all, however, be directly due to the primary defect (e.g. in PBP2). If the mutation has a drastic effect on the outer surface of the cell, the cell may only grow well by the acquisition of secondary mutations. Alternatively, the absence of PBP2 may require a compensating system to operate (e.g. PBP3), which is responsible for these effects.

IV(d) The Mechanism Of Action Of Mecillinam

The amidinopenicillanic acid, mecillinam, was first described by Lund and Tybring (1972). They observed that E.coli cells treated with mecillinam form spherical cells, and eventually lyse without forming spheroplasts. Spratt (1977a) showed that [^{14}C] mecillinam binds exclusively to PBP2. 50% saturation of PBP2 was achieved at very low concentrations (e.g. 0.023 µg/ml for 10 minutes at 30°C). At concentrations up to 1 mg/ml there was virtually no difference. However, high concentrations of mecillinam cause cell lysis. Spratt (1977d) suggests that this is due to the inhibition of division caused by a high concentration of the antibiotic. Such an inhibition has been shown by James et al. (1975). Mass continues to increase, and after several hours, the physical pressure causes lysis. This might be helped by increased autolytic activity, as described earlier. Electron micro-

graphy has shown that cells make abortive attempts to septate (Melchior et al., 1973). It is therefore not unlikely that any mutation which causes slow growth might allow successful septation, so that the cells survive. Resistant mutants appear rather frequently (10^{-5}), grow slowly (Matsuhashi et al., 1974) and survive better on less rich media.

The pH-conditional K.pneumoniae mutant is very resistant to killing by mecillinam, and the mutation appears to have no effect on growth rate, although the cells become spherical. An elaborate model to explain this was proposed (Satta et al., 1980), but there is really too little data available to comment on it.

IV(e) Peptidoglycan Synthesis In The Cell Cycle

The cell-shape of E.coli must reflect the way in which certain metabolic processes vary through the cell-cycle. It is therefore of great importance to study how the factors involved in cell-shape determination change and interact throughout the cell-cycle.

Several groups have investigated the final stages of peptidoglycan synthesis in this way. Hoffmann et al. (1972) generated synchronous cultures of E.coli B/r using the membrane elution method, and measured the rate of synthesis of peptidoglycan in cells of different ages by pulse-labelling with radioactive D-glutamate. They concluded that the rate of peptidoglycan synthesis was constant, with a doubling in rate near the time of septation.

Mirelman et al. (1976) measured PSE activities in synchronous cultures of E.coli ftsZ. At 30°C this strain grows and divides normally, but transfer to 42°C causes division to cease and the cells to filament. Shifting the cells back to 30°C after 60 minutes incubation at 42°C resulted in a 30 minute lag followed by a sharp increase in cell number,

indicating some synchronization of division. Pulse-labelling with [^{3}H] DAP after the shift-down showed that there was a peak of incorporation just before division, in agreement with the results of Hoffmann et al. (1972). Cells were taken at intervals after the shift-down to 30°C and made permeable to peptidoglycan precursors by treatment with ether. This allowed transpeptidase and DD-carboxypeptidase activities to be measured, and Mirelman et al. found that the transpeptidase : carboxypeptidase ratio was much higher at 42°C than at 30°C, mainly due to a fall in carboxypeptidase activity. Furthermore, the peptidoglycan was found to be 40% more crosslinked at the higher temperature. They suggested that the extent of cross-linking is regulated by controlling the number of pentapeptides in the nascent peptidoglycan (which act as donors in transpeptidation). This in turn is presumed to be regulated by the level of DD-carboxypeptidase activity. Thus the decrease in DD-carboxypeptidase activity at 42°C in the ftsA mutant leads to a net increase in pentapeptides in the nascent peptidoglycan. This local increase might account for the increased incorporation of nascent peptidoglycan into the sacculus, and for the higher degree of cross-linking. In keeping with this hypothesis was the finding that the use of low concentrations of ampicillin to specifically inhibit DD-carboxypeptidase activity (and therefore increase the number of pentapeptide side chains) stimulated the rate of peptidoglycan incorporation in the ftsA mutant at both 30°C and 42°C, and increased the level of cross-linking in both nascent peptidoglycan and in the sacculus.

In further studies, Mirelman et al. (1977) induced filamentation in a number of different ways. The ftsA mutant described above was treated at 30°C with nalidixic acid or cephalexin, or was grown at 42°C. In addition, a pbpB mutant, which contains a thermolabile PBP3 (see Section

III(f)), was grown at the restrictive temperature. All these treatments produced a higher level of cross-linking and reduced DD-carboxypeptidase activity, leading to an increased transpeptidase : carboxypeptidase ratio.

Mirelman et al. (1978) synchronised an E.coli B strain by a 60 minute period of isoleucine starvation. The cells divided about 20 minutes after the readdition of isoleucine, and again 45 minutes later. Samples were taken at intervals, the cells were treated with ether, and peptidoglycan synthesis was analysed. They found that the transpeptidase : carboxypeptidase ratio and hence the level of cross-linking in the sacculus increased just after division. As the cells approached the next division, the ratio gradually fell. It fell sharply during D (approximately 20 minutes before division) leading to a reduction in peptidoglycan incorporation and cross-linking.

The activities of other enzymes involved in peptidoglycan synthesis have also been analysed. Hakenbeck and Messer (1977) showed that membrane bound hydrolases increased to a peak about 15 minutes before division. Beck and Park (1977) reported that the level of LD-carboxypeptidase increased at division, and James and Gudas (1976) showed that synthesis of free lipoprotein peaked just before division. Bound lipoprotein showed no comparable increase, so the ratio of bound : free lipoprotein apparently decreases just before division. LD-carboxypeptidase has in the past been proposed as the in vivo transpeptidase which forms covalent bands between peptidoglycan and lipoprotein (e.g. Daneo-Moore and Shockman, 1977). The correlation just described suggests the opposite - that LD-carboxypeptidase acts as a carboxypeptidase in vivo. An increase in this activity will therefore reduce the number of tetrapeptides, which act as donors in the transpeptidation, and so reduce the proportion of bound lipoprotein.

IV(f) Peptidoglycan Synthesis And The Stringent Response

When the availability of any aminoacyl-tRNA becomes limiting (e.g. through amino acid starvation) there is a major readjustment of cellular activity, known as the stringent response (see review by Gallant, 1979). This includes a reduction in the rate of ribosomal RNA accumulation, an increase in the rate of protein turnover, reductions in the synthesis of carbohydrates, lipids and peptidoglycan, and the appearance of two unusual guanine nucleotides, ppGpp and pppGpp. A mutation in the relA gene abolishes these responses, resulting in a "relaxed" phenotype.

Ramey and Ishiguro (1978) showed that the site of inhibition of peptidoglycan synthesis during the stringent response is the incorporation of disaccharide-pentapeptide into the sacculus. Harkness et al. (1981) demonstrated that transpeptidase and DD-carboxypeptidase activities are under stringent control. Amino-acid deprivation led to a reduction in both activities in a relA$^+$ strain, but not in a relA$^-$ strain.

This reduction in DD-carboxypeptidase activity in amino-acid starved bacteria is not due to the disappearance or permanent inactivation of the enzyme, since sonication of the cells results in reappearance of the activity at levels even higher than before. The same reappearance of cryptic DD-carboxypeptidase activity was found on sonication of the ftsA mutants which filamented due to growth at 42^{o}C or to the presence of nalidixic acid.

Interestingly, whereas only half the TCA-insoluble peptidoglycan synthesized from a normal growing culture was SDS-insoluble (i.e. covalently linked to the sacculus; see Section II(d)), virtually all the TCA-insoluble peptidoglycan synthesized after amino-acid deprivation was SDS-insoluble. This lends support to the idea that the level of DD-carboxypeptidase regulates the level of cross-linking.

IV(g) Functions Of PBPs 4, 5 and 6 *in vivo*

As I described earlier (Section III(g)), the carboxypeptidase IA fraction corresponds to the combined activities of PBPs 5 and 6, while carboxypeptidase IB and the penicillin-sensitive endopeptidase activity correspond to PBP4. De Pedro *et al.* (1980) showed that a *dacA* mutant (which lacks PBP5) accumulated pentapeptide sidechains in the intact sacculi, showing that PBP5 acts as a DD-carboxypeptidase *in vivo*. A *dacB* mutant (which lacks PBP4) also showed an increase in pentapeptides, although not as much as in the *dacA* mutant. However, de Pedro and Schwarz (1981) provided evidence that the main function of PBP4 *in vivo* is as a transpeptidase, although the purified enzyme shows no significant transpeptidase activity *in vitro* (Nguyen-Disteche *et al.*, 1974). This will be discussed further in Chapter 8.

V. AIMS OF THE PRESENT STUDY

While there has been progress in the understanding of the final stages of peptidoglycan synthesis and the determination of cell-shape, little is known at the molecular level about the structure and organization of the genes involved. Furthermore, apart from the penicillin-binding proteins, the enzymatic functions of the proteins involved remain a mystery.

In an attempt to learn more about some of these genes and their products, the region carrying *pbpA*, *rodA* and *dacA*, as well as the *leuS* and *lip* genes, was cloned onto a specialized transducing phage (Spratt *et al.*, 1980). My aim was to investigate the cloned region, and in particular the *rodA* gene and its product. *rodA* is interesting because it is so closely linked to two genes encoding PBPs, and *rodA* mutants

have an almost identical phenotype to pbpA mutants, tempting one to speculate that their functions might be closely related. Therefore investigation into rodA might also elucidate more clearly the function of PBP2. This should eventually lead to a greater understanding of the processes controlling the shape of the peptidoglycan synthesized, and hence of the cell.

Chapter 2

MATERIALS AND METHODS

1. BACTERIAL STRAINS

All the bacterial strains were E.coli K-12 derivatives, and are listed in Table 2.1. Strains were kept at 4°C on agar plates, or at -80°C in nutrient broth containing 30% v/v glycerol.

2. BACTERIOPHAGE STRAINS

Bacteriophage strains used are listed in Table 2.2. Phage stocks were stored in λ buffer containing a few drops of chloroform in McCartney bottles. For long-term phage stocks, bottles were soaked in concentrated H_2SO_4 in order to remove any traces of detergent, and thoroughly rinsed before use.

3. MEDIA

Nutrient broth : 2.5% w/v lab M nutrient broth E powder.

Luria broth : 1% w/v Oxoid Tryptone, 0.5% w/v Oxoid yeast extract, 0.5% w/v NaCl (pH7.4).

M9-minimal medium : 40 mM Na_2HPO_4, 20 mM KH_2PO_4, 8 mM NaCl, 20 mM NH_4Cl, 1 mM $CaCl_2$, 10 mM $MgSO_4$.

Added as required : glucose (0.4%), maltose (0.2%), L-amino acids (50 μg/ml), adenine (20 μg/ml), DL-α-lipoic acid (1 μg/ml), thiamine (2 μg/ml), thymine (2 μg/ml), Difco casamino acids (0.2%).

Solid media : Nutrient broth, Luria broth or minimal medium were solidified with 1.45% Oxoid No.3 agar.

Table 2.1

Bacterial strains

Strain	Genotype	Reference or Source
AB1325 *lip9*	*lip9 his4 proA2 purB15 thi1 mtl1 xyl5 galK2 lacY1 tsx rpsL supE44*	Herbert and Guest (1968)
SP5211	AB1325 *lip*$^+$ *rodA52* (Ts)	Spratt *et al.* (1980)
SP5211 *zbe*::Tn*10*	SP5211 *zbe*::Tn*10*	B.G. Spratt
SP5211 *recA*	SP5211 *srl*::Tn*10 recA56*	Spratt *et al.* (1980)
SP13711	AB1325 *lip*$^+$ *pbpA137* (Ts)	Spratt *et al.* (1980)
SP45	*pbpA45* (Ts) *tyr* (Am) *trp* (Am) *ilv supD126* (Ts)	Spratt (1978a)
W3110	prototrophic	W.J. Brammar
W3110 *pbpA6*	W3110 *zbe*::Tn*10 pbpA6*	B.G. Spratt
C600	*thr1 leuB6 thi1 supE44 tonA21 lacY1*	W.J. Brammar
C600 *rpsL*	C600 *rpsL*	B.G. Spratt

Table 2.1 Continued

Strain	Genotype	Reference or Source
ED3184	his lacΔX74 tsx supF	W.J. Brammar
TMRL-12	leuS (Ts) argG proA his purB thi galK lacY rpsL	Matsuhashi et al. (1979)
TMRL-12 rodA52	TMRL-12 zbe::Tn10 rodA52 (Ts)	This thesis
159	gal uvrA rpsL	G.S. Plastow
DS410	minA minB lacY gal tonA rpsL	D.J. Sherratt
RB308	F^+ deoC thyA lacY	R. Buxton
CSR603	phr1 uvrA6 recA1 thr1 leuB6 proA2 argE3 thi1 ara14 lacY1 galK2 xyl5 mtl1 rpsL31 tsx33 supE44 gyrA96	Sancar and Rupert (1978)
MC4100	rpsL thi araD139 Δ(lacIPOZYA) U169	Casadaban (1976)

Table 2.2

Bacteriophage strains

Phage		Source
λ^+		W.J. Brammar
λ*imm*434		"
λ*c*I26		"
λ*vir*		"
λ*c*I857 *S*am7		"
λ*ind*		G. Plastow
λL41	*chi*A131 (*srI*λ1-2)$^{\Delta}$ *imm*434 *c*I *srI*λ4^{o} *nin*5 *shn*λ6^{o} *srI*λ5^{o}	W. Loenen
λNM627	(*srI*λ1-2)$^{\Delta}$ *c*I857 *srI*λ4^{o} *nin*5 *srI*λ5^{o} *S*am7	N. Murray
λ*dlip*5		B.G. Spratt
λ*ddacA*51		"
λ*drodA*1		"
λ*dpbpA*108		"
λpBS10		"
λpBS99		"
P1*vir*		"

Trypticase agar : 1% w/v Trypticase peptone (Baltimore Biological Laboratories), 0.5% w/v NaCl, 1.5% agar.

Soft Trypticase agar only contained 0.7% agar.

Antibiotics were added to the following concentrations :

Sodium ampicillin	25 μg/ml
Kanamycin sulphate	25 μg/ml
Tetracycline	10 μg/ml
Chloramphenicol	25 μg/ml
Streptomycin sulphate	200 μg/ml

Hershey salts (sulphur-free) : 90 mM NaCl, 40 mM KCl, 20 mM NH_4Cl, 0.6 mM KH_2PO_4, 100 mM Tris, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.001 mM $FeCl_3$ (pH7.4).

Hershey medium : Hershey salts, 0.4% w/v glucose, amino acid requirements.

Bacterial buffer : 20 mM KH_2PO_4, 50 mM Na_2HPO_4, 70 mM NaCl, 0.4 mM $MgSO_4$ (pH7.0).

λ buffer : 6 mM Tris HCl pH7.2, 10 mM $MgSO_4$, 0.05% w/v gelatin.

Sodium phosphate buffer : 10 mM Na_2HPO_4 and 10 mM NaH_2PO_4 titrated together to pH7.2.

4. GROWTH OF BACTERIAL CULTURES

Bacterial liquid cultures in nutrient broth, Luria broth or M9-minimal medium were shaken in a New Brunswick Gyrotatory Shaker, at 37°C unless otherwise indicated in the text. Bacterial cell mass was monitored by measuring absorbance using a Gilford Microsample spectrophotometer 300N. Alternatively, cultures were grown in test tubes in a non-shaking waterbath. They were aerated by bubbling filtered air

through the medium. Absorbance was measured using a Bausch and Lomb Spectronic 20 spectrophotometer. In general, cultures grown in minimal medium were monitored using A_{450}, and cultures grown in nutrient broth were monitored using A_{600}.

5. UV-IRRADIATION OF BACTERIA

Cultures of bacteria grown in M9-minimal medium were irradiated in an 18 cm glass petri dish, with gentle swirling, under a Hanovia Bacteriocidal lamp (254 nm). The dose rate was calibrated using a Latarjet dosimeter.

6. GROWTH OF BACTERIOPHAGE λ

(a) Titration of phage stocks

An overnight culture of C600 was supplemented with 10 mM $MgCl_2$. 0.05 ml was added to 0.1 ml of serial phage dilutions in λ buffer, and left at room temperature for ten minutes to allow adsorption. 3 ml molten Trypticase soft agar were added, briefly mixed and overlaid on Trypticase agar plates. The plates were incubated at 37°C overnight.

If the phage carried the Sam7 mutation, ED3184 (supF) was used as host strain.

(b) Preparation of phage stocks

(i) Plate lysates

Dilutions of phage were plated as described above. The Trypticase agar plates were freshly poured. The plate on which the plaques were just confluent was taken and the soft agar scraped into a McCartney bottle. The plate was rinsed with 3 ml λ buffer, which was also transferred to the bottle. A few drops of $CHCl_3$ were added, and the bottle

vortexed. The debris was pelleted by centrifugation, and the supernatant transferred to another McCartney bottle. A few drops of $CHCl_3$ were added, and the lysate was stored at $4^{\circ}C$. Titres obtained were usually greater than 10^{10} pfu/ml.

(ii) Liquid lysate

Large amounts of phage were prepared by the method described by Blattner et al. (1977). A fresh plaque or about 10^6 phage from a lysate were mixed with 0.3 ml to 1.0 ml of an overnight culture of C600 supplemented with 10 mM $MgCl_2$, and allowed to adsorb for ten minutes. This was diluted into 500 ml prewarmed Luria broth (supplemented with $MgCl_2$) and grown with vigorous shaking overnight at $37^{\circ}C$. Ideally, several rounds of lysis occurred, with all the bacteria eventually being lysed. The exact balance between phage and bacteria in the inoculum was determined empirically. Up to 10^{13} pfu/litre could be obtained in this way. If desired, the phage were concentrated by polyethylene glycol precipitation (see section 6b (v)).

(iii) Thermoinduction of λcI857 Sam7

λ phage carrying cI857 Sam7 mutations were prepared by thermoinduction of lysogens that were not able to suppress the Sam mutation. An overnight culture was grown in Luria broth at $30^{\circ}C$. This was diluted 100-fold into 500 ml prewarmed Luria broth containing 0.5% w/v glucose, and was grown at $30^{\circ}C$ to A_{650} 0.35. The culture was transferred to a waterbath at $44^{\circ}C$ for 10 minutes, and then shaken at $37^{\circ}C$ for three to six hours. The cells were chilled, centrifuged (Sorvall GS3 rotor, 5000 rpm, 10 minutes, $4^{\circ}C$), resuspended in 10 ml λ buffer and transferred to a McCartney bottle. 1 ml $CHCl_3$ was added, and the cells were shaken for 3 hrs at $37^{\circ}C$. After centrifugation to remove the cell debris,

the supernatant was decanted into a fresh bottle, and the phage were titred using ED3184.

(iv) UV-induction of lysogenic phage

Prophage with a *cI*$^+$ gene were induced by UV-irradiation. An overnight culture of the lysogenic bacteria was diluted in Luria broth and grown to A_{600} 0.5. The cells were harvested and resuspended in λ buffer. They were UV-irradiated with 30 Jm^{-2}, harvested, resuspended in fresh warm Luria broth; and grown at 37°C. After the cells began to lyse (monitored by eye), a few drops of $CHCl_3$ were added and the culture shaken for 10 minutes. Debris was removed by centrifugation and the supernatant titred.

(v) Polyethylene glycol precipitation of phage

Phage were precipitated as described by Yamamoto *et al.* (1970). NaCl was added to the lysate to 0.5 M. After incubation on ice for one hour, polyethylene glycol 6000 was added to 10% (w/v) and dissolved. After overnight incubation at 4°C, the precipitate was collected by centrifugation (Sorvall GS3 rotor, 5,000 rpm, 10 minutes, 4°C) and resuspended in λ buffer.

7. ISOLATION OF LYSOGENS

Lysogens were isolated by dropping phage onto a bacterial lawn on a Trypticase agar plate. After overnight incubation, cells were taken from the turbid area of lysis and streaked to single colonies on nutrient agar. These were tested for lysogeny by streaking a liquid culture across streaks of λ*vir* and λ*c*26 on nutrient agar. If testing for the presence of a λ*imm*434 prophage, cultures were streaked across λL41, which carries 434 immunity and has a mutant repressor. Lysis caused by λ*vir* showed the strain to be λ sensitive, while lysogeny was indicated by immunity to λ*c*26.

8. GENERALIZED TRANSDUCTION USING P1vir

P1vir plate lysates were prepared as for λ (see section 6b (i)) except that 5 mM $CaCl_2$ was used instead of 10 mM $MgCl_2$.

0.2 ml of an overnight culture of bacteria in nutrient broth, supplemented with 1 mM $CaCl_2$, was added to 0.2 ml of a P1vir preparation. After 15 minutes adsorption at room temperature, 0.1 ml 1 M sodium citrate was added, to prevent any secondary infection. The bacteria were plated onto selective medium, with appropriate controls.

Transduction of cells to rodA52 was performed using P1vir grown on SP5211 zbe::Tn10, selecting tetracycline resistance and screening for round cells at 42°C.

9. TESTS FOR TRANSDUCTION OF CHROMOSOMAL MARKERS CARRIED BY λ

The presence of leuS$^+$, pbpA$^+$ or rodA$^+$ on λ transducing phage was tested by the following methods:

(a) leuS

The leuS mutation produces a thermolabile leucyl-tRNA synthetase. Cells carrying this mutation grew at 30°C, but not at 42°C.

0.1 ml of an overnight culture of TMRL-12 (leuS) grown at 30°C was plated onto minimal agar, and phage dilutions were spotted on the surface. Plates were incubated at 42°C, selecting leuS$^+$.

(b) pbpA

Spherical cells tend to lyse at fast growth rates. Thus a pbpA derivative of a fast growing strain such as W3110 only survives on minimal medium.

0.1 ml of an overnight culture of W3110 pbpA6 (constitutively round) grown in minimal medium was plated onto penassay agar (Difco

antibiotic medium No.2), and phage dilutions were spotted on the surface. Plates were incubated at 37°C, selecting *pbpA*$^+$.

(c) *rodA*

(i) Round cells are supersensitive to detergents (see Chapter 1 IV(c)) 0.1 ml of an overnight culture of SP5211 (*rodA*Ts) grown at 30°C was plated onto penassay agar supplemented with 0.1% w/v sarkosyl NL97, and phage dilutions were spotted onto the surface. The plates were incubated at 30°C for three hours, and then transferred to 42°C, selecting *rodA*$^+$.

(ii) Transduction of *rodA*$^+$ and *leuS*$^+$ could be tested at the same time using the double mutant TMRL-12 *leuS rodA52*. Dilutions of phage were tested as in (a), the plates being incubated at 42°C, selecting *leuS*$^+$. *leuS*$^+$ transductants were then screened under the microscope for the presence or absence of *rodA*$^+$. Even with *leuS*$^-$ transductants, residual growth allowed identification of *rodA*$^+$ transductants. This test was more clear-cut than (i), and was used in preference.

When testing transduction of markers carried by λpBS10 and its derivatives, λ*imm434* lysogens were used. When testing transduction at 42°C of markers carried by λ*cI857* derivatives (e.g. λpBS99), appropriate λ$^+$ lysogens were used.

10. ISOLATION OF DELETION DERIVATIVES OF λpBS10

Deletions of λpBS10 were obtained using the method of Saito and Uchida (1978). Trypticase agar plates containing between 0.8 and 1.4 mM EDTA were prepared. Dilutions of λpBS10 were plated with C600 on Luria agar as described previously (section 6a), except that neither the

soft agar overlay nor the bacterial culture were supplemented with $MgCl_2$. It was essential that the overlay was absolutely even. With increasing EDTA concentration, the plaques formed by λpBS10 became smaller. At 1.1 to 1.2 mM EDTA, a number of larger plaques appeared on a lawn of pinprick plaques. These were picked, purified twice and tested for transduction of the genes carried by λpBS10.

11. TESTING THE SENSITIVITY OF λ PHAGE TO EDTA

This method was taken from Parkinson and Huskey (1971). Lysates of phage were diluted 100-fold into 10 mM Tris-HCl pH8.0, 10 mM EDTA and incubated at 42°C. After various time intervals, 0.1 ml was removed and diluted 100-fold into λ buffer. The viable phage were titred and survival plotted against time.

12. MOBILIZATION EXPERIMENTS

Cells were grown overnight in Luria broth and diluted 50-fold into fresh broth. They were grown for four to six generations (with at least one dilution) to 2-3 x 10^8 cells/ml. 0.3 ml of the donor was added to 3 ml of the recipient, and the mating mixture incubated with very gentle shaking at 37°C for one hour. Conjugation was terminated by vortexing the mixture for 30 seconds, and dilutions were plated on selective media.

13. PREPARATION OF PHAGE DNA

A phage lysate was prepared as described in section 6b, and concentrated with PEG 6000 if necessary. Solutions with densities of 1.7, 1.5 and 1.3 (refractive indeces of 1.3952, 1.380 and 1.3687 respectively) were prepared by mixing saturated caesium chloride solution with λ buffer,

and 5 ml of each were carefully layered in a 1" x $3\frac{1}{2}$" cellulose nitrate Beckman tube. The phage lysate was layered on top of the CsCl. The block gradient was centrifuged in a Beckman SW27 rotor (22,000 rpm, 3 hrs, 15°C). λ phage particles formed a sharp band in the 1.5 density layer, and were removed through the side of the tube with a syringe into a siliconized glass tube. RNase was added to 2 μg/ml and left at room temperature for 30 minutes. The CsCl was diluted by the addition of an equal volume of TE buffer, so as to allow it to form the upper phase when phenol-extracted. An equal volume of phenol mix was added and mixed thoroughly. This was centrifuged (Sorvall HB4 rotor, 10,000 rpm, 20 minutes, 8°C) and the aqueous layer transferred to a fresh tube. The phenol extraction was repeated, and the DNA was thoroughly dialysed against TE buffer. Sodium acetate pH5.6 was added to 0.3 M, followed by two volumes of absolute ethanol. The tube was mixed well and stored for 1 hour at -80°C, or overnight at -20°C. The DNA was pelleted by centrifugation (Sorvall HB4 rotor, 10,000 rpm, 30 minutes, -10°C), the supernatant carefully decanted and the pellet dried under vacuum. The DNA was resuspended in a small volume of TE buffer and stored at 4°C.

If defective and helper phage needed to be separated, the phage band was removed from the block gradient and transferred to a Beckman 3" x 5/8" cellulose nitrate tube. The tube was filled with CsCl -λ buffer solution with a density of 1.5, mixed and centrifuged to equilibrium (Beckman 50Ti rotor, 35,000 rpm, 40 hours, 15°C). The phage bands were removed separately from the side of the tube, and treated as described above.

Table 2.3

Reagents used in the preparation of DNA

Tris-sucrose	:	50 mM Tris-HCl pH8.0
		25% sucrose (w/v)
TES buffer	:	50 mM Tris-HCl pH8.0
		5 mM EDTA
		50 mM NaCl
TE buffer	:	10 mM Tris-HCl pH7.5
		1 mM EDTA
STET buffer	:	8% sucrose (w/v)
		5% Triton X-100 (v/v)
		50 mM EDTA
		50 mM Tris-HCl pH8.0
Brij lysis mix	:	1% Brij-58
		62.5 mM EDTA
		50 mM Tris
		0.4% sodium deoxycholate (w/v)
		- adjusted to pH8.0 with NaOH
Triton lysis mix	:	2% Triton X-100 (v/v)
		50 mM Tris-HCl pH8.0

Table 2.3 Continued

PEG-NaCl solution	:	25% polyethylene glycol 6000
		1.25 M NaCl
Phenol mix	:	100 g phenol dissolved in 100 ml $CHCl_3$
		4 ml isoamyl alcohol
		0.1 g 8-hydroxyquinoline
		Stored under 10 mM Tris pH7.5 at $4^{\circ}C$

All glassware used with DNA was siliconized beforehand. Tubes, tips and solutions were autoclaved, and gloves were worn, to prevent nuclease contamination.

14. PREPARATION OF PLASMID DNA

To prepare DNA from an amplifiable plasmid, a 400 ml culture in nutrient broth containing appropriate antibiotics was grown to late log phase at 37°C. Chloramphenicol (170 μg/ml) or spectinomycin (300 μg/ml) were added and the culture was shaken overnight. If the plasmid was not amplifiable, a 400 ml culture was grown overnight. The cells were then chilled, harvested and washed with bacterial buffer. They were then lysed using either Brij 58 or Triton X-100.

(a) Brij lysis

After harvesting, the cells were resuspended in 22 ml Tris-sucrose and divided into two tubes. To each, 5 ml lysozyme (5 mg/ml)/RNase (15 μg/ml) solution were added and incubated for five minutes on ice. 4.3 ml 0.25 M EDTA were added and again incubated for five minutes on ice. 18 ml Brij lysis mix were added and the tube gently inverted until the cells had lysed. The lysate was cleared by centrifugation (Sorvall SS34 rotor, 18,000 rpm, 30 minutes, 4°C) and the supernatant decanted into a Sorvall GSA bottle. Two-thirds of a volume of PEG/NaCl solution was added and left at 4°C overnight. The precipitate was centrifuged (Sorvall GSA rotor, 5,000 rpm, 10 minutes, 4°C), drained and resuspended in exactly 1.1 ml TES buffer.

(b) Triton lysis

After harvesting, the cells were resuspended in 3 ml Tris-sucrose, and 0.5 ml lysozyme (10 mg/ml)/RNase (300 μg/ml) solution was added. This was incubated at room temperature for five minutes. 1 ml 0.25 M EDTA was added, and the mixture again incubated for five minutes. 4 ml Triton lysis mix were added and the tube inverted until lysis was complete. The lysate was cleared by centrifugation and the DNA precipitated with

PEG as for lysis with Brij. The precipitate was resuspended in 1.1 ml TES buffer.

The 1.1 ml lysate was transferred to a Beckman VTi65 self-sealing tube, and underlaid with 4 ml CsCl-EtBr solution. The tube was centrifuged to equilibrium (Beckman VTi65 rotor, 55,000 rpm, 3 hours, 15°C, with slow acceleration). Chromosomal and plasmid bands were clearly visible, and were removed with syringes from the top of the tube, the chromosomal layer first. The plasmid band was transferred to a siliconized glass tube. Ethidium bromide was removed by extraction with CsCl-saturated propan-2-ol. The DNA was diluted, phenol extracted, dialysed and ethanol precipitated as for λ DNA.

15. RAPID PLASMID DNA PREPARATION

Small amounts of DNA from multicopy plasmids could be prepared for restriction enzyme analysis and transformation using the method described by Holmes and Quigley (1981).

An overnight culture was grown in nutrient broth. 1.5 ml was transferred to an Eppendorf tube and the cells pelleted in an Eppendorf microfuge. The pellet was resuspended in 75 µl STET buffer, and 6 µl lysozyme (10 mg/ml) were added. The tube was placed in a boiling water-bath for one minute, followed by centrifugation for ten minutes. The supernatant was transferred to a fresh tube, and an equal volume of propan-2-ol was added. The nucleic acid was precipitated in a dry ice-IMS bath for ten minutes and centrifuged for ten minutes. The supernatant was discarded, and the pellet resuspended in 100 µl 0.3 M sodium acetate pH5.6. 300 µl absolute ethanol were added, and the nucleic acid

precipitated and pelleted as before. The pellet was dried under vacuum, and resuspended in 30 µl TE buffer. 5 µl were used for digestion with restriction enzymes. If looking for fragments smaller than 1 kb, 1 µl RNase (5 mg/ml) was included with the digestion.

16. SDS-CLEARED LYSATES FOR RAPID PLASMID SCREENING

Plasmid size was quickly screened using a method developed by Broome-Smith (1980). This method was suitable for low and high copy number plasmids.

Cells were scraped off a plate using a spatula, and were resuspended (approximately 10^{10}/ml) in 150 µl TSE buffer (10 mM Tris-HCl pH8.0, 1 mM EDTA, 25% w/v sucrose). 15 µl 10% w/v SDS were added, and the lysate was cleared by centrifugation (Sorvall SM24 rotor, 18,000 rpm, 20 minutes, 8°C). 100 µl supernatant were transferred to an Eppendorf tube, loading buffer added, and about 35 µl loaded onto an agarose gel. Neither the gel nor the electrophoresis buffer contained ethidium bromide. After electrophoresis, the gel was stained in ethidium bromide solution (1.5 µg/ml) for one hour and photographed under UV-transillumination as described in section 17.

17. AGAROSE GEL ELECTROPHORESIS

Horizontal slab gels were prepared by boiling agarose in Tris-acetate electrophoresis buffer (40 mM Tris, 1 mM EDTA, adjusted to pH7.5 with glacial acetic acid), adding ethidium bromide (0.5 µg/ml) and pouring into a mould. Samples were prepared by adding 1/5-volume loading buffer (0.1% w/v bromophenol blue, 20% v/v glycerol, 20 mM EDTA). Digestions of λ DNA were heated at 65°C for 10 minutes before loading.

Samples were electrophoresed at 100 V with the gel submerged in Tris-acetate electrophoresis buffer containing 0.5 μg/ml ethidium bromide. DNA was visualized by transillumination with short wave UV light (260 nm) and photographed through an orange filter using a Nikon F camera with Kodak AHU microfile 35 mm film, or using a Polaroid MP-3 Land camera with Polaroid 4 x 5 Land film (Type 57).

18. RESTRICTION ENDONUCLEASE DIGESTION

Restriction endonuclease digests were performed in the buffers shown in Table 2.4. They were incubated at 37°C for one hour, with the exception of digestion with TaqI, when incubation was at 65°C. The reaction was terminated by heating at 65°C for ten minutes, or by addition of one-fifth volume 0.1 M EDTA pH8.0. All manipulations with DNA were performed using sterile buffers and Eppendorf tips and tubes, in order to minimize the risk of nuclease digestion.

Double digests were incubated with both enzymes together if buffers were similar. Otherwise, the enzyme requiring the lower salt concentration was added first and the tube incubated for one hour. The reaction was stopped by heating at 65°C. The salt concentration was then adjusted and the second enzyme added.

Size standards used were λ^+ x HindIII and PBR322 x TaqI.

Fragment sizes in λ^+ x HindIII digest (in kb):

23.51, 9.59, 6.76, 4.45, 2.29, 1.95, 0.59.

Fragment sizes produced in pBR322 x TaqI digest (Sutcliffe, 1979):

1.44, 1.31, 0.62, 0.37, 0.32, 0.31.

Table 2.4 Restriction enzyme buffers (5 x)

	Tris (mM)	$MgCl_2$	NaCl	KCl	DTT	mercaptoethanol	EDTA	pH
BglII, KpnI, PstI, PvuII	30	30	-	-	2.5	-	-	7.4
BamHI, SphI	30	30	250	-	-	30	-	7.4
EcoRI	500	50	250	-	2.5	-	-	7.4
HindIII	50	50	250	-	2.5	-	-	7.4
SalI, TaqI, XhoI	40	30	750	-	-	-	1	7.6
SmaI	75	30	-	75	-	-	-	7.6
HincII	50	5	150	-	5	-	-	7.9
HpaI	50	50	-	250	2.5	-	-	7.4

19. ALKALINE PHOSPHATASE TREATMENT

(a) Preparation of the alkaline phosphatase:

The ammonium sulphate suspension of calf intestinal alkaline phosphatase was mixed well. 50 units were removed and pelleted by centrifugation (Eppendorf centrifuge, 1 minute). The supernatant was discarded and the pellet was resuspended in 0.5 ml TE buffer containing 2 mM $ZnCl_2$. This was dialysed against 200 ml TE containing 0.1 mM $ZnCl_2$ for 30 minutes at 4°C, aliquoted and stored at 4°C.

(b) Use of alkaline phosphatase:

DNA was digested with restriction endonucleases, and the enzyme heat-inactivated. 5 µl (0.5 unit) alkaline phosphatase were added and the tube incubated for 30 minutes at 37°C. The alkaline phosphatase was inactivated by incubation at 65°C for ten minutes.

20. LIGATION

DNA samples were digested with restriction enconucleases, phenol extracted, ether extracted and ethanol precipitated. The DNA pellet was resuspended in a small volume of TE buffer. The samples to be ligated were mixed and made up to volume with TE buffer. This was heated at 65°C for five minutes. Ligation buffer (10 x : 1 M Tris HCl pH7.4, 1 M $MgCl_2$, 100 mM EDTA, 10 mM ATP, 5 mg/ml gelatin, 100 mM DTT) and T4 DNA ligase were added and the mixture was incubated at 12°C overnight. Ligation of the DNA was monitored by agarose gel electrophoresis.

21. TRANSFORMATION

An overnight culture of cells was diluted 50-fold into nutrient broth and grown to A_{650} 0.2. 30 ml were chilled, pelleted and resuspended in 15 ml ice-cold 100 mM $CaCl_2$. After 20 minutes incubation on ice,

they were pelleted and resuspended in 1 ml ice-cold 100 mM $CaCl_2$. These competent cells were kept at 4°C until used (generally, not longer than 24 hrs). 200 µl of the cells were transferred to an Eppendorf tube and DNA was added. After 1 hour on ice, the cells were heat-pulsed at 42°C for five minutes. The cells were diluted into 2 ml nutrient broth and incubated with shaking for 1 to 2 hours, when dilutions were plated onto nutrient agar containing antibiotics. A control with no DNA added was always performed.

22. PURIFICATION OF RESTRICTION FRAGMENTS

DNA restriction fragments were purified according to the method published by Dretzen et al. (1981). Whatman DE81 chromatography paper was cut to a convenient size, and pieces were soaked for several hours in 2.5 M NaCl. After being rinsed in distilled water, they were stored in 1 mM EDTA at 4°C until used. DNA was digested as required, loaded onto an agarose gel, and electrophoresed until the bands had separated. The bands were visualized under long wave UV light (366 nm). The gel was cut in front of the band to be purified, and DE81 paper was inserted. If any fragments ran close behind, paper was also inserted behind the band to be purified in order to prevent contamination. The DNA was electrophoresed onto the paper. The paper was removed, rinsed well in distilled water and blotted dry. It was transferred to a 1.5 ml Eppendorf tube and 450 µl high salt buffer (1 M NaCl, 50 mM Tris-HCl pH8.0, 1 mM EDTA) were added. The paper was shredded by vortexing and incubated for 2 hours at 37°C. The buffer (containing the DNA) was transferred into a 2" x ½" Beckman polyallomer tube by making a hole in the bottom of the Eppendorf tube followed by brief centrifugation. The liquid was passed through a

polyallomer wool plug in an Eppendorf tip in order to remove any paper fibres, and was extracted once with butan-2-ol to remove ethidium bromide. 50 µg tRNA were added as a carrier if desired, and 2 volumes absolute ethanol were added. The nucleic acid was precipitated in a dry ice/IMS bath for 10 minutes, centrifuged for 5 minutes and the supernatant discarded. The pellet was resuspended in 250 µl 0.3 M sodium acetate pH5.6 and three volumes of ethanol were added. The nucleic acid was precipitated and pelleted as before. The pellet was gently rinsed with 70% ethanol, dried under vacuum and resuspended in a small volume of TE buffer.

23. IDENTIFICATION OF BACTERIAL PROTEINS SPECIFIED BY λ TRANSDUCING PHAGE

Bacterial strain *E.coli* 159, 159 (λ*ind*$^-$) or 159 [pGY101] was grown in M9 minimal medium containing 0.2% maltose as carbon source at 37°C to A_{450} 0.64 (approximately 2 x 10^8 cells/ml) and chilled. 16 ml were transferred to a large glass petri dish and UV-irradiated with 1,200 Jm^{-2}. The cells were pelleted by centrifugation and resuspended in 3.2 ml prewarmed M9 minimal medium supplemented with 10 mM $MgCl_2$. For each sample, 1 ml was transferred to a Sorvall 15 ml glass centrifuge tube, and phage were added to a multiplicity of infection of 5 to 10. The phage had previously been exhaustively dialysed against λ buffer to remove methionine. For a control, λ buffer was added instead of phage. After ten minutes adsorption at 37°C, the cells were diluted by the addition of 4 ml prewarmed minimal medium and shaken for ten minutes at 37°C to allow host mRNA to decay. 10 to 100 µCi [^{35}S]-methionine (specific activity approximately 1500 Ci/mmol) were added, and the cells were shaken for 30 minutes at 37°C. Labelling was ended by the addition of 5 ml

chloramphenicol (0.6 mg/ml)/methionine (4 mg/ml) solution. The cells were chilled, harvested and resuspended in 1 ml sodium phosphate pH7.2. They were transferred to an Eppendorf tube, pelleted and resuspended in 50 µl sodium phosphate buffer. 50 µl SDS-lysis buffer were added, and the samples were incubated in a boiling waterbath for five minutes, and stored at -20°C until used. 20 µl were loaded onto a polyacrylamide gel. After electrophoresis, the gel was fluorographed, dried down and exposed to X-ray film at -80°C.

24. POLYACRYLAMIDE GEL ELECTROPHORESIS AND FLUOROGRAPHY

Polyacrylamide gel electrophoresis (PAGE) was carried out using a vertical slab gel apparatus according to the system of Laemmli (1970). A 14 cm separating gel was poured (see Table 2.5) and overlaid with 0.1% SDS to prevent a meniscus forming. When the gel had set, the SDS was poured off, and a stacking gel of 6% acrylamide was poured, in which the gel slots were formed, with at least 1 cm stacking gel below the bottom of the slots.

Samples (5 to 50 µl) were boiled for 5 minutes, loaded under electrophoresis buffer, and run at 25 mA until the dye front had reached the bottom of the separating gel. The gel was removed, fixed in 5% methanol, 10% acetic acid (v/v) for at least 1 hour, and fluorographed according to the method of Bonner and Lasky (1974). The gel was dried down onto Whatman chromatography paper No.17 using a Biorad Model 224 slab gel drier connected to a vacuum pump.

The dried gel was placed in an X-ray cassette with a sheet of Kodak X-Omat RP X-ray film and stored at -80°C for times varying from a few hours to several weeks. When gels contained very low amounts of radio-

Table 2.5

Solutions and buffers used in SDS-PAGE

A. Acrylamide solution :

30% w/v acrylamide : 0.4% w/v N,N'-methylene-bisacrylamide. This solution was purified by adding a little activated charcoal and leaving for 30 minutes. This was passed through a coarse and then a fine filter, and stored in the dark at $4^{\circ}C$.

B. Separating gel buffer :

1.5 M Tris-HCl pH8.8

C. 10% SDS solution

D. Stacking gel buffer :

0.5 M Tris-HCl pH6.8

E. Electrophoresis buffer :

25 mM Tris, 192 mM glycine, 0.1% w/v SDS pH8.8

F. Sample buffer :

125 mM Tris pH6.8, 20% v/v glycerol, 2% w/v SDS, 5% v/v mercaptoethanol, 0.001% w/v bromophenol blue

Table 2.5 Continued

G. Gel composition :

Separating gel	10%	13%	15%
Acrylamide	16.7 ml	21.7 ml	25 ml
Buffer B	12.5 ml	12.5 ml	12.5 ml
SDS solution	0.5 ml	0.5 ml	0.5 ml
distilled water to:	49.5 ml	49.5 ml	49.5 ml
ammonium persulphate (10% w/v)	0.5 ml	0.5 ml	0.5 ml
TEMED	50 µl	50 µl	50 µl

Stacking gel, 2 ml acrylamide solution, 2.5 ml buffer D, 0.1 ml SDS solution, 5.3 ml distilled water, 50 µl ammonium persulphate, 10 µl TEMED

activity, the film was prefogged by limited exposure to a photographic flash, which increased the sensitivity of the film (Laskey and Mills, 1975).

Films were developed in darkness by immersion in Kodak DX-80 developer for 3 minutes followed by a brief wash in 1% acetic acid and immersion for 3 minutes in Kodak FX-40 fixer.

A [^{14}C]-methylated protein mixture was used as molecular weight markers. The mixture contained myosin (200,000), phosphorylase B (92,500), bovine serum albumin (69,000), ovalbumin (46,000 - apparent MW 43,000), carbonic anhydrase (30,000) and lysozyme (14,300).

25. IDENTIFICATION OF PLASMID-SPECIFIED PROTEINS IN MINICELLS

(a) Purification of minicells

A 400 ml culture of E.coli DS410 containing a plasmid was grown overnight in nutrient broth. Some of the whole cells were removed by centrifugation (Sorvall GS3 rotor, 2000 rpm, 5 minutes, 4°C), the supernatant decanted into a fresh bottle and the minicells pelleted by centrifugation (Sorvall GS3 rotor, 8000 rpm, 15 minutes, 4°C). The supernatant was discarded and the pellet resuspended in 6 ml M9-minimal medium. 3 ml were layered onto each of two 30 ml 10-30% sucrose gradients (see section 25b). These were centrifuged (Sorvall HB4 rotor, 5000 rpm, 18 minutes, 4°C) and the top three-quarters of the minicell bands were taken off and pooled. An equal volume of M9-minimal medium was added to dilute the sucrose, and the minicells were pelleted (Sorvall HB4 rotor, 10,000 rpm, 10 minutes, 4°C). The pellet was resuspended in 4 ml M9-minimal medium and layered onto another sucrose gradient. This was centrifuged and sampled as before. The minicells were diluted and the

A_{600} read in a Bausch and Lomb Spectronic 20 spectrophotometer. They were centrifuged (Sorvall HB4 rotor, 10,000 rpm, 10 minutes, 4°C), resuspended in M9-minimal medium containing 30% glycerol (v/v) at A_{600} 2.0, and stored at -80°C.

(b) Formation of sucrose gradients

30 ml M9-minimal medium containing 20% sucrose (w/v) were transferred into a Sorvall polysulfone tube, and frozen at -80°C for at least two hours. It was thawed out at 4°C overnight, forming approximately a 10-30% gradient.

(c) Labelling of minicells

The minicells were completely thawed out, and 100 µl were transferred to an Eppendorf tube. The minicells were pelleted and resuspended in 200 µl M9-minimal medium containing 1.5 mg/ml Difco methionine assay medium. After 90 minutes incubation at 37°C to allow long-lived mRNA to decay, 75 µCi [^{35}S]-methionine (specific activity approximately 1500 Ci/mmol) were added and the minicells were incubated for 15 minutes at 37°C. They were pelleted, resuspended in M9-minimal medium containing 200 µg/ml methionine and incubated for 15 minutes. They were pelleted again, resuspended in 50 µl 10 mM sodium phosphate pH7.2, and 50 µl SDS-lysis buffer were added. The samples were incubated in a boiling waterbath for five minutes and stored at -20°C. 25 µl aliquots were analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography.

26. IDENTIFICATION OF PLASMID-SPECIFIED PROTEINS IN MAXICELLS

A 5 ml culture of E.coli CSR603 containing a plasmid was grown in M9-medium containing casamino acids at 37°C overnight. 0.1 ml was inoculated into 10 ml fresh medium and grown at 37°C to A_{450} 0.5. 2.5 ml were transferred to a petri dish and UV-irradiated (3.75 Jm^{-2}). This culture was incubated for 1 hour at 37°C and cycloserine added to 100 µg/ml. The culture was incubated overnight. 0.4 ml was transferred to an Eppendorf tube and the cells were pelleted. They were washed twice in Hershey salts, resuspended in 200 µl Hershey medium and incubated for 1 hour at 37°C. 25 µCi [^{35}S]-methionine (specific activity 1500 Ci/mmol) and 3 µl Difco methionine assay medium (100 mg/ml) were added and the cells were incubated for 1 hour at 37°C. They were pelleted, resuspended in Hershey medium containing 40 mg/ml methionine and incubated for a further five minutes. The cells were pelleted and resuspended in 25 µl 10 mM sodium phosphate pH7.2. 25 µl SDS-lysis buffer were added and the sample was incubated for five minutes in a boiling waterbath and stored at -20°C. 25 µl aliquots were analysed by SDS-PAGE followed by fluorography.

27. PREPARATION OF BACTERIAL CELL ENVELOPES FROM SONIC LYSATES

Labelled cells were transferred to a 25 ml beaker and unlabelled carrier cells were added immediately prior to sonication to give 10 A_{450} units. The combined sample (final volume adjusted to at least 8 ml by addition of ice-cold 10 mM phosphate buffer pH7.2) was sonicated for three 30 second intervals, with 30 second cooling periods, using the 3/4 inch end diameter probe in a 150 watt MSE ultrasonic disintegrator.

The sonicated sample was transferred to a centrifuge tube and centrifuged (Sorvall SS34 rotor, 7000 rpm, 5 minutes, 4°C) to remove unlysed cells. The supernatant was transferred to a Beckman 50Ti centrifuge tube and centrifuged (Beckman 50Ti rotor, 30,000 rpm, 30 minutes, 4°C). The supernatant was discarded, the pellet resuspended in sodium phosphate buffer and the envelopes repelleted (Beckman 50Ti rotor, 35,000 rpm, 30 minutes, 4°C). The washed envelope pellet was drained, the tube walls dried carefully, and the pellet resuspended in 50 µl 0.5% w/v sarkosyl NL97. This was incubated at room temperature for 30 minutes and centrifuged (Beckman 50Ti rotor, 35,000 rpm, 1½ hours, 4°C). The supernatant, representing the cytoplasmic membrane, was transferred to an Eppendorf tube. The outer membrane pellet was gently rinsed with sodium phosphate buffer, drained and the tube walls carefully dried. The pellet was resuspended in 50 µl sodium phosphate and transferred to an Eppendorf tube. Both membrane fractions were prepared for SDS-PAGE by addition of 50 µl SDS lysis buffer and boiling for 5 minutes. Samples were stored at -20°C, and reboiled before loading onto a gel.

28. ISOLATION OF PERIPLASMIC FRACTION

(a) Osmotic shock

Labelled cells were resuspended in 150 µl ice-cold 10 mM Tris HCl pH7.5, 20% w/v sucrose. 5 µl 0.5 M EDTA pH8.0 were added and the cells were incubated on ice for ten minutes. 50 µl were removed as the untreated control. The remaining cells were pelleted, the supernatant quickly removed and the pellet was resuspended in 100 µl ice-cold distilled water. The cells were incubated on ice for ten minutes,

pelleted and the supernatant saved as the periplasmic fraction. Residual fluid was removed, and the pellet was resuspended in 100 µl distilled water. This was saved as the cytoplasm/membrane sample. SDS-lysis buffer was added and the samples were boiled for 5 minutes and analysed by SDS-PAGE followed by fluorography.

(b) Spheroplasting

Labelled cells were resuspended in 75 µl 100 mM Tris HCl pH8.0, 0.5 mM EDTA, 17% w/v sucrose. 7.5 µl lysozyme solution (2 mg/ml) were added, followed by 75 µl ice-cold distilled water. The cells were incubated for 25 minutes on ice, and spheroplasting was checked under the microscope. 50 µl were saved as the untreated control. 3 µl 1 M $MgCl_2$ were added to the remaining cells, to stabilize the spheroplasts. The cells were pelleted and the supernatant saved as the periplasmic fraction. Residual fluid was removed and the pellet resuspended in 100 µl distilled water. This was saved as the cytoplasm/membrane sample. SDS-lysis buffer was added and the samples were boiled for five minutes and analysed by SDS-PAGE followed by fluorography.

29. IN VITRO PROTEIN SYNTHESIS

An in vitro transcription - translation system was prepared as described by Pratt et al. (1981a). Proteins synthesized from a DNA template were labelled with [^{35}S] methionine and analysed by SDS-PAGE and autoradiography. The system was prepared by and the incubation of DNA was carried out by Dr J.M. Pratt.

30. CHEMICALS AND ENZYMES

Chemicals and enzymes used are listed in Table 2.6.

Table 2.6

Chemicals and enzymes

	Source	Catalogue Number
1. Media and agar :		
Nutrient broth	London Analytical and Bacteriological Media Ltd	Lab 68
Tryptone	Oxoid	L42
Yeast extract	"	L21
Agar No.3	"	L13
Casamino acids	Difco	0288-01
Methionine assay medium	"	0423-15
Gelatin	"	0143-01
Antibiotic medium No.2	"	0270-17
Trypticase peptone	Baltimore Biological Laboratories	11921
2. Chemicals :		
Common chemicals and solvents	Fisons	
PEG 6000	BDH	29577
Trizma base	Sigma	T-1503
Triton X-100	"	T-6878
Sarkosyl	"	L-5125
2-mercaptoethanol	"	M-6250
DTT	"	D.0632

Table 2.6 Continued

	Source	Catalogue Number
3. Biochemicals :		
L-amino acids	Sigma	
thiamine	"	T-4625
DL-α-Lipoic acid	"	T-5625
thymine	"	T-0376
adenine sulphate	BDH	42002
deoxyguanosine	Sigma	D-9000
ATP	"	A-5394
lysozyme (egg white)	"	L-6876
Bovine pancreatic ribonuclease A	"	R-5000
E.coli tRNA	"	R-4251
chloramphenicol	"	C-0378
kanamycin sulphate	"	K-4000
tetracycline hydrochloride	"	T-3383
streptomycin sulphate	Glaxo	
sodium ampicillin	"	
mecillinam	Leo laboratories	

Table 2.6 Continued

	Source	Catalogue Number
4. Electrophoresis :		
Acrylamide	Kodak	5521
N,N'-Methylenebis-acrylamide	"	8383
TEMED	"	8178
Ammonium persulphate	"	11151
SDS	Biorad	161-0302
SeaKem ME agarose	FMC Marine Colloids, Division	50011
5. Photographic chemicals :		
DX-80 developer	Kodak	3006616
FX-40 X-ray liquid fixer	"	345 7694
HX-40 X-ray liquid hardener	"	310 1730
D19 developer	"	333 1972
Hypam fixer	Ilford	
6. Radiochemicals :		
L-[^{35}S] methionine	The Radiochemical Centre Amersham	SJ204
[^{14}C] methylated protein mixture	"	CFA626
[^{3}H] amino acid mixture	"	TRK440
[2-^{14}C] thymine	"	CFA182

Table 2.6 Continued

		Source	Catalogue Number
7.	Enzymes		
	BAL 31 nuclease	New England Biolabs	213
	DNA polymerase I Klenow fragment	"	210
	Calf Intestinal alkaline phosphatase	Boehringer Mannheim	108138
	EcoRI	"	606189
	Other restriction enzymes	BRL	

31. ABBREVIATIONS

Amp^R	ampicillin-resistant
ATP	adenosine 5'-triphosphate
cAMP	cyclic adenosine 3',5'-monophosphate
Cm^R	chloramphenicol-resistant
DAP	diaminopimelic acid
DTT	DL-dithiothreitol
EDTA	ethane diamine tetraacetic acid

Genetic nomenclature: rodA = gene

rodA = gene product

The gene symbols used are taken from Bachmann and Low (1980).

GlcNAc	N-acetyl glucosamine
kb	kilobase
Km^R	kanamycin-resistant
LPS	lipopolysaccharide
m.o.i.	multiplicity of infection
MurNAc	N-acetyl muramic acid
MW	molecular weight (daltons)
	(e.g. MW of 30 Kd = 30,000 daltons)
PAGE	polyacrylamide gel electrophoresis
PBP	penicillin-binding protein
PEG	polyethylene glycol
pfu	plaque-forming units
PPO	2,5-diphenyloxazole
PSE	penicillin-sensitive enzyme
RNase	ribonuclease
SDS	sodium dodecyl sulphate

ABBREVIATIONS CONTINUED

Tc^R	tetracycline-resistant
TEMED	N,N,N',N'-Tetramethylethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
UDP	uridine 5'-diphosphate
UV	ultraviolet

Chapter 3

CHARACTERIZATION OF λpBS10 AND λpBS99

I. INTRODUCTION

The series of λ transducing phage isolated by Spratt et al. (1980; Figure 3.1), which carried different parts of the lip-leuS region of the E.coli chromosome was used to determine the order of the five known genes in that region. Analysis of polypeptides synthesized in UV-irradiated bacteria by these phage showed that nine non-phage proteins were encoded. By comparing the different phage, these proteins could be approximately correlated with the physical map and the leuS, pbpA and dacA gene products could be identified. Two candidates for the lip gene product were identified, but no protein corresponding to the rodA gene product was found.

Further manipulation of these phage was limited by their defective phenotype. Plaque-forming transducing phage were therefore constructed. Most of the transducing DNA in λdlip5 was encompassed within a single HindIII fragment, approximately 15 kb in size (Figure 3.2). This was cloned into a λ replacement rector, λL41 (Loenen and Brammar, 1980; Figure 3.2). HindIII digests of λdlip5 and λL41 were ligated together and used to transfect $CaCl_2$-treated cells of C600 (P2). The resulting clear plaque, spi$^-$ recombinant phage were tested for their ability to transduce leuS, pbpA, rodA and lip genes. λpBS10 was found to transduce leuS, pbpA and rodA, but not lip.

In the same way, SalI fragments of λdlip5 were cloned into the vector λNM627 (Figure 3.2), and phage carrying genes from the lip-leuS

Figure 3.1

Structure of defective transducing phage carrying genes from the *lip-leuS* region.

(A) λ^+

(B) λ*dlip5*

(C) λ*ddacA51*

(D) λ*drodA1*

(E) λ*dpbpA108*

Restriction enzyme sites : B, *Bam*H1; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; S, *Sal*I. Targets in the right arm of the transducing phage are not shown as they are identical to those in λ^+. The precise junction points between the left end of the chromosomal DNA and the remainder of the λ left arm are unknown, and are indicated approximately by broken lines. (Taken from Spratt *et al.*, 1980).

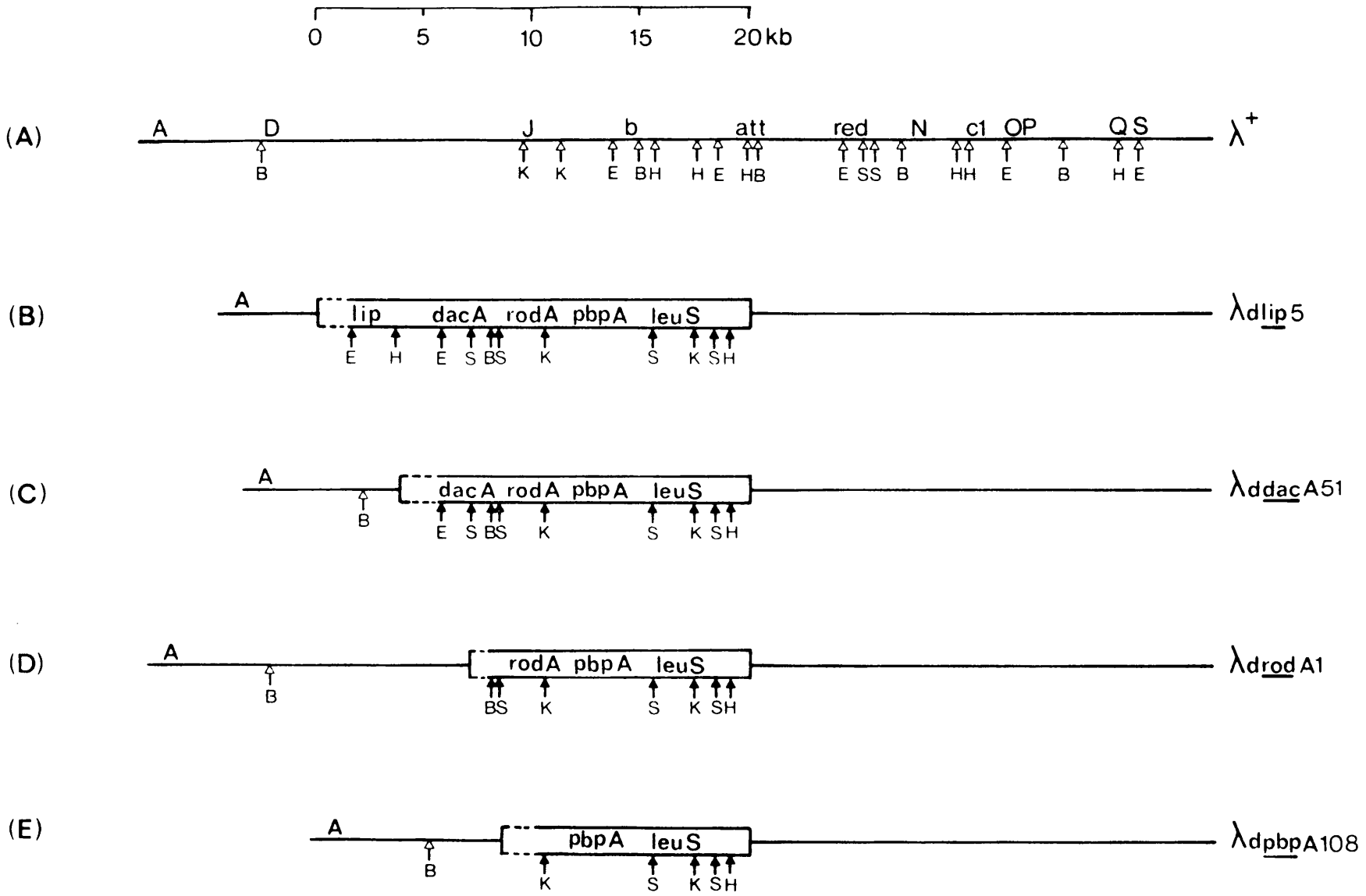

0
5
10
15
20kb
(A)
A
D
J
b
att
red
N
c1
OP
Q S
λ+
B
K K E BH H E HB E SS B HH E B H E
(B)
A
lip dacA rodA pbpA leuS
λdlip5
E H E S BS K S K SH
(C)
A
dacA rodA pbpA leuS
λddacA51
B
E S BS K S K SH
(D)
A
rodA pbpA leuS
λdrodA1
B
BS K S K SH
(E)
A
pbpA leuS
λdpbpA108
B
K S K SH

Figure 3.2

Structure of plaque-forming phage carrying genes from the *lip-leuS* region.

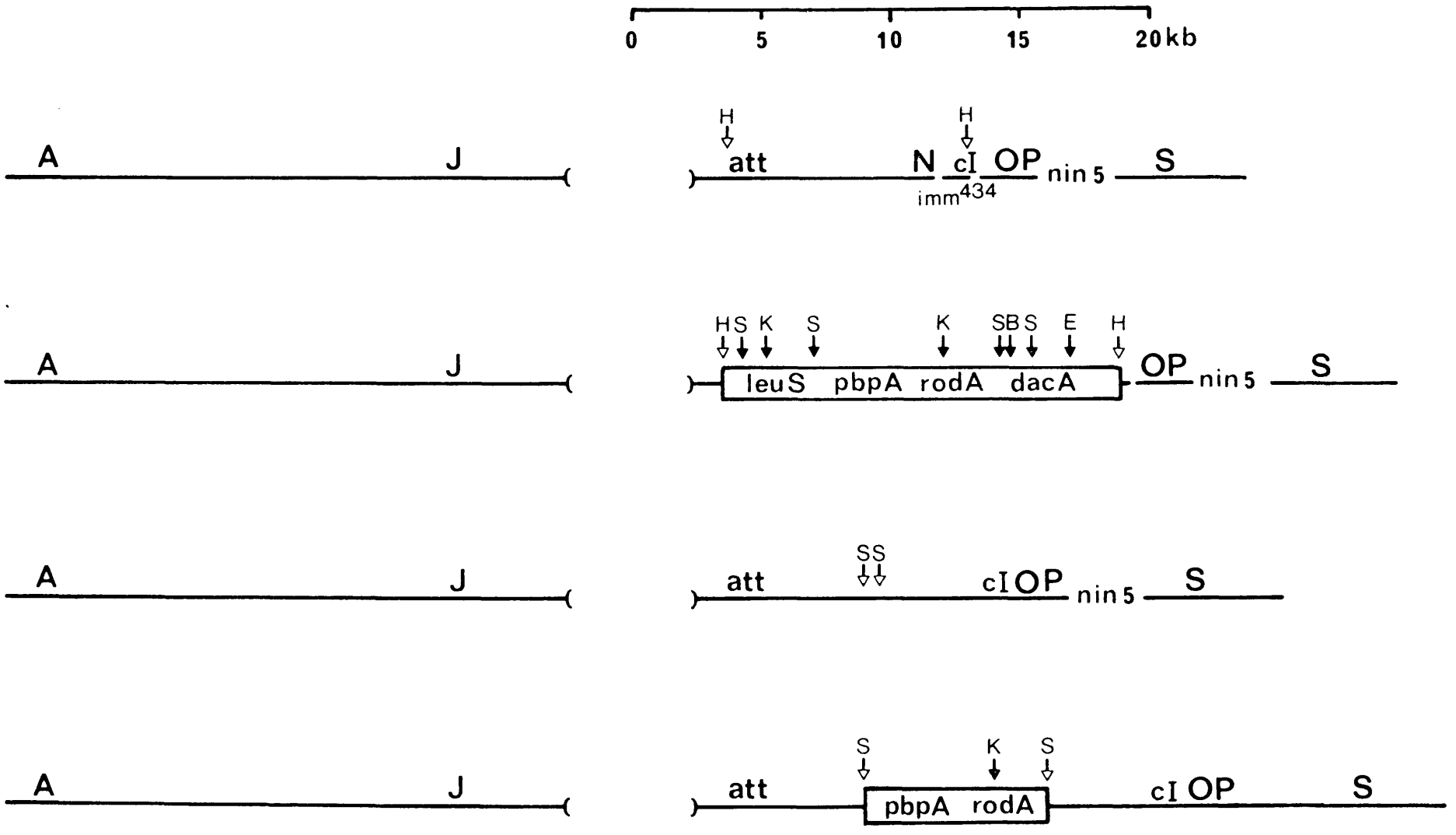
(A) λL41

(B) λpBS10

(C) λNM627

(D) λpBS99

Restriction enzyme sites : B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; S, *Sal*I. The targets in the λ DNA of the transducing phage are not shown. The space between brackets in the left arm of the phage represents the deletion of DNA between *Eco*RI sites 1 and 2 of λ. (Taken from Spratt *et al.*, 1980).


0
5
10
15
20kb
A
J
H
H
att
N
cI
OP
nin 5
S
imm434
HS K
S
K
SB S
E
H
leuS pbpA rodA dacA
OP
nin 5
S
SS
att
cI OP
nin 5
S
S
K
S
att
pbpA rodA
cI OP
S

region were detected by transduction of λ^+ lysogens of TMRL-12, SP13711, SP5211 and AB1325 lip9. The resulting dilysogens were induced by UV-irradiation and the lysates plated on ED3184. Single plaques were tested for transduction of the chromosomal markers, and purified λpBS99 was found to transduce pbpA and rodA, but not leuS or lip. No λ phage that transduced leuS or lip were found, presumably because neither gene could be cloned on a SalI fragment. In λdlip5, there was no SalI site between lip and the λ left arm (Figure 3.1), and lip was therefore uncloneable using SalI. It was confirmed later that the leuS gene crossed a SalI site (unpublished data).

II. INITIAL CHARACTERIZATION OF λpBS10 AND λpBS99

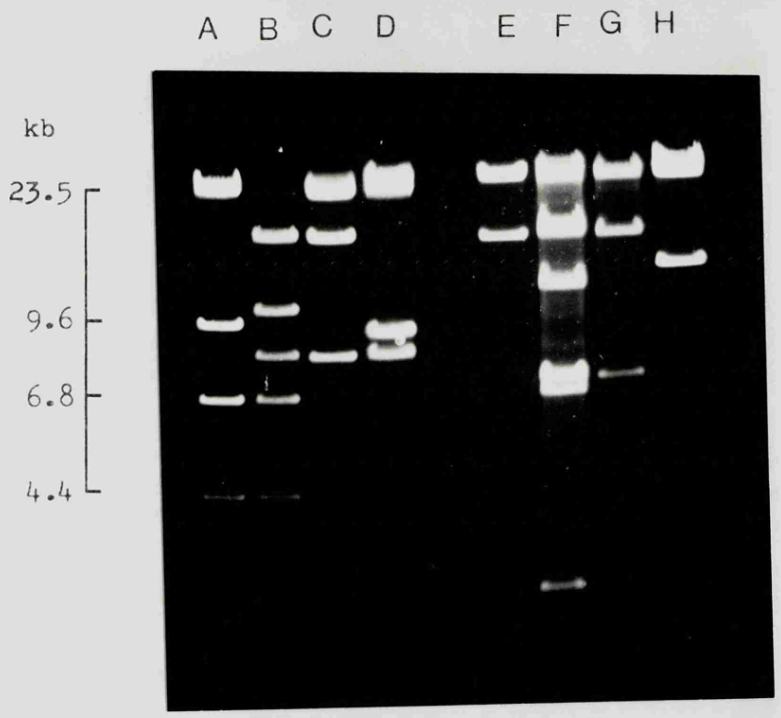
DNA was prepared from plate lysates of λpBS10. Digestion of the DNA with HindIII confirmed that the 15 kb fragment from λdlip5 had been cloned (Figure 3.3). The orientation of the cloned fragment relative to the λ DNA was determined by use of the asymmetric BamHI sites within the HindIII fragment. Digestion with BamHI showed that the orientation of the rodA-leuS genes was the reverse of that in λdlip5 (Figure 3.2; Table 3.2).

λpBS99 was red$^-$ gam$^-$ owing to the loss of the 0.53 kb region between the SalI targets of the vector phage, and since the phage lacked a chi site, it grew very poorly. In order to prepare a large quantity of DNA, it was grown with a helper phage, by thermoinduction of strain AB1325 pbpA6 (λcI857 Sam7 λpBS99). Phage were purified on a CsCl-block gradient, and λpBS99 was separated from the helper phage on an equilibrium gradient. DNA was prepared and digested with SalI, showing that the region between the SalI targets in λNM627 had been replaced with

Figure 3.3

Electrophoretic analysis of restriction enzyme digests of DNA from plaque-forming λ transducing phage.

DNA from (A) λcI857 Sam7, (B) λdlip5, (C) λpBS10 and (D) λL41 was digested with HindIII. DNA from (E) λcI857 Sam7, (F) λdlip5, (G) λpBS99 and (H) λNM627 was digested with SalI. The fragments were separated on a 0.5% agarose gel. DNA fragments smaller than 4 kb are not visible in the photograph. The largest fragment in track F is from contaminating λcI857 Sam7 helper phage present in this preparation of λdlip5 DNA.


A B C D E F G H
kb
23.5
9.6
6.8
4.4

the 7 kb SalI fragment from λdlip5 (Figure 3.3). The right arm of λpBS99 was larger than that in the rector, λNM627, as the nin5 deletion present in the vector had been lost in λpBS99, presumably by in vivo recombination with λ^+ during the isolation of the phage.

The 7 kb SalI fragment which had been cloned contained an asymmetric KpnI site. Digestion of the DNA with a combination of KpnI and EcoRI showed that the orientation of rodA and pbpA in λpBS99 was the same as in λpBS10 (Figure 3.2; Table 3.1).

III. FURTHER RESTRICTION MAPPING OF λpBS10

λpBS10 DNA was digested with a variety of restriction endonucleases and a physical map was constructed (Figure 3.4; Table 3.2). Fragments within the λDNA were sized using the map published by Daniels et al. (1980). The remaining part of the 434 immunity region was sized using the DNA sequence (R. Pastrana, personal communication). The map shown in Figure 4 differs slightly from that published (Spratt et al., 1980; Figure 3.2) in which some small fragments were missed.

IV. IDENTIFICATION OF PROTEINS SYNTHESIZED BY λpBS10 and λpBS99

The proteins encoded by λpBS10 and λpBS99 were investigated using the UV-irradiated host system. In this system, bacteria are heavily UV-irradiated to prevent any chromosomal transcription. The cells are then infected with λ transducing phage and pulse-labelled with [^{35}S] methionine. The only proteins synthesized and labelled are those encoded by the phage. In addition, by using a lysogenic strain, cI protein present in the cell represses transcription of all λ genes except for cI and rex, while all bacterial genes carried by the phage

Table 3.1

Fragments produced in restriction enzyme digests of λpBS99 (in kb)

x	SalI	:	28.2, 15.5, 7.0
x	KpnI	:	17.7, 17.3, 14.2, 1.5
x	EcoRI	:	21.5, 14.0, 5.9, 5.7, 3.6
x	SalI x KpnI	:	17.3, 15.5, 9.5, 4.8*, 2.2*, 1.5
x	EcoRI x KpnI	:	17.3, 8.2, 5.9, 5.8, 5.7, 3.6, 2.8, 1.5

In double digests, fragments not found in single digests are underlined. In the SalI-KpnI double digest, non-λ fragments are indicated with an asterisk (*).

Figure 3.4

Restriction map of λpBS10.

The map was constructed by digestion of λpBS10 DNA with combinations of restriction endonucleases. The sizes of fragments produced are shown in Table 3.2.

Restriction enzyme sites : B, BamH1; Bg, BglII; E, EcoR1; H, HindIII; K, KpnI; S, SalI.

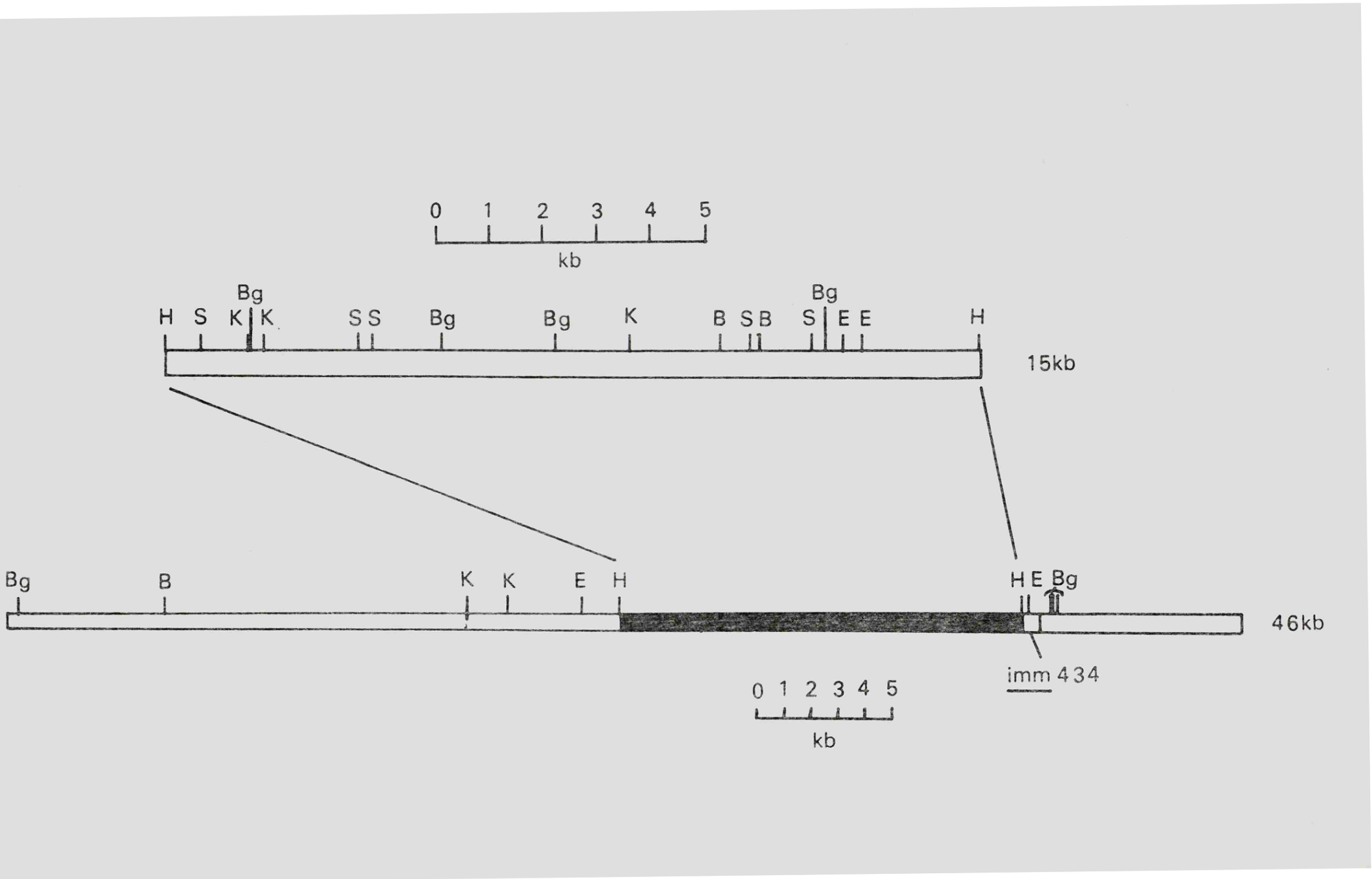

0 1 2 3 4 5
kb
Bg
H S K K S S Bg Bg K B S B S Bg E E H
15kb
Bg B K K E H H E Bg
46kb
imm 434
0 1 2 3 4 5
kb

Table 3.2

Fragments produced in restriction enzyme digests of λpBS10 (in kb)

x HindIII	: 22.9, 15.0, 8.1
x BamHI	: 32.0, 12.2, 5.8, 0.8
x SalI	: 23.5, 11.2, 7.1, 2.9, 1.2, 0.3
x KpnI	: 17.3, 14.8, 7.2, 5.6, 1.5, 0.3
x EcoR1	: 21.5, 13.9, 7.8, 2.5, 0.4
x BglII	: 24.0, 6.9, 5.0, 4.0, 3.6, 2.1, 0.5, 0.1
x HindIII x BamHI	: 18.0, 10.3*, 8.1, 5.8, 4.1*, 0.8*
x HindIII x SalI	: 22.9, 8.1, 7.1*, 3.2*, 2.9*, 1.2*, 0.6*, 0.3*
x HindIII x KpnI	: 17.3, 8.1, 7.2*, 6.7*, 4.1, 1.5*, 1.5, 0.3*
x HindIII x EcoRI	: 21.5, 12.7*, 7.8, 2.1*, 1.2, 0.4*, 0.3
x HindIII x BglII	: 22.4, 6.9, 5.0*, 3.6*, 3.0*, 2.1*, 1.53*, 1.1, 0.5, 0.1
x SalI x BamHI	: 17.7, 11.2, 6.4, 5.8, 2.9, 1.0, 0.6, 0.3, 0.2
x SalI x KpnI	: 17.3, 11.2, 4.8, 4.8, 2.1, 1.7, 1.5, 1.2, 0.9, 0.3, 0.3
x SalI x BglII	: 23.4, 6.9, 4.0, 3.6, 2.1, 2.0, 1.3, 1.2, 1.0, 0.5, 0.3, 0.3, 0.1
x KpnI X BglII	: 16.8, 6.9, 5.6, 4.0, 3.6, 3.3, 2.1, 1.5, 1.3 0.5, 0.3, 0.1

In double digests, fragments not found in single digests are underlined. In the HindIII double digests, non-λ fragments are indicated with an asterisk (*).

which have their own promoter are transcribed. For immλ phage (e.g. λdlip5, λpBS99), a strain carrying the non-inducible λind$^-$ prophage was used. For the imm434 phage, λpBS10, it was found that a λimm434 prophage did not provide complete repression of the λ rightwards promoter, p_R (p_L had been deleted during construction of the phage). Instead, plasmid pGY101 was used, which carries the phage 434 cI gene (Levine et al., 1979). The 434 cI gene carried by the plasmid was derived from λimm434T, which carries a mutation, probably in the cI promoter region, causing the phage to produce repressor at five times the normal level. Combining this with the high plasmid copy number, cells carrying the plasmid contain approximately 70 times as much repressor as λimm434 lysogens.

The proteins synthesized by λdlip5 are shown in Figure 3.5. Nine proteins were consistently detected from the transducing fragment (Spratt et al., 1980; A. Boyd, personal communication). Comparison with the proteins synthesized by λddacA51, λdrodA1 and λdpbpA108, and with published data, allowed the leuS, pbpA and dacA gene products to be identified. These experiments were repeated and confirmed.

λpBS10 synthesized eight of the nine bacterial proteins synthesized by λdlip5. λdlip5 transduced the lip gene, while λpBS10 did not. There is no published information about the lip gene product, so the 35 Kd protein not synthesized by λpBS10, which was cytoplasmic, was tentatively assigned as the lip gene product.

λpBS99 transduced the pbpA and rodA genes. It synthesized two proteins which were not synthesized by λ^+, of 66 Kd and 11 Kd (Figure 3.6). The 66 Kd protein has been identified as PBP2. The 11 Kd protein, how-

Figure 3.5

Proteins synthesized by λdlip5.

E.coli 159 (λind$^-$) cells were UV-irradiated, infected with phage and the phage proteins labelled with [^{35}S] methionine. Whole cell lysates were electrophoresed in a 16% SDS-polyacrylamide gel.

(A) control

(B) λ^+

(C) λdlip5

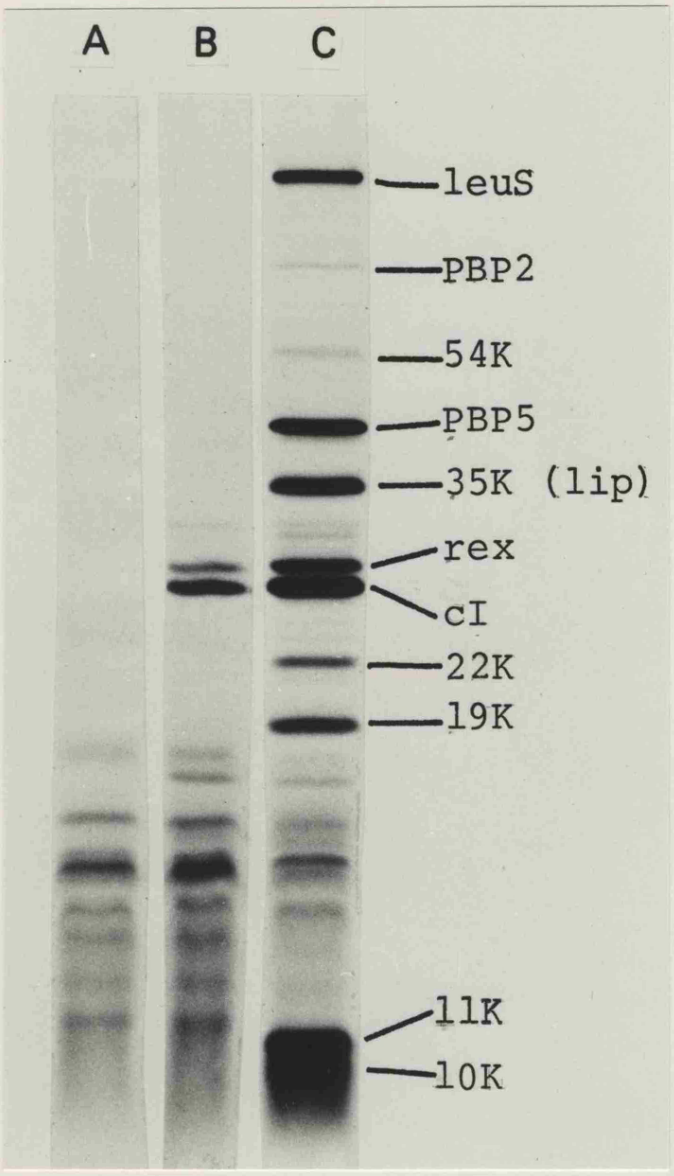

A
B
C
leuS
PBP2
54K
PBP5
35K (lip)
rex
cI
22K
19K
11K
10K

Figure 3.6

Proteins synthesized by λpBS99 and λdlip5.

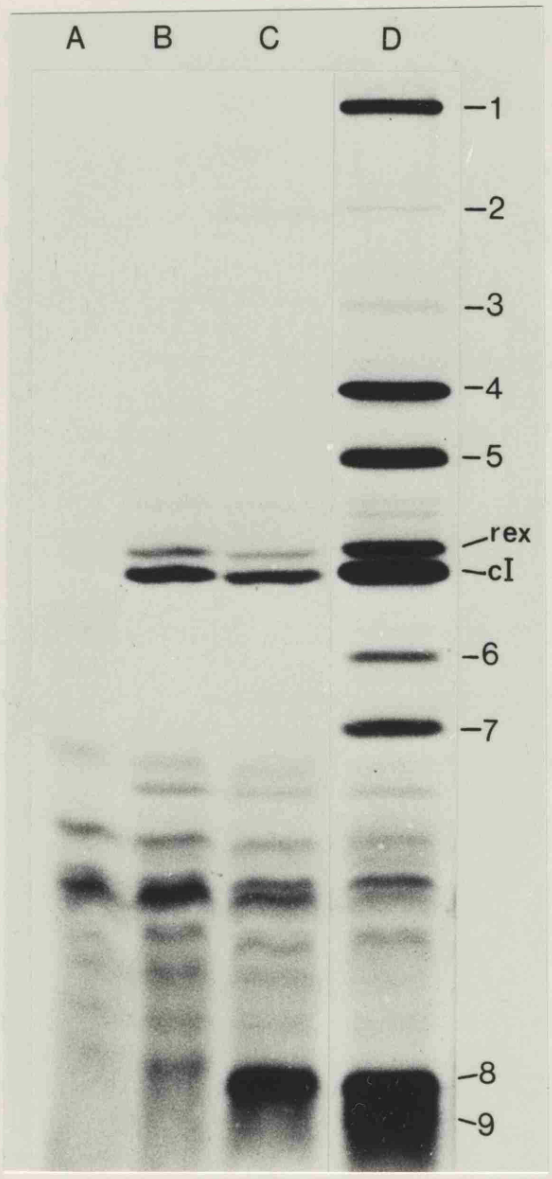
E.coli 159 (λind$^-$) cells were UV-irradiated, infected with phage and the phage proteins were labelled with [^{35}S] methionine. Whole cell lysates were electrophoresed in a 16% SDS-polyacrylamide gel.

(A) control

(B) λ^+

(C) λpBS99

(D) λdlip5


A
B
C
D
-1
-2
-3
-4
-5
rex
cI
-6
-7
-8
-9

ever, could not be the rodA gene product as it was also synthesized by λdpbpA108, which did not transduce the rodA gene. The use of a non-lysogen, to allow expression from p_L, might have helped if rodA was very weakly expressed, and was in the appropriate orientation. However, no candidate protein was found using a non-lysogen. Another trivial explanation might be that the rodA gene product lacked methionine, and so would not be labelled. To test this a [^{3}H]-amino acid mixture was used to label proteins synthesized by λpBS10, but no new protein appeared.

This data was used to construct a preliminary map showing the positions of the genes in relation to the physical map (Figure 3.7).

V. CELLULAR LOCATION OF PROTEINS SYNTHESIZED BY λpBS10

Irradiated cells were infected with λpBS10 and pulse-labelled. Inner and outer membranes were isolated and the proteins in these fractions compared with those in the whole cell lysates (Figure 3.8). The 66 Kd (PBP2), 54 Kd, PBP5 and 19 Kd proteins were all present in the inner membrane fraction. The 105 Kd (leuS), 22 Kd, 11 Kd and 10 Kd proteins were all present in the soluble part of the cell (cytoplasm/periplasm). No labelled proteins appeared in the periplasmic fraction, extracted by osmotic shock or spheroplasting of cells (data not shown), so these four proteins must be cytoplasmic. The 35 Kd protein which may be the lip gene product was also cytoplasmic (A. Boyd, personal communication).

Figure 3.7

Alignment of the genes, gene products, and restriction enzyme targets in the *lip-leuS* region.

The proteins synthesized from the *lip-leuS* region of the chromosome are numbered according to Figure 3.6. The boxes above the numbers represent the estimated size of the genes required to code for each protein. It was assumed that 1 kb codes for a 37 Kd protein. The order of pairs of proteins between brackets is undetermined. (Taken from Spratt *et al.*, 1980).

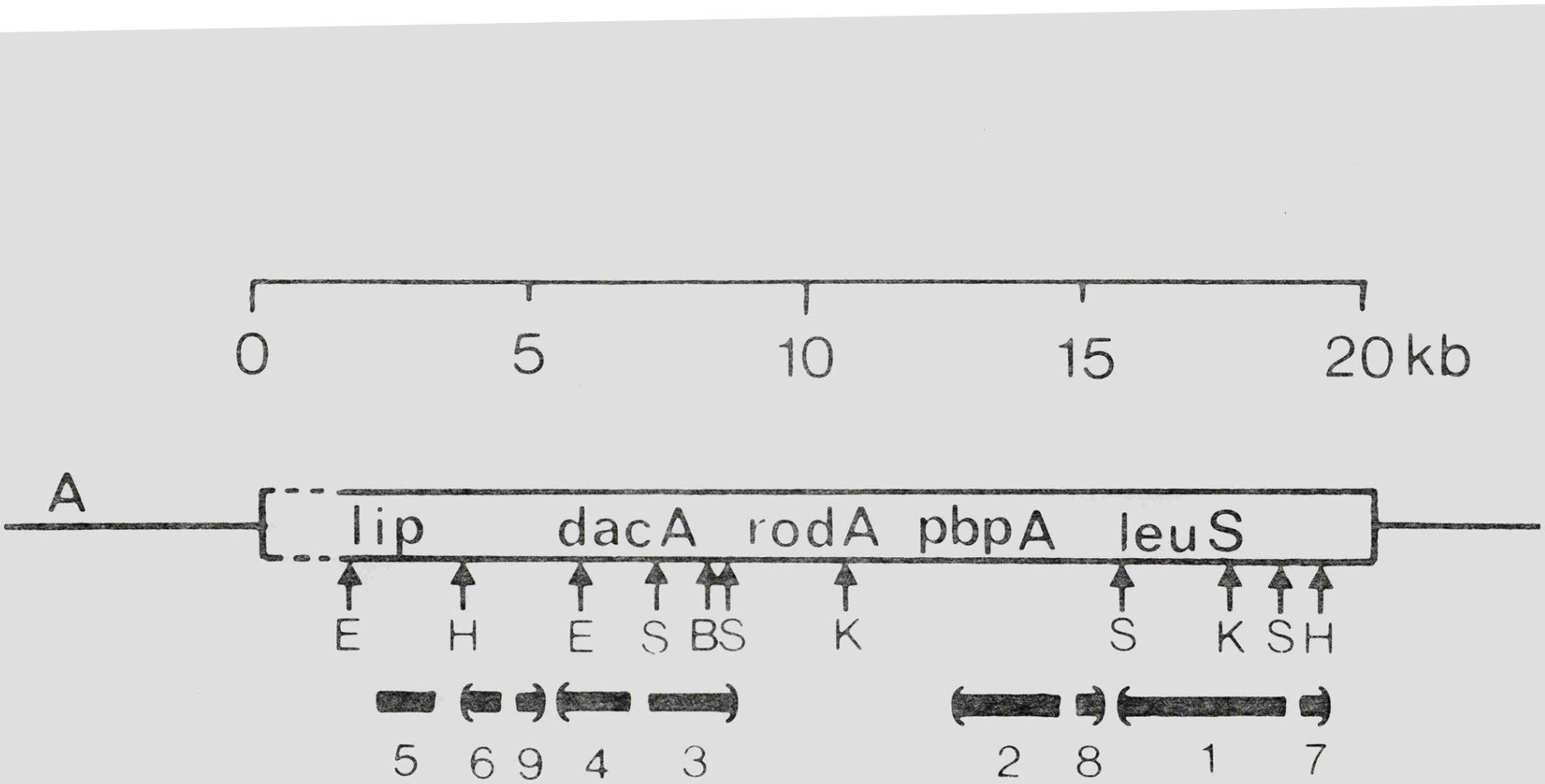

0
5
10
15
20kb
A
lip
dacA
rodA
pbpA
leuS
E
H
E
S
BS
K
S
K
S
H
5
6
9
4
3
2
8
1
7

Figure 3.8

Cellular localization of proteins encoded by λpBS10.

E.coli 159 [pGY101] cells were UV-irradiated, infected with λpBS10 and the phage-encoded proteins were labelled with [^{35}S] methionine. Part of the sample was sonicated and fractionated into sarkosyl-soluble and sarkosyl-insoluble membranes. Whole cell lysates and membrane fractions were electrophoresed in a 15% polyacrylamide gel.

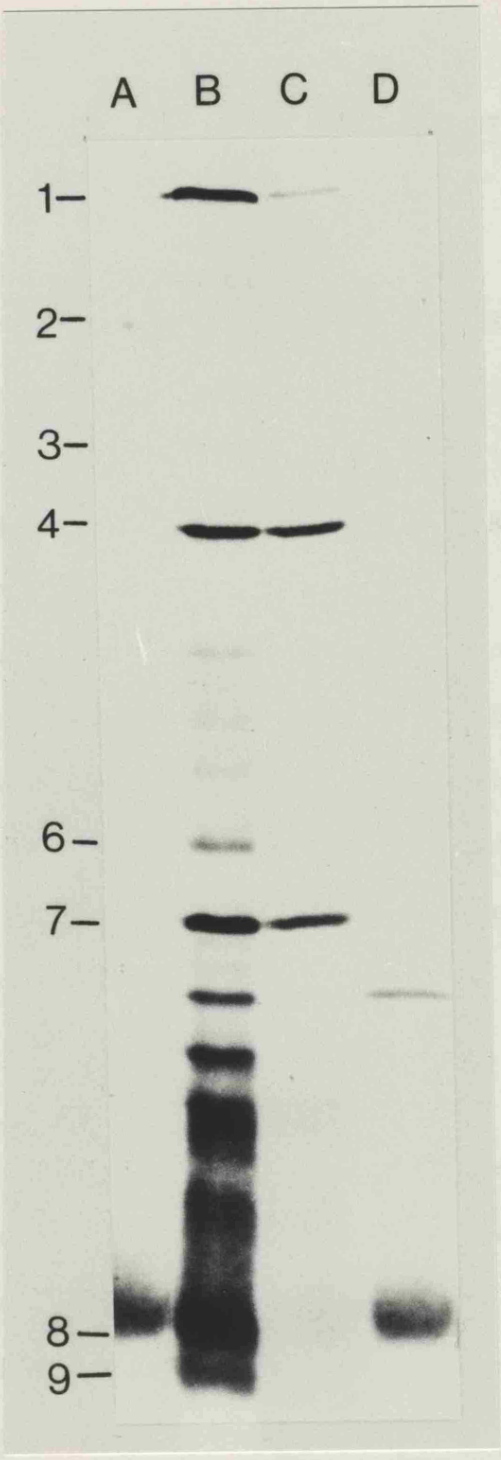
(A) control

(B) λpBS10 (whole cell lysate)

(C) sarkosyl-soluble (cytoplasmic) membrane

(D) sarkosyl-insoluble (outer) membrane

The proteins are numbered according to Figure 3.6 and there is consequently no protein 5. Proteins 2 and 3 cannot be clearly seen in this figure, but are found in the cytoplasmic membrane fraction (Spratt et al., 1980; A. Boyd, personal communication).


A
B
C
D
1
2
3
4
6
7
8
9

Chapter 4

ISOLATION AND CHARACTERIZATION OF DELETION DERIVATIVES OF λpBS10

I. INTRODUCTION

In Chapter 3, the proteins encoded by λpBS10 and λpBS99 were analysed and compared with those encoded by λdlip5 and the other defective λ transducing phage. A 35 Kd protein was identified as possibly being the lip gene product. However, no rodA gene product was identified. One explanation was that the rodA gene carried by λpBS99 had lost its promoter, although some positive transduction of rodA could still be seen due to recombination between the gene carried by the phage and the mutant gene in the chromosome. In order to remove ambiguity of this kind, and also to determine more accurately the order and exact location of genes coding for the other proteins, a series of deletion derivatives of λpBS10 was isolated and analysed.

II. ISOLATION OF λpBS10 DELETIONS

Parkinson and Huskey (1971) showed that treatment of λ phage with EDTA allows the selection of deletion derivatives. The smaller the phage genome (and hence, the lower the density of the phage), the more resistant to EDTA it is. The deletions were isolated by plating λpBS10 with C600 on Luria agar plates containing EDTA and picking large plaques. After purification, the phage were tested for transduction of leuS, pbpA and rodA.

In order to ensure that the large-plaque phage were deletion derivatives of λpBS10, four were tested for sensitivity to EDTA. Isolates

number 2 and 6 had lost transductional markers, whereas numbers 1 and 4 had not. λpBS10 (40 kb) and λNM627 (41.3 kb) were also tested. The results showed clearly that numbers 2 and 6 were more resistant to EDTA than λpBS10 (Figure 4.1). It was possible that number 1 had a small deletion although it still transduced leuS, pbpA and rodA.

III. ANALYSIS OF PROTEINS SYNTHESIZED BY λpBS10 DELETIONS

Six derivatives of λpBS10 which no longer transduced rodA were chosen for further study (Table 4.1). The deletions were approximately mapped by restriction enzyme analysis. DNA was prepared from each phage, and digested with a number of restriction enzymes. The fragments produced were compared with those produced by digestion of λpBS10 (see Figure 4.2). Determination of the restriction enzyme sites which had been deleted and calculation of the sizes of new fragments allowed the sizes and approximate end-points of the deletions to be estimated. These are shown in Figure 4.3.

The proteins synthesized by these phage were analysed in UV-irradiated cells, (Figure 4.4; Table 4.2). By comparing the proteins missing in the profiles, some of the ambiguities remaining in the protein order (see Figure 3.7) could be removed.

(a) dacA

The gene encoding protein 3 (54 Kd) has been shown to map next to dacA, but their relative positions were not known. λpBS10 Δ 8 and Δ 13 were rodA$^-$ and synthesized protein 4 (PBP5) but not protein 3. Therefore, either the gene which codes for protein 3 is rodA, or it maps between rodA and dacA. In either case, it must map on the pbpA- side of dacA.

Figure 4.1

EDTA sensitivity of λ phage with different genome sizes.

The phage was incubated in 10 mM Tris-HCl pH8.0, 10 mM EDTA at 42°C, and samples were removed at intervals and titred. The phage tested were λNM627 (□), λpBS10 (■), and λpBS10 deletion derivatives Δ1 (▲), Δ2 (Δ), Δ4 (●) and Δ6 (o).

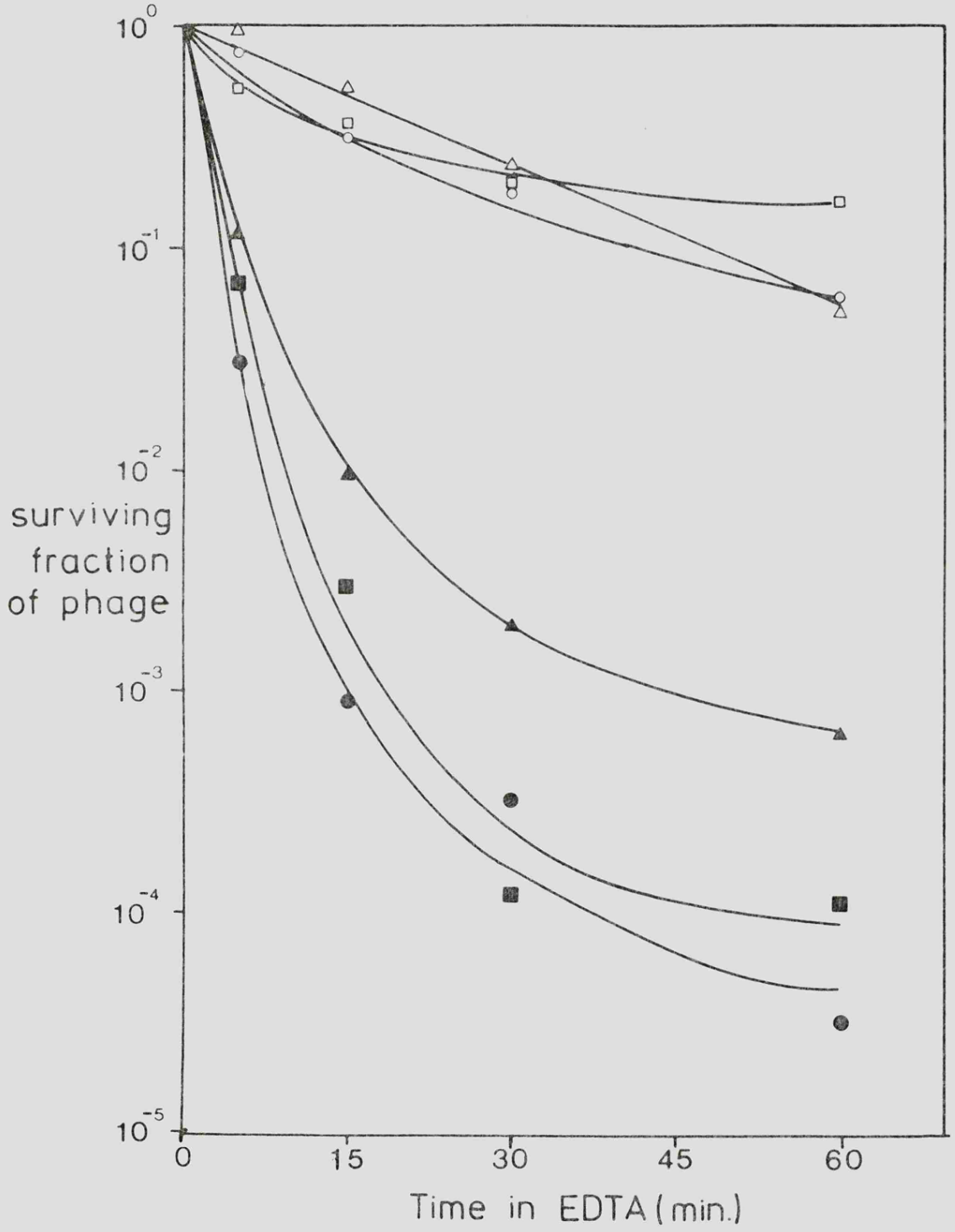

10^{0}
10^{-1}
10^{-2}
surviving fraction of phage
10^{-3}
10^{-4}
10^{-5}
0
15
30
45
60
Time in EDTA (min.)

Table 4.1

Genes Transduced By Deletion Derivatives of λpBS10

	λpBS10	Δ2	Δ6	Δ8	Δ13	Δ34	Δ51
leuS	+	+	+	+	+	+	+
pbpA	+	-	+	+	+	-	-
rodA	+	-	-	-	-	-	-

Figure 4.2

Restriction endonuclease digests of DNA from λpBS10 (λ^*), λpBS10 Δ2 (2), λpBS10 Δ6 (6) and λpBS10 Δ34 (34). λ^+ DNA was digested with HindIII as a size marker. The digests were electrophoresed in a 0.5% agarose gel.

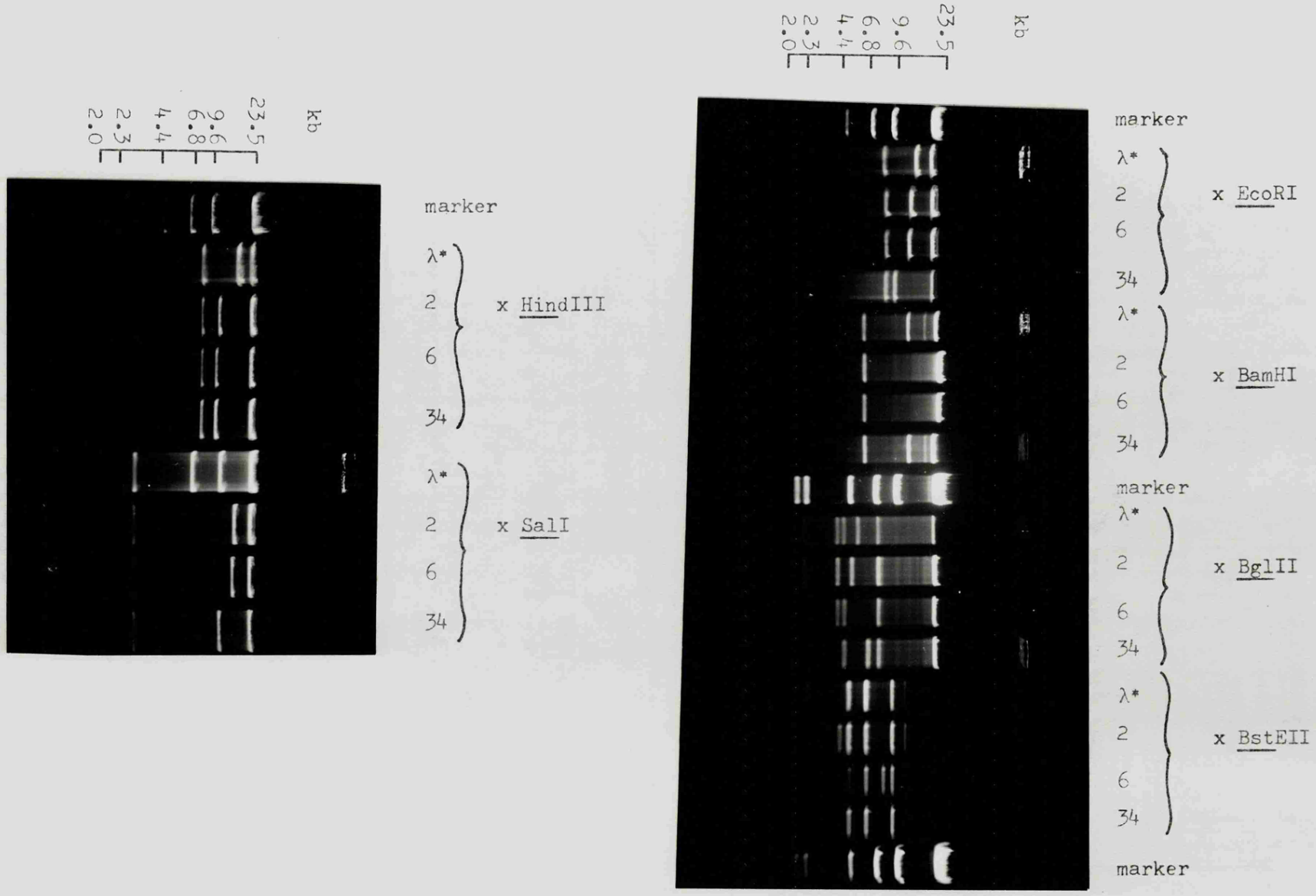

kb
23.5
9.6
6.8
4.4
2.3
2.0
marker
λ*
2
6
34
x HindIII
x SalI
x EcoRI
x BamHI
x BglII
x BstEII

Figure 4.3

Map of the 15 kb transducing fragment of λpBS10 showing the regions deleted in the deletion derivatives, and the approximate locations of the genes identified. The dotted lines indicate ambiguities in the mapping of the deletions. The genes are numbered as in Table 4.2. For the purposes of mapping the genes, it was assumed that 1 kb codes for a 37Kd protein. The order of genes in brackets is undetermined.

Restriction enzyme sites : B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; K, KpnI; S, SalI.

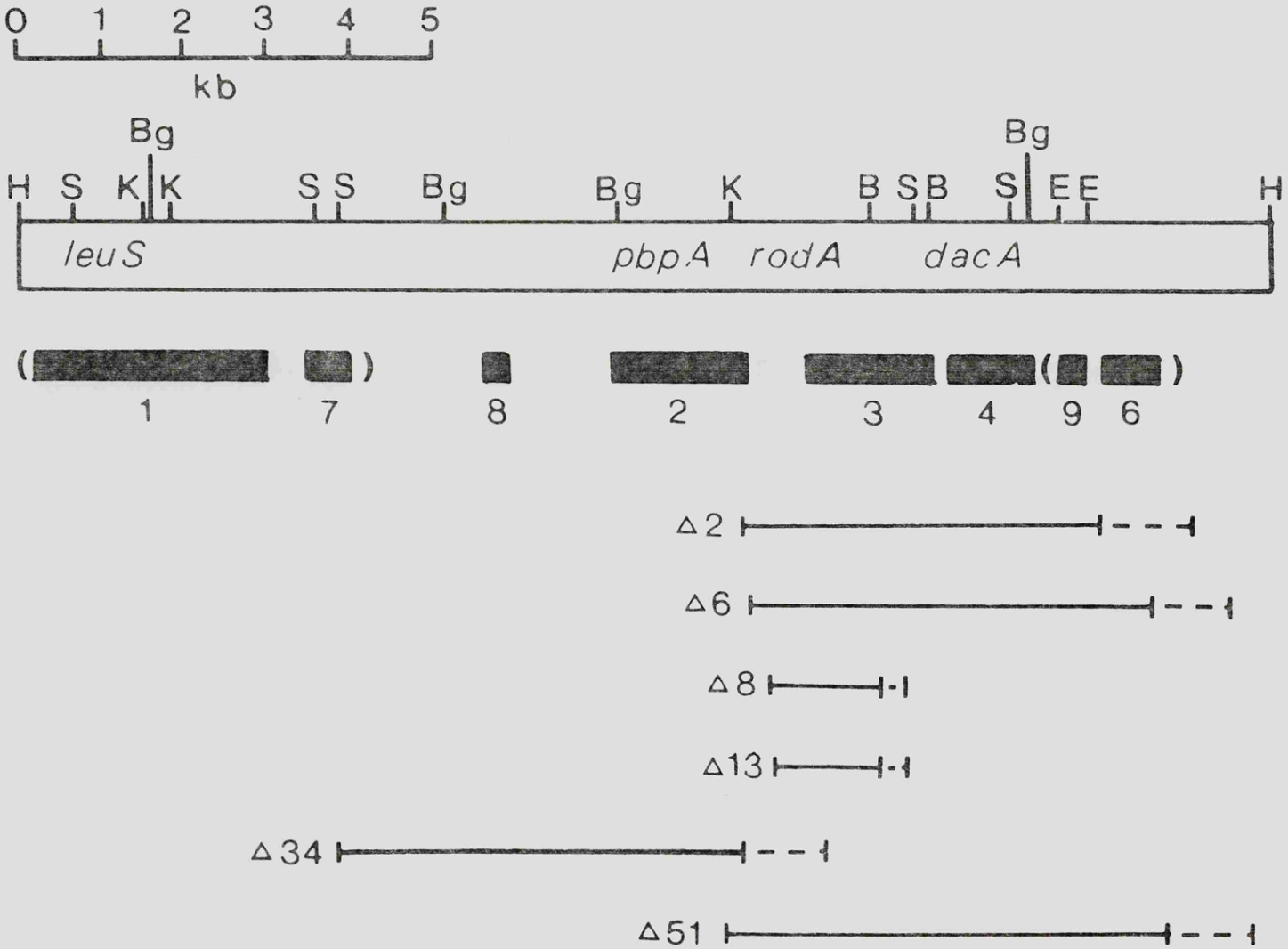

0 1 2 3 4 5
kb
Bg
Bg
H S K K S S Bg Bg K B S B S E E H
leuS
pbpA rodA dacA
1 7 8 2 3 4 9 6
Δ2
Δ6
Δ8
Δ13
Δ34
Δ51

Figure 4.4

Proteins encoded by λpBS10 and its deletion derivatives.

The phage were adsorbed to heavily UV-irradiated E.coli 159 [pGY101]. The proteins synthesized were pulse-labelled with [^{35}S] methionine, followed by electrophoresis in a 15% polyacrylamide gel and fluorography.

A control (no phage added)

B λpBS10

C " Δ2

D " Δ6

E " Δ8

F " Δ13

G " Δ34

H " Δ51

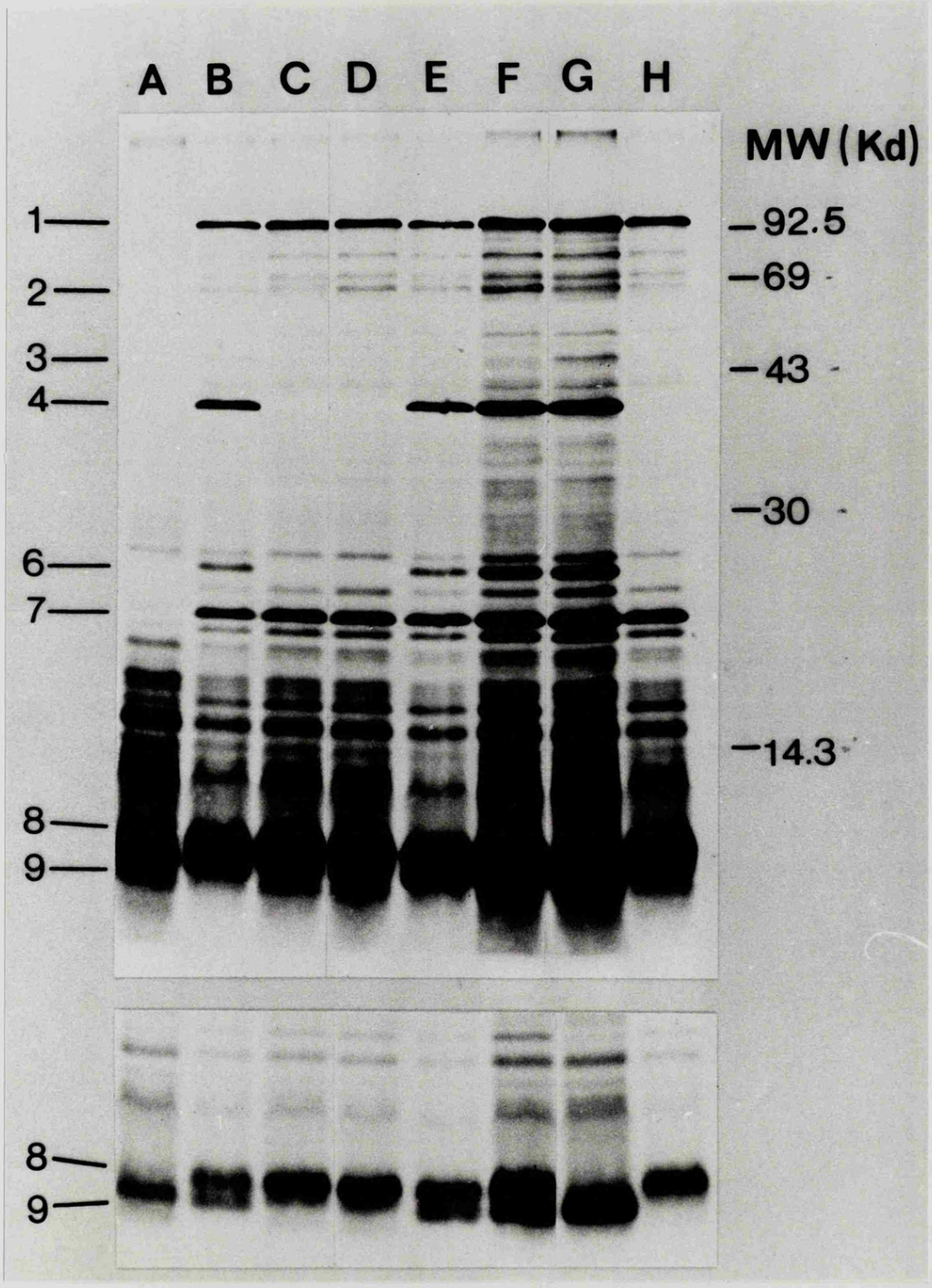

A B C D E F G H
MW (Kd)
1
2
3
4
6
7
8
9
92.5
69
43
30
14.3
8
9

Table 4.2 Proteins Encoded By Deletion Derivatives Of λpBS10

Protein	MW (Kd)	Gene	λpBS10	Δ2	Δ6	Δ8	Δ13	Δ34	Δ51	Cellular location
1	105	leuS	+	+	+	+	+	+	+	C
2	66	pbpA	+	-	+	+	+	-	-	IM
3	54	?	+	-	-	-	-	+	-	IM
4	41	dacA	+	-	-	+	+	+	-	IM
6	22	?	+	-	-	+	+	+	-	C
7	19	?	+	+	+	+	+	+	+	IM
8	11	?	+	+	+	+	+	-	+	C
9	10	?	+	-	-	+	+	+	-	C

The proteins are numbered according to Figure 3.6, so there is no protein 5.

C, cytoplasmic; IM, inner membrane

(b) pbpA

The gene encoding protein 8 (11 Kd) mapped next to pbpA. λpBS10 Δ 2, in which dacA, rodA and pbpA had been deleted, still synthesized the 11 Kd protein. Therefore, the gene encoding this protein maps on the leuS-side of pbpA.

(c) rodA

rodA mapped between pbpA and dacA. The only protein synthesized from this region that had been identified so far, was protein 3 (54 Kd). However, λpBS10 Δ 34, which did not transduce pbpA or rodA, clearly synthesized protein 3. Therefore, rodA maps between pbpA and the gene which codes for protein 3.

The ambiguities in the positions of proteins 1 and 7, and of proteins 6 and 9, were not resolved. Using these data and the location of the deletions, the sizes and positions of the genes encoding these proteins were calculated (Figure 4.3).

Having obtained evidence that none of the nine proteins shown in Figure 3.5 was the rodA gene product, it was decided to subclone rodA onto a plasmid vector in order to allow more detailed analysis of the gene.

Chapter 5

SUBCLONING THE *rodA* GENE

I. INTRODUCTION

It was decided to subclone the *rodA* gene into a plasmid vector. Previous experiments had not precluded the possibility that *rodA* was part of an operon transcribed from *pbpA*. Cloning *rodA* without its neighbouring genes would discount this, and would also allow more detailed analysis of the gene.

II. CLONING *rodA* INTO pSC105

The 5 kb *Bgl*II fragment from λpBS10 was cloned into the plasmid vector pSC105 (Cohen *et al.*, 1973). This *Bgl*II fragment was chosen because it carried the entire *rodA* gene as well as the gene coding for the 54 Kd protein and most or all of *pbpA* and *dacA*. pSC105 was chosen as the vector because previous attempts to clone several fragments of λ*dlip5* onto a multicopy plasmid had resulted in recombinants which grew with bizarre morphologies (B.G. Spratt and A. Zaritsky, unpublished data). This was possibly due to the deleterious effects of overproduction of one or more of the proteins encoded by this region. pSC105 has a copy number of only about five molecules per chromosome. It carries resistance genes to kanamycin and tetracycline. There was a single *Bam*HI site within the tetracycline resistance gene. Since *Bgl*II produces a 5'-extension in DNA identical to that produced by *Bam*HI, the *Bgl*II fragment could be cloned into the *Bam*HI site, although the hybrid sites created would not be cut by either enzyme.

λpBS10 was digested with BglII, and the 5 kb fragment was purified. This fragment was ligated with a BamHI digest of pSC105. The ligation mix was transformed into the temperature-sensitive rodA mutant SP5211. After incubation for one hour to allow expression of the antibiotic resistance, the cells were plated onto nutrient agar containing kanamycin, and incubated overnight at 42°C. Colonies of two distinct sizes appeared, some being much larger than others. It was confirmed under the microscope that the larger colonies contained rod-shaped cells, while the smaller colonies contained spherical cells.

SDS-cleared lysates of the rod-shaped cells were run on an agarose gel. They all contained an identically sized plasmid, larger than pSC105. Four colonies were purified and plasmid DNA was prepared. Digestion with BamHI showed the presence of the 0.8 kb BamHI fragment, as expected. Digestion with SalI produced two different patterns, representing the fragment cloned in both possible orientations. A representative of each class was taken, and they were named pLG340 and pLG341 (Figure 5.1). As both pLG340 and pLG341 complemented rodA, the rodA gene was transcribed from a promoter within the cloned fragment rather than from the promoter of the tetracycline-resistance gene, into which the fragment had been cloned.

It was now tested whether these plasmids carried the pbpA gene as well as rodA. They were transformed into the temperature-sensitive pbpA mutant SP45. The transformants remained spherical at 42°C, indicating that the plasmids did not carry the whole pbpA gene. This was the first time that rodA had been cloned separately from pbpA, and showed that rodA was not dependent upon the pbpA promoter for expression.

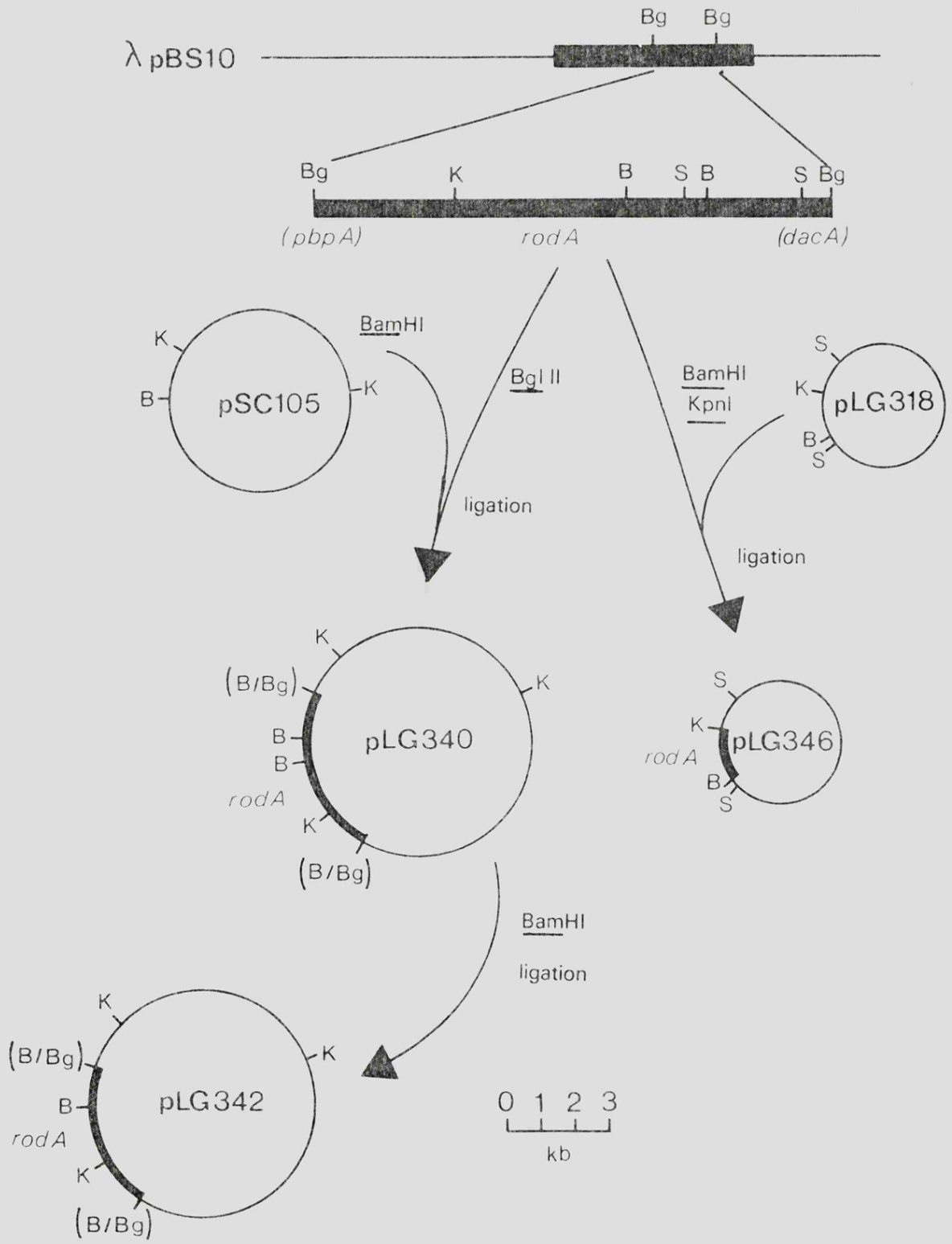
Figure 5.1

Diagram showing the subcloning of *rodA*.

Restriction enzyme sites : B, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I; S, *Sal*I;

B/Bg, *Bam*HI-*Bgl*II hybrid site.

The scale shown refers only to the plasmids in the figure.


λ pBS10
Bg
Bg
Bg
K
B
S
B
S
Bg
(pbpA)
rodA
(dacA)
BamHI
BglII
ligation
pSC105
K
B
K
BamHI
KpnI
ligation
pLG318
S
K
B
S
pLG340
(B/Bg)
K
B
B
rodA
K
(B/Bg)
pLG346
S
K
rodA
B
S
BamHI
ligation
pLG342
K
(B/Bg)
B
rodA
K
(B/Bg)
0 1 2 3
kb

Other work showed that the BglII site at the dacA-end of the fragment actually lies within dacA (B.G. Spratt and J.K. Broome-Smith, unpublished data), so the plasmids only carried part of the dacA gene.

III. FURTHER SUBCLONING OF rodA : CONSTRUCTION OF pLG346

Digestion of pLG340 and pLG341 with BamHI, followed by religation, removed the 0.8 kb BamHI fragment from the cloned DNA (Figure 5.1). The plasmids produced, pLG342 and pLG344, still complemented the rodA mutation. It was known that rodA did not lie between this BamHI site and dacA, since λpBS99 (rodA$^+$) did not carry this DNA. λpBS10 Δ 2 carried a deletion of the pbpA, rodA and dacA genes (see Chapter 4). However it retained the KpnI site which lies within the 5 kb BglII fragment from λpBS10 which had been cloned. Therefore, one end of the pbpA gene extends past the BglII site while the other end extends past the KpnI site. It was therefore anticipated that rodA lay within the 1.65 kb KpnI-BamHI fragment in λpBS10.

This KpnI-BamHI fragment was cloned into pLG318 (a derivative of pSC105 with a single KpnI site - see Chapter 9). λpBS10 and pLG318 were digested with KpnI and BamHI, mixed and ligated. The ligation mixture was transformed into SP5211 and plated onto nutrient agar containing kanamycin at 42°C as before. Again, large colonies appeared which grew as rod-shaped cells at high temperature. DNA from one transformant was prepared, and the plasmid (pLG346) had the expected restriction pattern. Therefore, the rodA gene lay within this 1.65 kb fragment.

Although pLG346 apparently complemented the rodA gene, it could still be argued that homologous recombination between the cloned fragment and the chromosome was allowing growth of rod-shaped cells at 42°C. To

discount this, pLG346 was transformed into SP5211 recA. The cells grew as rods at 42°C, confirming that it was true complementation.

IV ANALYSIS OF PROTEINS SYNTHESIZED BY PLASMIDS CARRYING rodA

Plasmids pLG340, pLG341, pLG342, pLG344 and pLG346 were transformed into the minicell-producing strain DS410. Minicells were isolated and labelled, and the proteins analysed by SDS-PAGE followed by autoradiography. The results are shown in Figure 5.2.

All six plasmids synthesized a major 28 Kd protein. This was kanamycin phosphotransferase, which has a reported molecular weight of 27 Kd (Meagher et al., 1977). A 33 Kd protein was synthesized by pSC105, but by none of the others. Presumably this was the 34 Kd tetracycline resistance protein described by Tait and Boyer (1978).

pLG340 and pLG341 synthesized the 54 Kd protein as expected. pLG340 synthesized a 36 Kd protein, while pLG341 synthesized a 35 Kd protein. These were probably truncated PBP5 polypeptides, since the 5 kb BglII fragment contained the proximal three-quarters of dacA (B.G. Spratt and J.K. Broome-Smith, unpublished data). The difference in size was presumably due to the formation of a slightly larger fusion protein when the BglII fragment was cloned in one orientation, than in the other. Approximately as much of the truncated PBP5 was synthesized as of the 54 Kd protein, whereas much more PBP5 was made by λpBS10 (see Figure 4.4). This may indicate that the truncated proteins were rather unstable, and some proteolysis occurred. A protein band which lies just above the 54 Kd protein is synthesized by pLG340 and pLG341. This may be an unprocessed precursor of the 54 Kd protein. Herrero et al. (1982) have shown that this protein is

Figure 5.2

Proteins synthesized in minicells by *rodA*$^+$ plasmids.

Minicells were pulse-labelled with [^{35}S] methionine and analysed on a 12% polyacrylamide gel followed by autoradiography. Kan, kanamycinphosphotransferase.

A : pSC105

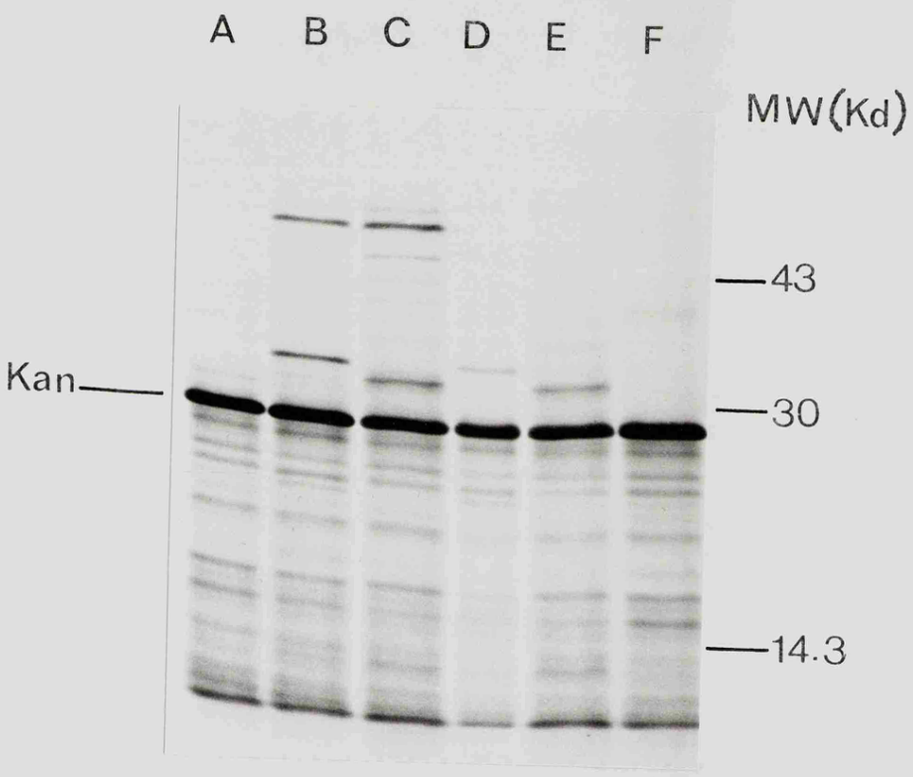
B : pLG340

C : pLG341

D : pLG342

E : pLG344

F : pLG346


A
B
C
D
E
F
MW(Kd)
43
30
14.3
Kan

synthesized as a larger protein, which is then processed, and it appears that processing of proteins is not always as efficient in minicells as in normal cells (e.g. Clement et al., 1982). A 56 Kd protein was synthesized by pLG341, which might be a truncated PBP2, but no such protein was seen with pLG340.

pLG342 and pLG344 no longer synthesized the 54 Kd protein and its putative precursor. pLG346 appeared to synthesize only one protein (40 Kd) not synthesized by pSC105. Presumably this was a fusion protein, because it was not made by the other clones. The rodA protein should be synthesized by all of the plasmids except for pSC105. No such protein was seen, so there was still no candidate for the rodA gene product.

Chapter 6

IDENTIFICATION OF THE rodA GENE PRODUCT

I. INTRODUCTION

Although rodA had been cloned on a 1.65 kb fragment into the plasmid pLG318, no protein was identified as its gene product. In order to help further attempts to identify the gene product, it was decided that it would be useful to obtain some idea of the size of the gene. If the gene was rather small, and therefore encoded a small protein, the protein might not have been detected partly because small proteins tend to form rather diffuse bands in the PAGE system used, and partly because the higher background in this region of the gel might have obscured a protein made in small amounts.

One way of estimating the size of the gene was by transposon mutagenesis. Transposons which had inserted into rodA and inactivated it could be selected, and the sites of insertion mapped by restriction analysis. Mapping several insertions would then provide a minimum size for the gene, and would give some indication of the size of protein to expect.

A simple method for isolating transposition mutants has been developed using the Tn3-like transposon Tn1000, formerly called γδ (Guyer, 1978). Tn1000 is carried by the conjugative plasmid F. A non-conjugative plasmid present in the same cell may be mobilized with high efficiency if it has an origin of transfer, and if, complemented by F-coded proteins, it can form a relaxation complex. Plasmids mobilized in this way are not altered by the transfer process. However, plasmids which are non-mobilizable can be transferred at a lower efficiency by integration into the F plasmid. The non-mobilizable plasmid is transferred passively

in this cointegrate form. Once in the recipient cell, the cointegrate is resolved, leaving the F factor and the non-mobilizable plasmid separated as before. The whole process is recA-independent. In more than 99% of cases, cointegrate formation occurs by transposition of Tn1000 (Figure 6.1), producing a plasmid carrying two copies of Tn1000 in direct repeat and separated by the non-mobilizable plasmid DNA. In the recipient, recombination between the two copies of Tn1000 separates the two plasmids, each carrying one copy of Tn1000. Therefore, virtually all non-mobilizable plasmids which are transferred by F carry a copy of Tn1000, inserted fairly randomly.

II. CLONING OF rodA INTO pACYC184 : CONSTRUCTION OF pLG355

For the mutagenesis, it was desirable, though not essential, to clone rodA into an amplifiable plasmid vector, in order to simplify restriction mapping of a large number of different plasmids. It was decided to try and clone rodA into pACYC184, a small amplifiable plasmid with a copy number of about 18 molecules per chromosome (Chang and Cohen, 1978; Figure 6.2).

The 1.65 kb KpnI-BamHI fragment carrying rodA which had been cloned to produce pLG346 was awkward to clone, since very few available amplifiable cloning vectors contained a KpnI site. However, rodA could be cloned as a 2.6 kb BamHI-SalI fragment from pLG346 (Figure 5.1). pLG346 and pACYC184 were digested with BamHI and SalI, mixed, ligated and transformed into SP5211 recA. The cells were plated onto nutrient agar containing chloramphenicol and incubated at 42°C. Large colonies made up of rod-shaped cells appeared, and DNA from one transformant was prepared. The plasmid (pLG355) had the expected restriction pattern (Figure 6.2).

Figure 6.1

Diagram illustrating Tn1000 mutagenesis.

A : Conjugative plasmid F carrying Tn1000, present in cell together with non-mobilizable plasmid.

B : Transposition of Tn1000 into another plasmid produces a co-integrate plasmid in which the non-mobilizable plasmid is flanked by two copies of Tn1000 in direct repeat.

C : Conjugation - the cointegrate plasmid is transferred. The non-mobilizable plasmids can only be transferred in this way.

D : Recombination between the two copies of Tn1000 resolves the co-integrate, forming the F plasmid in its original state, and the non-mobilizable plasmid carrying a copy of Tn1000.

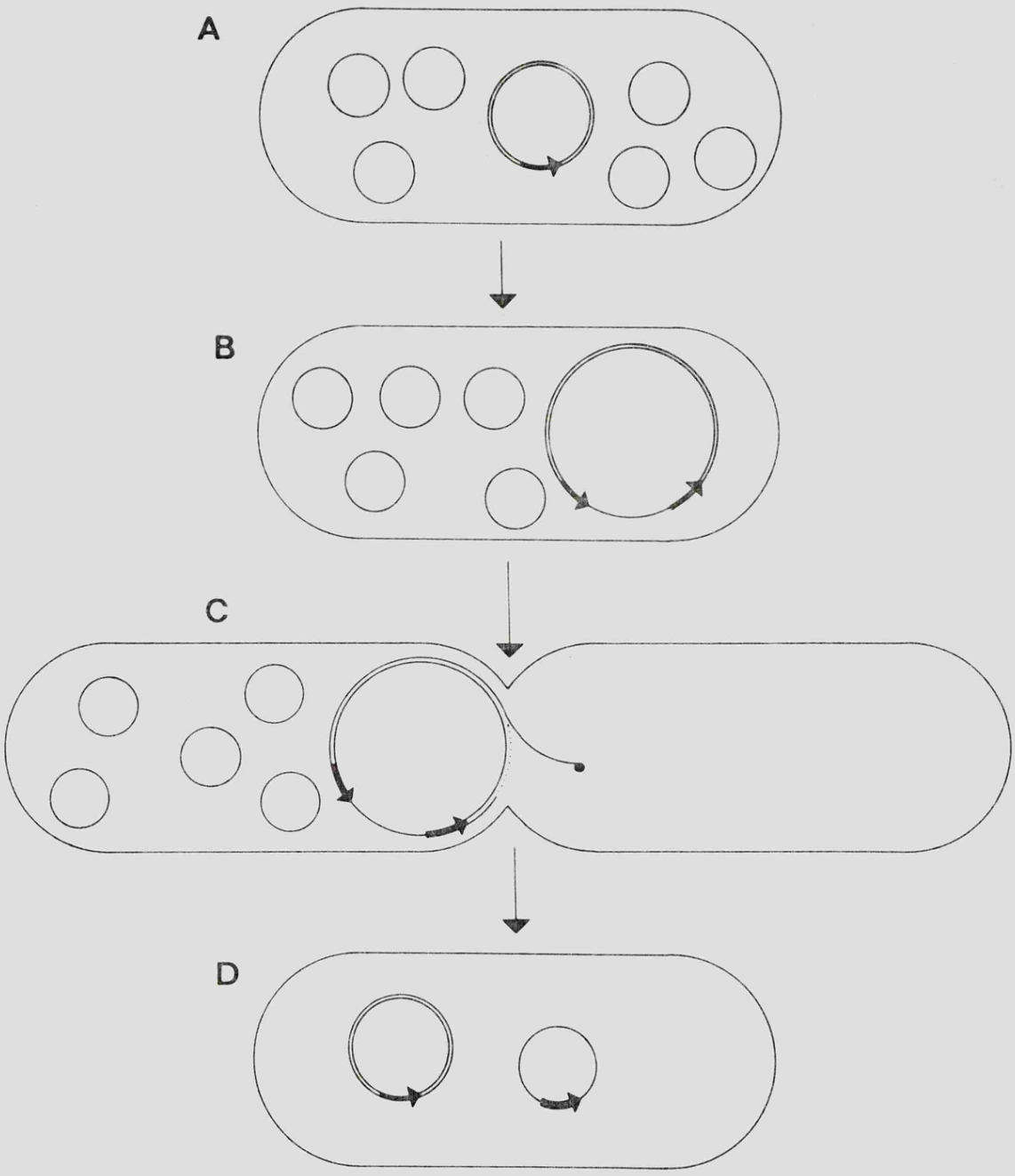

A
B
C
D

Figure 6.2

Diagram showing the construction of pLG355.

Restriction enzyme sites : B, BamHI; K, KpnI; S, SalI.

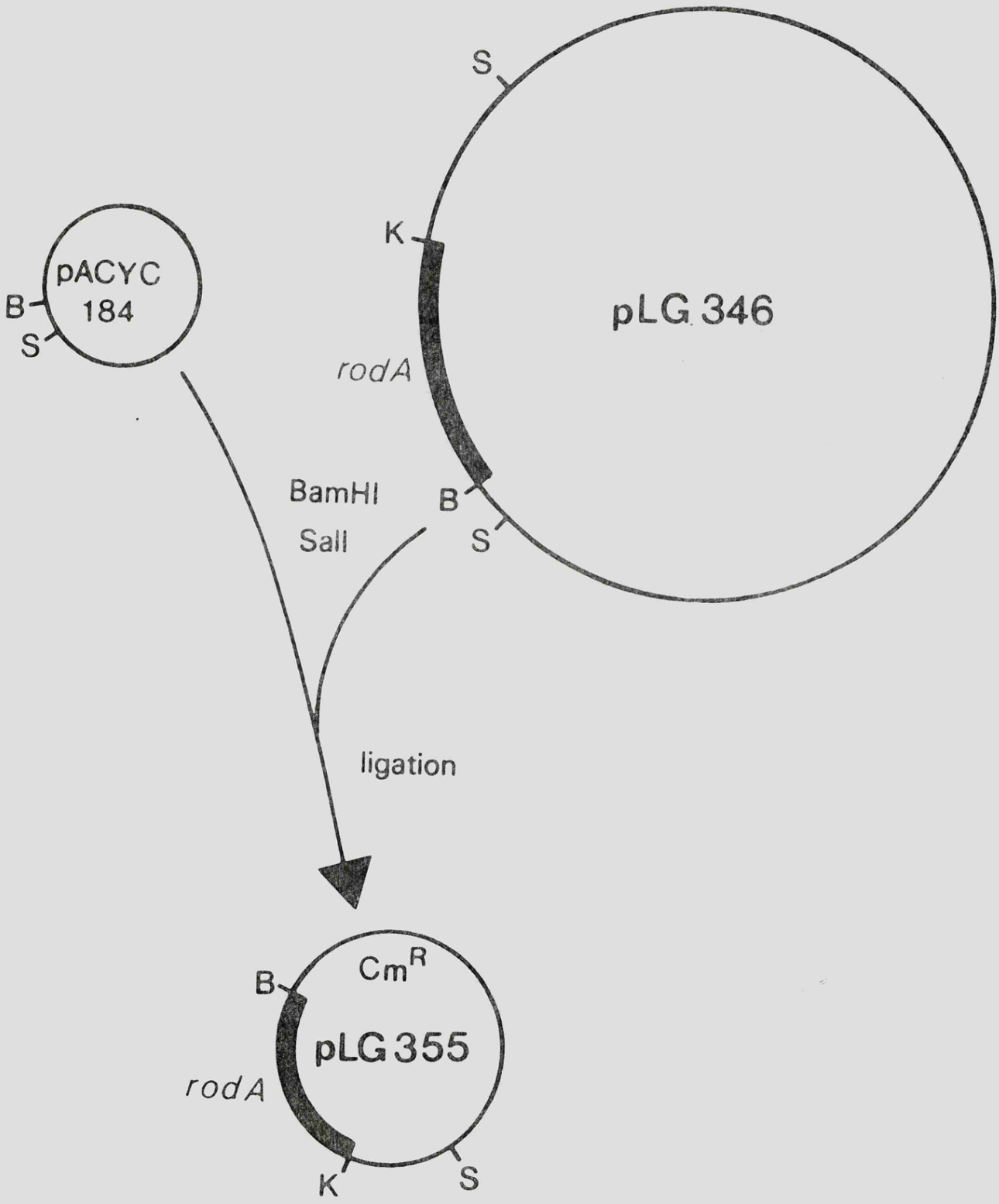
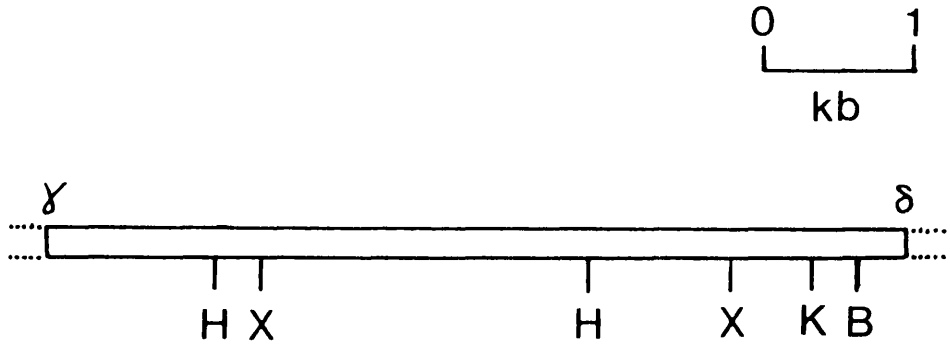

S
K
pACYC
184
B
S
pLG 346
rodA
BamHI
SalI
B
S
ligation
B
Cm^R
pLG 355
rodA
K
S

Figure 6.3

Restriction map of Tn1000.

Restriction enzyme sites : B, BamHI; H, HindIII; K, KpnI; X, XhoI.

Taken from Guyer (1978).



III. Tn1000 MUTAGENESIS OF pACYC184

In order to make sure that the experiment was feasible, it was tried out using pACYC184. pACYC184 was transformed into the F^+ strain, RB308. This was mated with C600 rpsL and the cells were plated onto nutrient agar containing streptomycin alone or with chloramphenicol or tetracycline. Cm^R and Tc^R transconjugants appeared with a frequency of about 3.5×10^{-7}. This was ten-fold higher than the efficiency of mobilization of pACYC184 by an Hfr strain reported by Chang and Cohen (1978). Hfr formation is frequently a consequence of Tn1000 transposition, presumably forming an F-chromosome cointegrate with Tn1000 at each end of F. Presumably co-integration with pACYC184 then occurred by transposition of either copy of Tn1000, only one of which would be transferred efficiently. This would halve the efficiency of mobilization of pACYC184. An additional explanation might be that a linear molecule containing two copies of Tn1000 is a less efficient substrate for the resolution of the cointegrate.

Transconjugants were tested for the drug resistance not selected. 2 out of 22 (9%) Tc^R colonies were Cm^S, and 7 out of 50 (14%) Cm^R colonies were Tc^S. Small plasmid preparations from four of these mutants were analysed. All plasmids were the same size, much larger than pACYC184. They were digested with BamHI and HindIII. All had gained one BamHI site, and contained a novel 2.9 kb HindIII fragment. This was consistent with the restriction map of Tn1000 (Figure 6.3). It was concluded that all four plasmids had gained a copy of Tn1000.

IV. Tn1000-MUTAGENESIS OF pLG355

RB308 [pLG355] was mated with SP5211 recA, and the cells plated onto nutrient agar containing streptomycin and chloramphenicol at 42°C. Colonies that appeared were heterogeneous in size. It was predicted that insertion of Tn1000 into rodA would make the cells grow more slowly than equivalent cells carrying a functional rodA gene. However, any insertion which partially affected plasmid replication and maintenance, or expression of chloramphenicol resistance, might have the same effect. Small colonies were streaked onto fresh plates and grown at 42°C. The cells were then examined under the microscope. 31 colonies which grew as round cells (about 5.5% of transconjugants) were purified and analysed further.

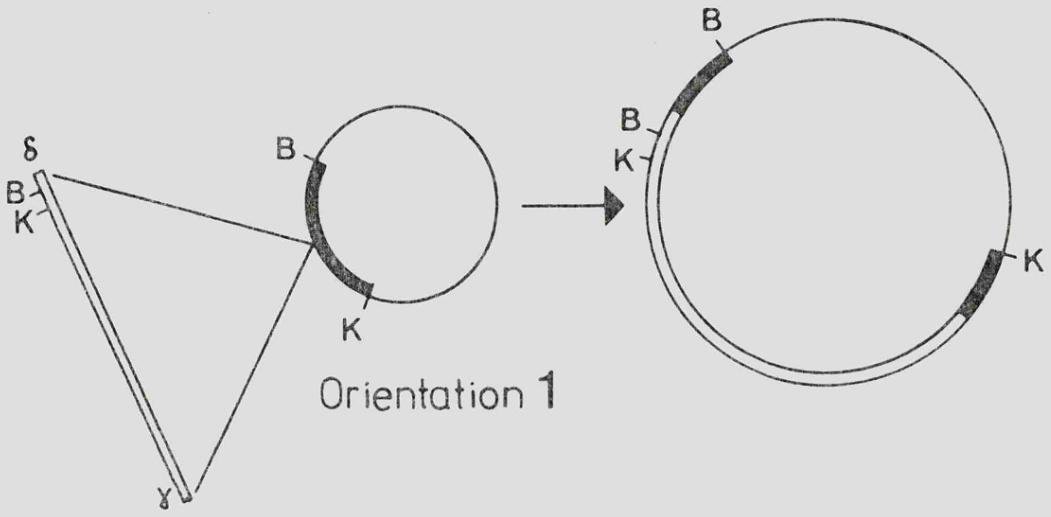
Plasmid DNA from the 31 strains was prepared and digested with various restriction enzymes. The DNA was firstly digested with XhoI. XhoI does not cut pACYC184, but cuts Tn1000 twice, producing a 3.5 kb fragment. Therefore every plasmid carrying Tn1000 should have an identical restriction pattern. This confirmed that all 31 plasmids contained Tn1000.

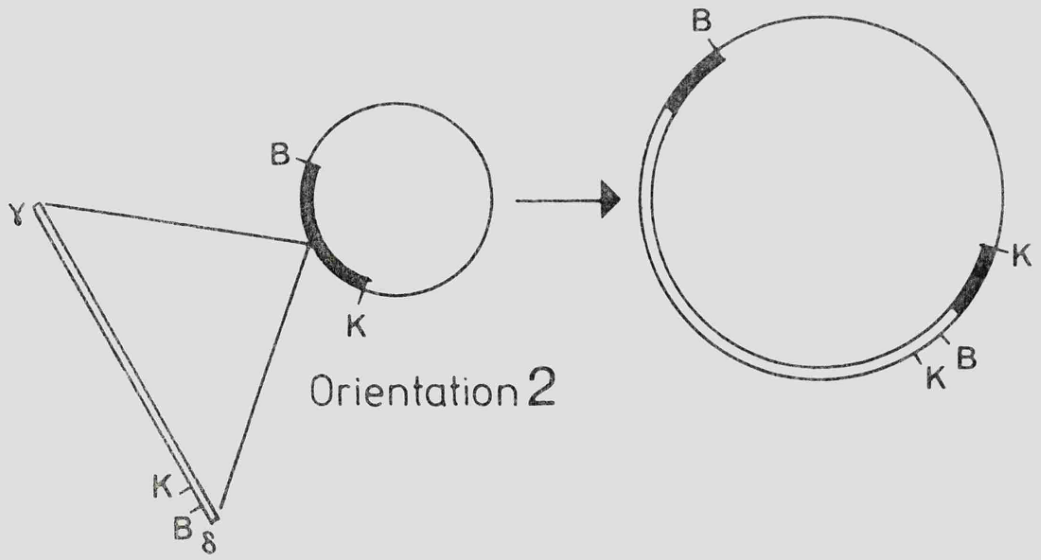
The plasmid DNA was then digested with BamHI. This established the orientation of Tn1000 within the rodA gene. Tn1000 (about 6.3 kb) has a BamHI site 0.3 kb from its δ-end. Since all the rodA$^-$ insertions must lie within the 1.65 kb BamHI-KpnI fragment in pLG355, insertion with the δ-end nearer the BamHI site (orientation 1) produced a plasmid digested by BamHI into a large fragment (over 10 kb) and a small fragment (less than 2 kb). Insertion in the opposite orientation, with the δ-end nearer the KpnI site (orientation 2) produced a plasmid digested by BamHI into two fragments of approximately equal size, between 6 and 8 kb (Figure 6.4). Of the 31 isolates, 12 inserts were in orientation 1, and 19 in orientation 2.

Figure 6.4

Diagram showing the insertion of Tn1000 into pLG355 in orientations 1 and 2.

Restriction enzyme sites : B, BamHI; K, KpnI.


δ
B
K
B
K
γ
B
B
K
K
Orientation 1


γ
B
K
B
K
K
B
K
B
δ
Orientation 2

Those insertions in orientation 1 could be immediately mapped by measuring the size of the small BamHI fragment and subtracting 0.3 kb (see Figure 6.5(a)). Insertions in orientation 2 could be mapped by digestion with KpnI. There is one KpnI site in Tn1000, 0.6 kb from the δ-end. Since in orientation 2 the δ-end lies nearer the KpnI site in pLG355, digestion with KpnI produced a large and a small fragment, similar to the digestion of a plasmid with an orientation 1-insert with BamHI (see Figure 6.5 (b)). The insertion site could be mapped by measuring the size of the small fragment and subtracting 0.6 kb.

The distribution of insertion sites is shown in Figure 6.6. The insertions in orientation 1 do not apparently cover quite the same region as those in orientation 2. Sources of error which would produce this effect are if the map of Tn1000 is not quite accurate, or if the sizing of the KpnI-BamHI fragment was not accurate. Also, the mapping was done using rapid plasmid preparations, where the mobility of fragments may differ slightly from that of pure DNA, leading to slight inaccuracies. However, although it is not easy to match insertions in opposite orientations, each group should be internally consistent. The spread of insertions in orientation 1 was 1.03 kb, while with those in orientation 2 it was 0.94 kb. This provides a minimum estimate for the size of rodA. An average gene of 1 kb would code for a protein of approximately 37 Kd. Therefore it was concluded that it was extremely unlikely that the rodA gene product was a very small protein.

Figure 6.5

Restriction enzyme digests of pLG355 rodA::Tn1000 isolates.

The samples were electrophoresed in a 1.5% agarose gel, alongside λ^+ x HindIII and pBR322 x TaqI markers.

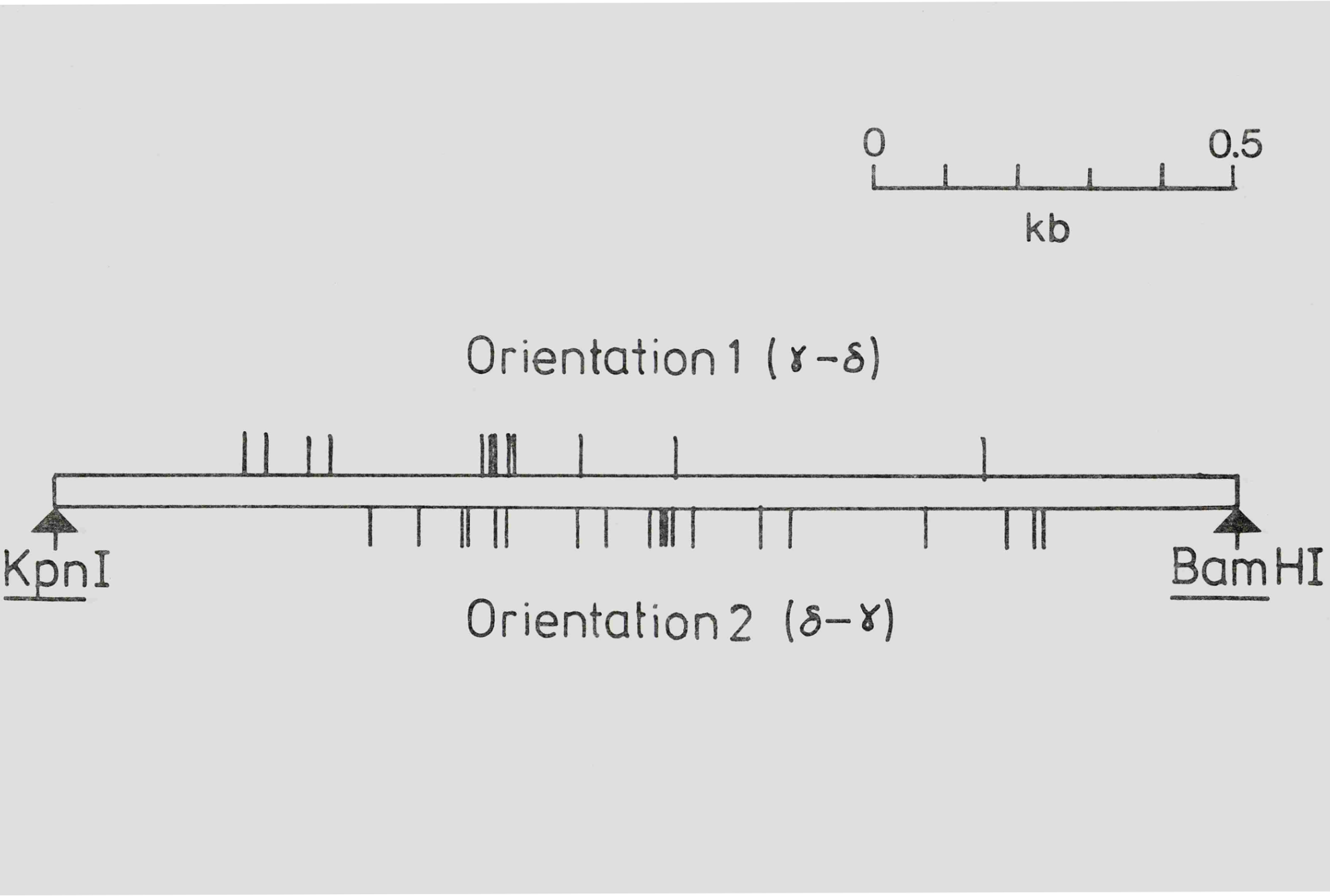
A : Plasmids carrying Tn1000 in orientation 1, digested with BamHI.

B : Plasmids carrying Tn1000 in orientation 2, digested with KpnI.

A
λ x HindIII
pBR322 x TaqI
x BamHI
λ x HindIII
kb
4.4
2.3
2.0
1.4
1.3
B
x KpnI
pBR322 x TaqI
λ x HindIII
kb
4.4
2.3
2.0
1.4
1.3

Figure 6.6

Diagram showing sites of insertion of Tn1000 into rodA.


0
0.5
kb
Orientation 1 (γ-δ)
KpnI
BamHI
Orientation 2 (δ-γ)

V. IDENTIFICATION OF THE *rodA* GENE PRODUCT

pACYC184, pLG355 and a representative pLG355 *rodA*::Tn*1000* isolate were transformed into DS410, and minicells were prepared, labelled and analysed by SDS-PAGE (Figure 6.7). No difference could be seen between the profiles of pACYC184 and of pLG355 except for the disappearance of the tetracycline resistance protein. Furthermore, the profile of pLG355 *rodA*::Tn*1000* showed additional proteins (presumably Tn*1000*-encoded) but no protein disappeared. Labelling with [^{3}H]-amino acid mixture produced the same result (data not shown).

Several theories could explain the absence of rodA protein:

(a) *rodA* could have a very weak promoter. However, *pbpA* which has a weak promoter produces easily-detectable quantities of PBP2.

(b) The protein could be extremely unstable, such that it is degraded before analysis by PAGE.

(c) The protein could behave abnormally in the gel system used.

(d) Production of the protein could be linked to a particular event in the cell cycle. Thus, in minicells or UV-irradiated cells, which are not going through a normal cell-cycle, conditions might be unsuitable for transcription of *rodA*.

One system with a reduced level of proteases is the *in vitro* transcription-translation system initially described by Zubay (1973). Furthermore, purified DNA restriction fragments provide suitable templates in this system (Pratt *et al.*, 1981a). Accordingly, several micrograms of the *Kpn*I-*Bam*HI fragment carrying *rodA* were prepared from pLG355 and were used as the template in this system (the extracts were prepared by, and the incubation carried out by Dr J.M. Pratt). However, no protein could be seen after SDS-PAGE and fluorography (data not shown).

Figure 6.7

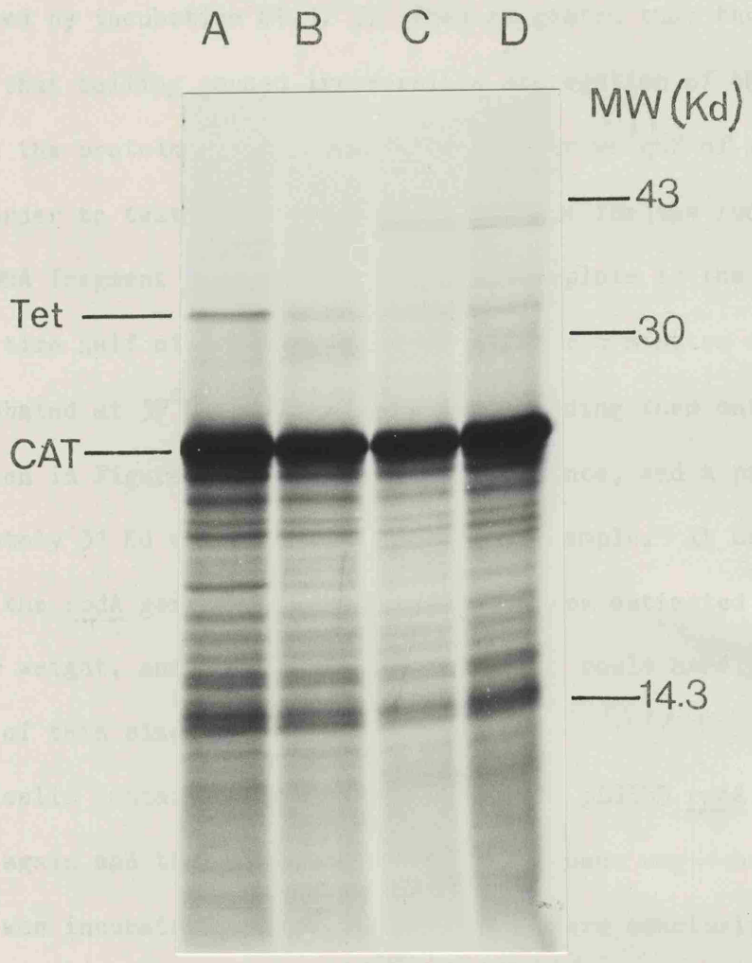
Proteins synthesized in minicells by pLG355 and pLG355 rodA::Tn1000 derivatives.

Minicells were pulse-labelled with [^{35}S] methionine and analysed on a 15% polyacrylamide gel followed by autoradiography. Tet, tetracycline resistance protein; CAT, chloramphenicol acetyl transferase.

A : pACYC184

B : pLG355

C and D : pLG355 rodA::Tn1000 isolates


A
B
C
D
MW (Kd)
43
30
14.3
Tet
CAT

Teather *et al.* (1978) reported that lactose permease, an inner membrane protein, was only detected on polyacrylamide gels if the sample was not boiled before loading onto the gel. Instead, the proteins were solubilized by incubation at 37°C. They suggested that the reason for this was that boiling caused irreversible aggregation of the permease, such that the protein had an apparent molecular weight of more than 200 Kd. In order to test whether the same was true for the rodA protein, the 1.65 kb DNA fragment was again used as the template in the *in vitro* system, but this time half of the sample was boiled for 5 minutes and the other half incubated at 37°C for one hour before loading them onto a gel. As can be seen in Figure 6.8, this made a difference, and a protein of approximately 31 Kd was seen in the unboiled sample. It seemed likely that this was the *rodA* gene product since it had been estimated to have a similar molecular weight, and the 1.65 kb DNA fragment could hardly code for two proteins of this size.

Minicells containing pACYC184, pLG355 and pLG355 *rodA*::Tn*1000* were labelled again and the samples treated in the same way - half was boiled and half was incubated at 37°C. The results were conclusive (see Figure 6.9). pACYC184 synthesized a large amount of the 25 Kd chloramphenicol acetyl transferase, and a rather smaller amount of the 33 Kd tetracycline-resistance protein. pLG355 no longer synthesized the tetracycline-resistance protein, but it did encode a 31 Kd protein. This protein was not synthesized by pLG355 *rodA*::Tn*1000*. Since this plasmid carries a transposon inserted into *rodA*, the 31 Kd protein must be the rodA gene product. In the boiled samples, no protein of this molecular weight was detected. It is interesting that the 33 Kd tetracycline-resistance protein (like lactose permease, an inner membrane protein) also appeared in greater amounts when

Figure 6.8

Proteins synthesized *in vitro* from the 1.65 kb fragment carrying *rodA* : effect of boiling the sample before electrophoresis.

2 µg DNA fragment were used as the template in an *in vitro* transcription-translation system. Proteins encoded by the fragment were labelled with [^{35}S] methionine, and analysed on a 15% polyacrylamide gel followed by fluorography. Samples A and B were boiled for five minutes in SDS-lysis buffer before electrophoresis, while samples C and D were incubated at 37°C for 1 hour.

A : Control

B : *rodA* fragment

} boiled samples

C : Control

D : *rodA* fragment

} samples incubated at 37°C


A
B
C
D
MW(Kd)
-43
rodA-
-30
-14.3

Figure 6.9

Proteins synthesized by pLG355 : effect of boiling.

Minicells were pulse-labelled with [^{35}S] methionine and analysed on a 13% polyacrylamide gel. Samples A, B and C were boiled before electrophoresis, while samples D, E and F were incubated at 37°C for 1 hour. Tet, tetracycline resistance protein; CAT, chloramphenicol acetyltransferase.

A : pACYC184

B : pLG355

C : pLG355 rodA::Tn1000

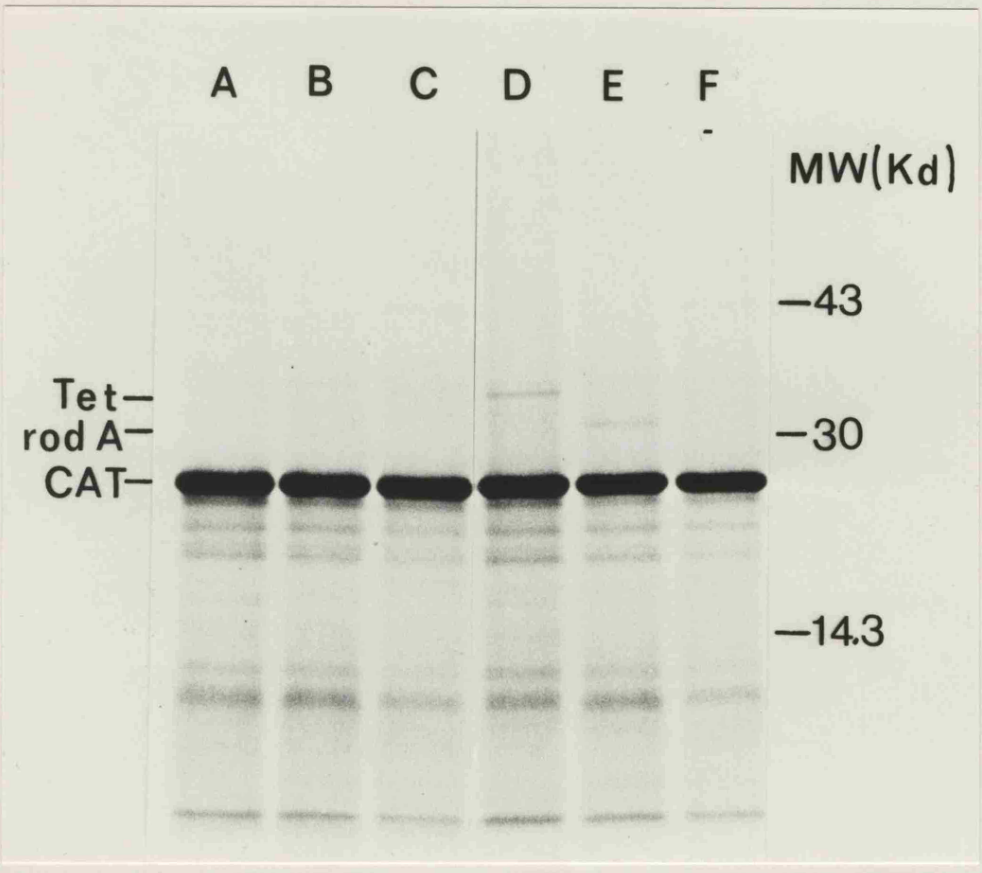
} samples boiled

D : pACYC184

E : pLG355

F : pLG355 rodA::Tn1000

} samples incubated at 37°C


A
B
C
D
E
F
MW(Kd)
—43
—30
—14.3
Tet—
rod A—
CAT—

the samples were incubated at 37°C rather than boiled. Reanalysis of the proteins synthesized by pLG340, pLG341, pLG342, pLG344 and pLG346 showed that all plasmids synthesized a 31 Kd protein (data not shown).

Chapter 7

CHARACTERIZATION OF *rodA* AND ITS GENE PRODUCT

I. INTRODUCTION

The identification of the *rodA* gene product allowed further characterization of the gene and its product to be undertaken.

Firstly, the localization of the rodA protein in the cell was examined. *rodA* is surrounded by other genes which code for inner membrane proteins (PBP2, PBP5 and the 54 Kd protein). Since, like *pbpA*, *rodA* is involved in cell shape determination, it was thought likely that the rodA protein would be found in the inner membrane. Secondly, if the rodA protein was shown to be either a membrane or periplasmic protein, the possibility would exist that it was synthesized as a precursor, which was processed during assembly into, or passage through, the membrane. This is true of PBP5 and the 54 Kd protein (Pratt *et al.*, 1981; Herrero *et al.*, 1982; M. Jackson, personal communication). Thirdly, I investigated the direction of transcription of *rodA*.

II. LOCALIZATION OF THE *rodA* GENE PRODUCT

The localization of the *rodA* gene product in the cell was examined. Since the fractionation of minicells to yield purified membranes is difficult, pACYC184 and pLG355 were transformed into CSR603, a strain which is extremely sensitive to UV-irradiation, and the plasmid-encoded proteins were labelled as described by Sancar *et al.* (1979; see Chapter 2.26).

The principle of this maxicell system is that the cells are exposed to a very low dose of UV-irradiation, such that only a small number of

pyrimidine dimers are formed per cell. In most cases these will be formed only in the chromosome, since it is a much larger target than the plasmid DNA. Since the cells are unable to repair the lesions, damaged DNA is degraded during overnight incubation of the culture. The only intact DNA molecules left in the cells are plasmids, and plasmid-encoded proteins can be identified by pulse-labelling with [^{35}S] methionine. Unlike minicells, these cells are relatively simple to fractionate.

CSR603[pACYC184] and CSR603[pLG355] were UV-irradiated and pulse-labelled, and inner and outer membrane fractions were isolated by sonication of the cells, followed by centrifugation and solubilization of the inner membrane with Sarkosyl, leaving the Sarkosyl-insoluble outer membrane fraction. The labelled proteins in these fractions and those in whole maxicells were compared using SDS-PAGE followed by autoradiography (Figure 7.1). There was very little contamination between fractions, as judged by the relative distribution of chloramphenicol acetyl transferase (a cytoplasmic protein) and of the tetracycline-resistance protein, which is found in the inner membrane. The rodA protein was found quantitatively in the inner membrane fraction. A faint band appeared in a similar position in the pACYC184 inner membrane track. However, this protein migrated slightly faster than the rodA protein, and did not appear consistently when the experiment was repeated. This was probably a proteolytic product of the tetracycline-resistance protein.

Figure 7.1

Localization of the rodA protein in the cell.

Plasmid-encoded proteins were labelled using the maxicell technique. Half of each sample was sonicated, and sarkosyl-soluble and -insoluble membrane fractions were prepared, representing the inner and outer membrane fractions respectively. The samples were analysed in a 13% polyacryamide gel, followed by autoradiography.

A : pACYC184 (whole cell lysate)

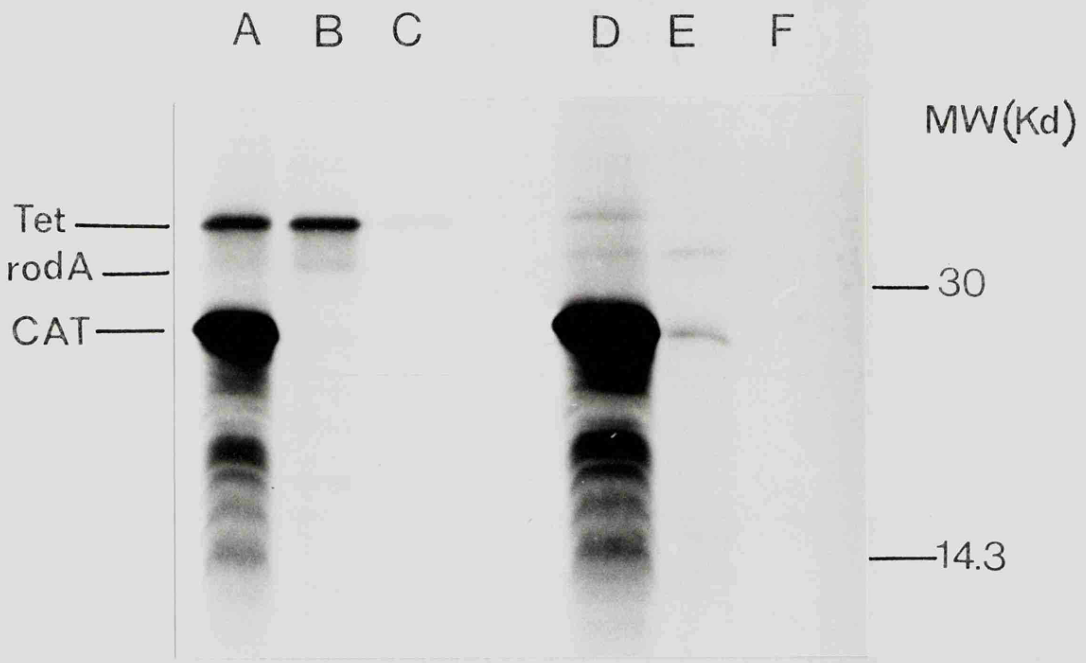
B : pACYC184 (inner membrane fraction)

C : pACYC184 (outer membrane fraction)

D : pLG355 (whole cell lysate)

E : pLG355 (inner membrane fraction)

F : pLG355 (outer membrane fraction)


A B C D E F
MW(Kd)
Tet
rodA
CAT
30
14.3

III. COMPARISON OF rodA PROTEIN SYNTHESIZED IN VIVO AND IN VITRO

Both PBP5 and the 54 Kd protein are synthesized as larger precursors with a NH_2-terminal signal sequence (Pratt et al., 1981b; Herrero et al., 1982; M. Jackson, personal communication). These signal sequences are proteolytically removed in the course of assembly of the proteins into the cytoplasmic membrane. Proteins synthesized in semi-in vivo systems such as minicell and maxicell systems are processed in the normal way, but proteins synthesized in the in vitro transcription-translation system are not, although the addition of inverted membrane vesicles may allow subsequent processing.

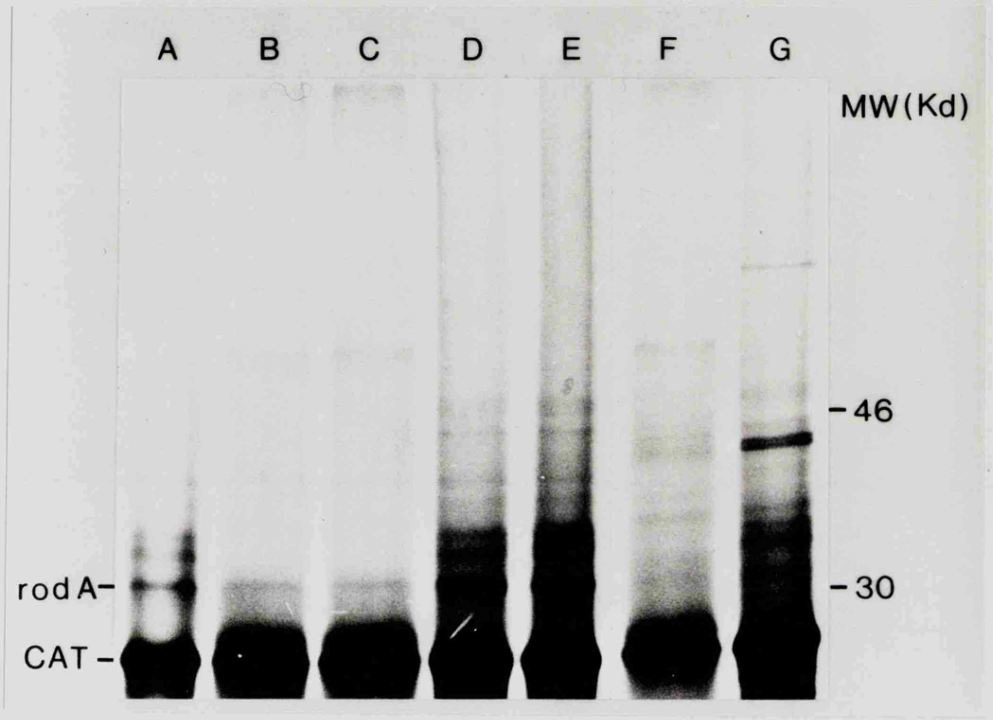
In order to determine whether or not rodA is synthesized with a signal sequence, the protein was labelled in minicell and in vitro systems and the molecular weights of the proteins were compared by SDS-PAGE. Proteins specified by pLG355 and pLG355 rodA::Tn1000 were labelled with [^{35}S] methionine as described previously, except that cAMP was omitted from the in vitro system, in order to decrease transcription of the chloramphenicol acetyl transferase gene (de Crombrugghe et al., 1973). The samples were solubilized in SDS-sample buffer by incubation at 37°C, and were analysed by SDS-PAGE followed by autoradiography (Figure 7.2). The rodA protein synthesized appeared to have the same molecular weight in both systems, suggesting that it is not made as a precursor. Representative pLG355 rodA ::Tn1000 isolates were used as controls. A band of approximately the same molecular weight as rodA was occasionally seen both in minicells and in vitro, but this protein was always much fainter than rodA, and was not consistently produced.

Figure 7.2

Comparison of rodA protein synthesized *in vivo* and *in vitro*.

pLG355-specified proteins were labelled with [^{35}S] methionine in minicells and in an *in vitro* transcription-translation system, and were fractionated in a 13% SDS-polyacrylamide gel, followed by autoradiography.

A	pLG355 minicells
B, C	pLG355 *in vitro*
D, E	pLG355 minicells
F	pLG355 *rodA*::Tn*1000* *in vitro*
G	pLG355 *rodA*::Tn*1000* minicells


A
B
C
D
E
F
G
MW(Kd)
46
30
rodA
CAT

IV. THE DIRECTION OF TRANSCRIPTION OF *rodA*

In order to determine the direction of transcription of *rodA*, it was decided to clone the gene into a plasmid expression vector. The plasmid pGWPL1 (Figure 7.3) consists of the λ p_L promoter and the *cI857* and *N* genes cloned between the *BamHI* and *EcoRI* sites of pBR322. A 4.7 kb *BamHI-EcoRI* fragment from λ*cI857* was cloned into pBR322. Subsequent partial digestion with *BglII* followed by religation deleted p_R and *cro* (A. Hall, personal communication). At 30°C, this plasmid synthesizes active cI repressor protein which prevents transcription from p_L. At 42°C, the *cI* protein is inactivated, allowing transcription from p_L. The *cro* gene was deleted because the cro protein normally switches off transcription from p_L. This system was chosen because the antitermination properties of the N protein mean that transcription from p_L will not stop at most terminator sequences (Ward and Gottesman, 1982). This is important since it allows fragments to be cloned without first ensuring that no terminators lie between p_L and the relevant cloned gene.

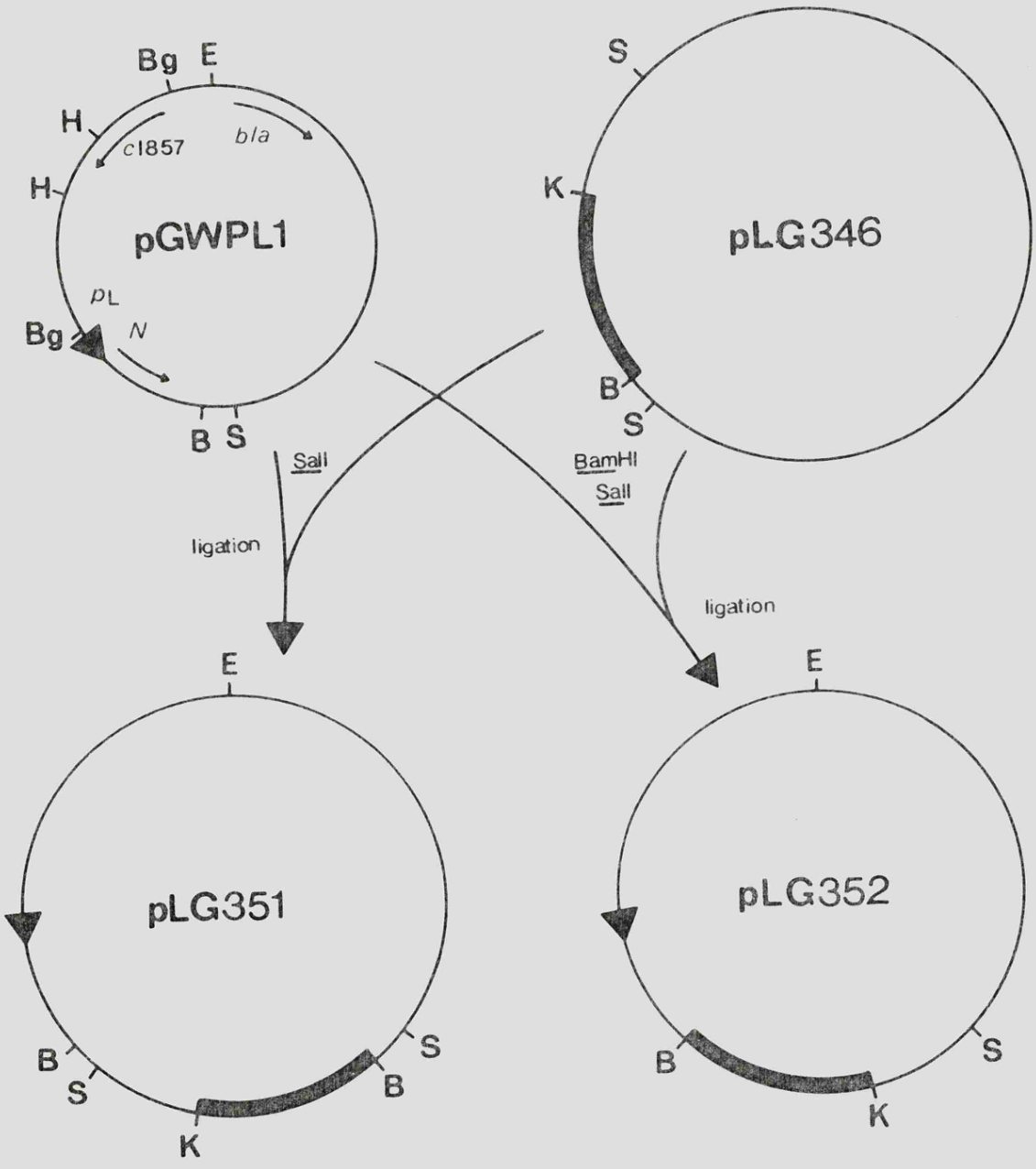
V. CLONING *rodA* INTO pGWPL1 : CONSTRUCTION OF pLG351 AND pLG352

One problem with using pGWPL1, with its temperature-sensitive promoter, as a vector for *rodA*, was how to select clones carrying *rodA*, since the only available *rodA* mutant was also temperature sensitive. It was possible that a clone carrying *rodA* in the correct orientation for transcription from p_L might be deleterious or lethal to the cell at 42°C. Furthermore, attempting to clone *rodA* in this way might result in the isolation of a clone carrying a mutant gene or promoter. However it was shown that a λ^+ lysogen carrying pGWPL1 was immune to λ at both 30°C and 42°C. Therefore the λ^+ prophage synthesized enough cI protein to repress p_L on pGWPL1 at 42°C, and a *rodA*$^+$ clone could be selected at 42°C without

Figure 7.3

Diagram showing construction of pLG351 and pLG352.

Restriction enzyme sites : B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; K, KpnI; S, SalI.


Bg
E
H
H
cI857
bla
pGWPL1
pL
N
Bg
B
S
S
K
pLG346
B
S
SalI
ligation
BamHI
SalI
ligation
E
pLG351
B
S
S
B
K
E
pLG352
B
S
K

switching on p_L. Remaut et al. (1981) have also shown that cI^+ repressor synthesized from a prophage can repress transcription from multiple copies of p_L.

pGWPL1 and pLG346 were digested with SalI, mixed, ligated and transformed into SP5211 recA (λ^+). A recA strain was used to minimize any possibility of DNA rearrangements. The cells were plated onto nutrient agar containing ampicillin at 42°C. Colonies of rod-shaped cells were purified, and plasmid DNA was isolated and digested with restriction enzymes. All clones contained the SalI fragment from pLG346 cloned in one orientation (Figure 7.3). This was not surprising, as an inverted repeat would be generated in the opposite orientation, which would probably make the plasmid unstable (Lilley, 1981). The plasmid isolated was called pLG351.

The rodA gene was cloned in the opposite orientation, by taking a BamHI-SalI fragment from pLG346 and cloning it between the BamHI and SalI sites of pGWPL1. The resultant plasmid was called pLG352 (Figure 7.3).

VI. EFFECTS OF pLG351 AND pLG352 ON CELL GROWTH AND MORPHOLOGY

pGWPL1, pLG351 and pLG352 were transformed into SP5211 recA, with the heat-pulsing step carried out at 30°C. The cells were plated onto nutrient agar containing ampicillin and incubated at 30°C. Transformants were purified at 30°C. The effect of a temperature-shift to 42°C on the growth and morphology of the cells was then tested.

Overnight cultures of SP5211 recA and of SP5211 recA carrying pGWPL1, pLG351 or pLG352 were grown in nutrient broth containing ampicillin where appropriate. These cultures were diluted into fresh nutrient broth, and grown at 30°C through at least two mass doublings. The cultures were then shifted to 42°C and shaken for several hours, during which time cell growth and morphology were monitored.

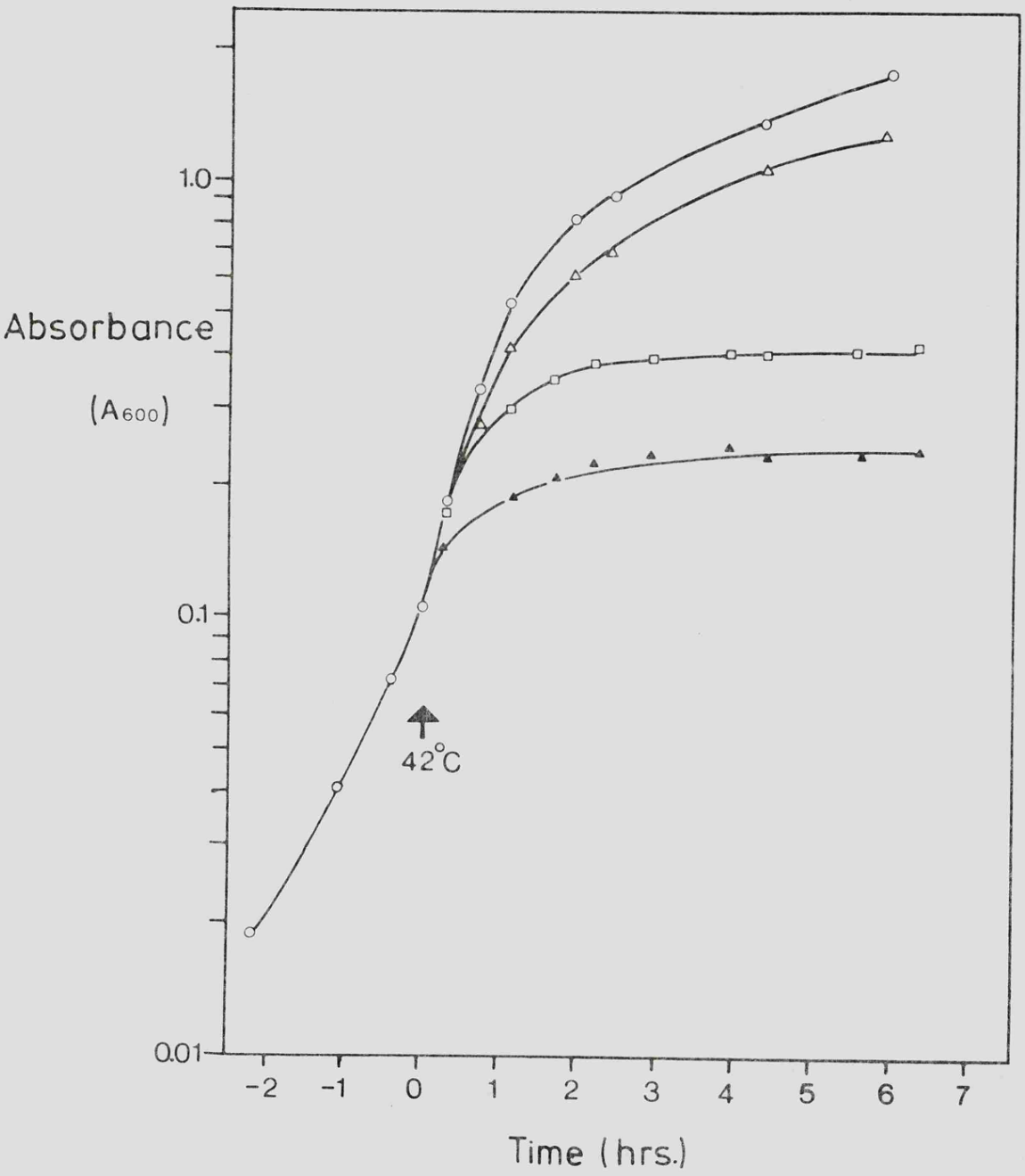
The effect of the temperature-shift on cell growth is shown in Figure 7.4. SP5211 recA [pGWPL1] grew rather poorly, doubling in mass approximately twice before ceasing to grow. A deleterious effect on the host is a common feature of plasmid vectors carrying a derepressed p_L promoter in the presence of the N protein (Remaut et al., 1981). SP5211 recA [pLG351] grew even less well, ceasing growth after one doubling in mass, while SP5211 recA [pLG352] grew virtually as well as the plasmid-free strain.

SP5211 recA cells, which were rod-shaped at 30°C, began to change shape almost immediately after the temperature shift, and after two hours most of the cells were spherical. SP5211 recA [pGWPL1] cells also became round quickly, but some lysis was visible. SP5211 recA [pLG351] cells remained rod-shaped and appeared to filament slightly. Many of the rods took on a granular appearance, and much lysis occurred. SP5211 recA [pLG352] cells remained short and gradually became spherical. After seven hours at 42°C, there were many spheres, while the other cells were fat rods or ovoid in shape. A little lysis was visible.

Some tentative conclusions may be drawn from these observations. Cells carrying pLG352 became spherical while those carrying pLG351 did not. This is consistent with what might be expected if transcription from p_L is the same as rodA in pLG351, but opposite to rodA in pLG352. Thus, in pLG351 more rodA protein is synthesized, while in pLG352, convergent transcription reduces and possibly stops transcription of rodA (see Ward and Murray, 1979). It would be expected that SP5211 recA [pLG352] cells would become spherical much more slowly than the host strain, because in SP5211 recA, a shift to 42°C would inactivate all the rodA protein in the cell, while in SP5211 recA [pLG352] only a proportion of the rodA protein

Figure 7.4

Graph showing effect of a temperature shift from 30°C to 42°C on the growth of SP5211 recA (o), SP5211 recA [pGWPL1] (□), SP5211 recA [pLG351] (▲) and SP5211 recA [pLG352] (Δ).


Absorbance
(A600)
1.0
0.1
0.01
42°C
-2
-1
0
1
2
3
4
5
6
7
Time (hrs.)

present in the cell when the temperature shift occurred would be thermolabile. Furthermore, even at 42°C, some rodA protein might be synthesized by the plasmid.

Few other conclusions can be drawn from these experiments because of the detrimental effect on the host cell of pGWPL1 at 42°C. This may be due to interference by transcription from p_L to plasmid replication. A similar effect was described for other plasmid expression vectors in which p_L had been cloned in the same orientation in relation to the pBR322 origin of replication as in pGWPL1 - the PLc vectors described by Remaut *et al.* (1981). It is strange that SP5211 *recA* [pLG352] grows so well. Perhaps there is a strong terminator sequence within the cloned DNA which, in this orientation, is not suppressed or is only partially suppressed by the N protein.

These experiments were devised purely to determine the direction of transcription of *rodA*. Once determined, and having identified the gene product, the rodA protein could be overproduced using a conditional promoter which does not in itself affect the cell. This could then be used to determine whether overproduction of rodA protein is deleterious to the cell. Cells carrying pLG351 doubled in mass once after shifting to 42°C, and appeared to filament slightly, suggesting that the overproduction of rodA protein may inhibit division. Furthermore, isolation of the purified protein, or of membranes containing large quantities of the protein would allow possible enzyme activities to be assayed.

VII. ANALYSIS OF PROTEINS SYNTHESIZED BY pLG351 AND pLG352

The proteins synthesized by pLG351 and pLG352 in minicells were analysed. pGWPL1, pLG351 and pLG352 were transformed into DS410, and minicells were isolated, and labelled at 30°C and 42°C, followed by SDS-PAGE and autoradiography. As before, boiled and unboiled samples were prepared and analysed. The results are shown in Figure 7.5.

A major protein present in each track had a molecular weight of 30 Kd. This was probably β-lactamase, which has an actual molecular weight of 29 Kd (Sutcliffe, 1978). A fainter band found above this protein in all tracks was probably pre-β-lactamase which has a molecular weight of 31.5 Kd. Below β-lactamase were two proteins, which were probably the cI and rex proteins (reported molecular weights 26 and 29 Kd respectively - see Szybalski and Szybalski, 1979). In the unboiled samples at 30°C, the rodA protein could be seen as a faint band between β-lactamase and pre-β-lactamase in both the pLG351 and pLG352 tracks. At 42°C, however, pLG351 synthesized rather more rodA protein, while none was detectable with pLG352. This confirmed the direction of transcription suggested by the morphological effects of induction of the p_L promoter. Therefore *rodA* is transcribed from the *KpnI* site towards the *BamHI* site (see Figure 8.1).

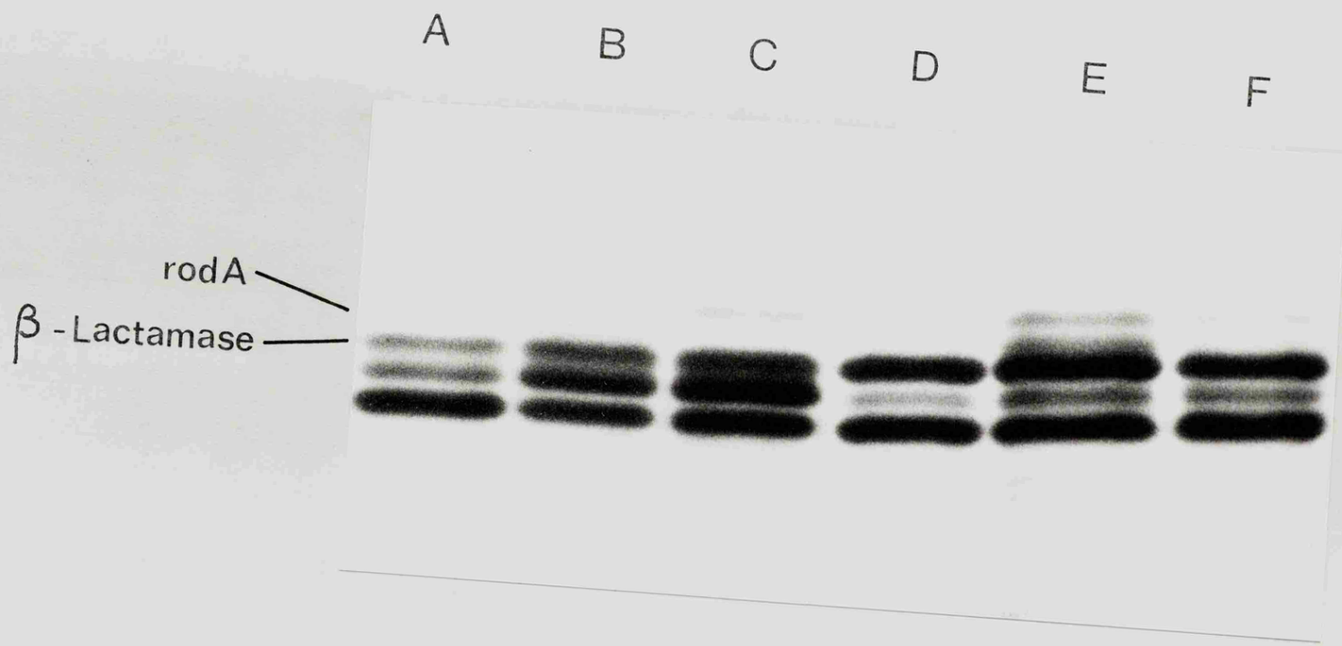
In retrospect, pGWPL1 was not an ideal vector to use for this experiment, because β-lactamase had a molecular weight so similar to that of the rodA protein. However, these experiments were begun before the rodA gene product had been identified. Fortunately, the rodA protein is just visible above the β-lactamase.

Figure 7.5

Determination of the direction of transcription of rodA.

Minicells were labelled at 30°C or 42°C, and the samples were solubilized in SDS-lysis buffer at 37°C and analysed in a 13% polyacrylamide gel followed by autoradiography. The small amount of rodA protein synthesized by pLG351 and pLG352 at 30°C is not visible in this photograph.

A	:	pGWPL1	30°C
B	:	pLG351	
C	:	pLG352	
D	:	pGWPL1	42°C
E	:	pLG351	
F	:	pLG352	


A
B
C
D
E
F
rodA
β - Lactamase

Chapter 8

DISCUSSION

I. THE *rodA* GENE AND ITS PRODUCT

The region of the *E.coli* chromosome, mapping at approximately 14', which extends from the *leuS* to the *lip* gene, has been cloned by specialized transduction. The cloned region contains a cluster of genes which are involved in the determination of cell shape and in peptidoglycan synthesis, and the aim of this thesis has been to further characterize these genes.

λpBS10, a phage carrying the *leuS*, *pbpA*, *rodA* and *dacA* genes, was constructed, and the proteins encoded by the cloned fragment were examined. The gene order within the *pbpA*-*rodA*-*dacA* region was clarified by isolation of derivatives of λpBS10 which carried deletions, followed by correlation of the loss of transducible markers with failure to programme the synthesis of particular proteins.

These experiments, however, failed to reveal a protein corresponding to the *rodA* gene. The only protein produced by a gene in the region between *pbpA* and *dacA* (where *rodA* was genetically mapped) was shown not to be the rodA protein.

The *rodA* gene was subcloned into a plasmid vector in order to isolate it from the other genes within the cluster. It was shown to be contained within a 1.65 kb *KpnI*-*BamHI* fragment, which also carried parts of the *pbpA* gene and of the gene encoding a 54 Kd protein. No protein corresponding to *rodA* appeared to be synthesized from plasmids carrying this fragment. Consequently, the region occupied by *rodA* was estimated by transposon mutagenesis in an attempt to determine its size. Since

the results indicated a minimal size of 1 kb, it appeared unlikely that rodA encoded a very small protein which would not have been detected in the gel system used.

The rodA protein was finally identified by solubilizing the proteins in SDS-lysis buffer at 37°C, rather than in a boiling waterbath. Under these conditions, a 31 Kd protein was detected by SDS-PAGE. This protein was programmed in an in vitro transcription-translation system by the 1.65 kb KpnI-BamHI fragment, and in minicells by all the plasmids which carried rodA. Insertion of Tn1000 into rodA caused this protein to disappear, confirming that it was indeed the rodA protein.

The 31 Kd protein was shown to be localized in the cytoplasmic membrane of the cell, as were PBP2, PBP5 and the 54 Kd protein. The apparent disappearance of the rodA protein when boiled in SDS-lysis buffer (see Chapter 6) may be due to irreversible aggregation of the protein resulting in its exclusion from the gels. The same appears to be true of the lactose permease (Teather et al., 1978), the glycerol-3-phosphate permease (Larson et al., 1982), and to some extent the 33 Kd tetracycline resistance protein encoded by pACYC184 (see Chapter 6). All four proteins are localized in the cytoplasmic membrane. The cause of the aggregation is not clear, but may be due to the emergence of highly hydrophobic regions of the proteins during boiling.

The rodA protein does not appear to be processed (see Chapter 7), a result deduced from comparison of proteins synthesized in vivo and in vitro. However, confirmation of this would require partial sequencing of the amino-terminal amino acids in the proteins synthesized in vivo and in vitro.

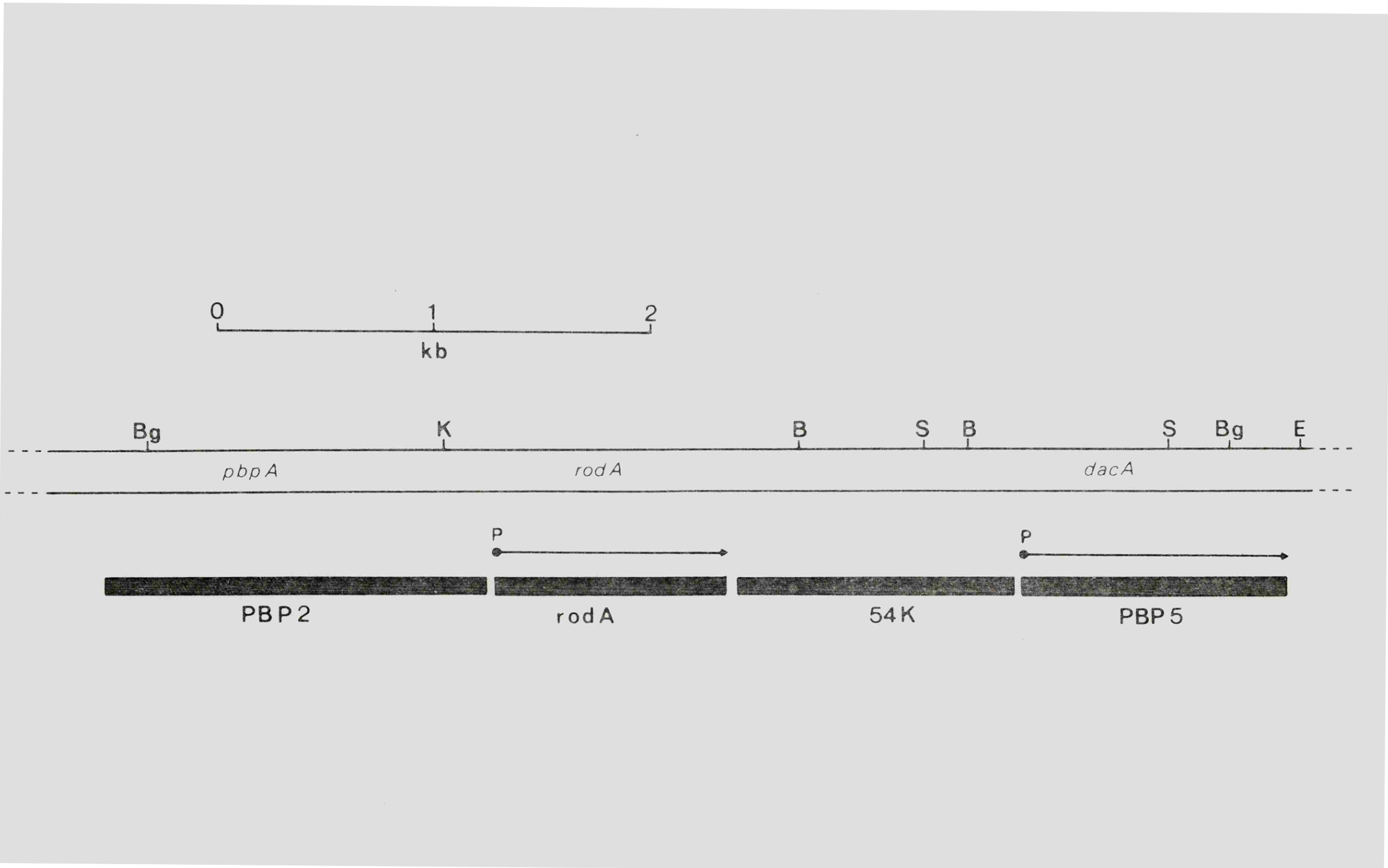
The direction of transcription of rodA was determined, showing that the gene is transcribed in the direction from the pbpA gene towards the dacA gene. Other work has shown that dacA is transcribed in the same direction (B.G. Spratt, unpublished results). Each gene in this cluster has its own promoter. rodA has been subcloned without adjacent genes into a plasmid vector. The possibility that the plasmid was providing a promoter was eliminated by showing that the purified DNA fragment carrying rodA synthesized rodA protein in vitro. dacA has also been subcloned without adjacent genes into a plasmid vector, and DNA sequencing has shown a promoter-like sequence to be present (J.K. Broome-Smith and B.G. Spratt, unpublished results). The 54 Kd protein was synthesized from λpBS10 derivatives in which either dacA or rodA had been deleted (see Chapter 4), suggesting that the gene has its own promoter. Similarly, PBP2 was synthesized from λpBS10 derivatives in which rodA or the gene encoding the 11 Kd protein had been deleted (see Chapter 4; unpublished results), again suggesting that pbpA has its own promoter.

We cannot exclude the possibility that one or more transcriptional units exist within this gene cluster, with distal genes having their own internal promoters. It would therefore be of interest to determine the direction of transcription of pbpA and the gene encoding the 54 Kd protein, to see if they are the same as for rodA and dacA. The pbpA-rodA-dacA region is shown in Figure 8.1.

Figure 8.1

Summary diagram showing the current status of knowledge about the pbpA-rodA-dacA region.

The proteins are drawn to a scale which assumes that a 37 Kd protein is encoded by 1 kb of DNA. The directions of transcription of rodA and dacA are shown.


0
1
2
kb
Bg
K
B
S
B
S
Bg
E
pbpA
rodA
dacA
P
P
PBP2
rodA
54K
PBP5

II. THE FUNCTIONS OF THE GENES IN THE pbpA-rodA-dacA GENE CLUSTER

(a) pbpA and rodA

The function of the rodA gene in cell-shape determination is unknown. Mutations which inactivate rodA produce a phenotype indistinguishable from that of pbpA mutants, except for the presence of a functional PBP2. Figure 8.1 shows that the coding capacity of the pbpA-rodA-dacA region is apparently saturated. It is therefore likely that pbpA and rodA are contiguous genes, although it is possible that a small gene we have not detected maps between them. Furthermore, both genes encode cytoplasmic membrane proteins. This leads one to speculate not only that both are involved in the same cellular process, but also that the genes might be co-ordinately expressed, and that the proteins might interact physically. Alternatively, the adjacent location of the genes might indicate an evolutionary relationship following, for example, a gene duplication.

Since the standard assay for PBPs involves boiling samples in SDS lysis buffer prior to electrophoresis (Spratt, 1977c), it was possible that the rodA protein was a PBP that had not been detected previously due to aggregation on boiling. However, no new PBPs appeared when samples were solubilized at 37°C (B.G. Spratt, unpublished results), showing that the rodA protein is not a PBP.

There is now evidence that PBP2 possesses transpeptidase activity (Ishino et al., 1982), but no enzymatic activity could be detected from purified PBP2 (Curtis and Strominger, 1981). Since the protein had been removed from its membrane environment, where it might be part of a multi-enzyme complex, this was not particularly surprising. Furthermore, it might be expected that a protein present in such low quantities in vivo

(10-20 molecules per cell; Spratt, 1977c), which may affect the incorporation of peptidoglycan into the sacculus in a rather subtle way, leading to the synthesis of peptidoglycan of a particular shape, would utilise a substrate which is difficult to predict or to mimic *in vitro*. Ishino *et al.* (1982) prepared membranes containing high levels of PBP2 (about 50-fold higher than normal) by inducing a lysogen carrying a λ*dlip5* *cI857* *Qam73* prophage (Spratt *et al.*, 1980). This resulted in elevated levels of PBP2 and PBP5. Cefmetazole, a β-lactam antibiotic which binds strongly to all PBPs except PBP2, was added. Membranes from these cells showed some transpeptidase activity. This activity was sensitive to mecillinam, strongly suggesting that PBP2 was catalysing the cross-linking.

Peptidoglycan transglycosylase activity was also elevated in these membrane preparations, suggesting that the enzyme responsible was encoded by λ*dlip5*. However, it is not known whether it is due to PBP2 or another protein (such as rodA). Now that the rodA protein has been identified, enzyme activities can be assayed in membranes in which rodA has been overproduced. Clearly, it would be useful to repeat the experiments of Ishino *et al.* having dissected the *pbpA-rodA-dacA* region into individual genes.

As regards the role of PBP2, Matsuhashi *et al.* (1982) suggested that it acts in concert with a nicking enzyme which breaks bonds in the peptidoglycan at incipient growth sites, producing acceptor chains to which new precursors can be cross-linked by PBP2. Such a nicking enzyme might be encoded by one of the other genes essential for a normal rod-shape (e.g. *rodA* or *envB*). It is interesting that James *et al.* (1975) reported that mecillinam only inhibited lateral wall formation if added

in the first few minutes of the cell cycle. They suggested that the mecillinam-sensitive target (presumably PBP2) appears in the cell coincident with chromosome initiation, and could be involved in initiation of a specific developmental sequence leading to division. This data is not easy to interpret, but Satta et al. (1982) have also indicated that when mecillinam was added during the first five minutes of the cell cycle, peptidoglycan synthesis was immediately reduced by 50%. After this time, peptidoglycan synthesis was not influenced by the addition of mecillinam until the end of the first synchronous division, when the rate fell rapidly. This may indicate that PBP2 acts transiently after division to recommence elongation of the sacculus. Presumably it could be involved in the activation of the synthesis of cylindrical peptidoglycan, or inhibition of the synthesis of hemispherical peptidoglycan at the newly formed poles. Buchanan (1981) compared the amount of PBP2 present in normal E.coli and in minicells, which consist mainly of polar material. The major difference she found was reduced levels of PBP2 in minicells, which is clearly compatible with the idea that PBP2 could be involved in activation of cell elongation. rodA is also a minor protein, and could be responsible for some of the functions proposed for PBP2.

(b) The 54 Kd protein

The function of the 54 Kd protein which is encoded by the gene mapping between rodA and dacA remains unknown. However, it seems likely that this protein has a role in peptidoglycan synthesis. A strain deleted for dacA has been constructed (Spratt, 1980a), which also lacks the 54 Kd protein gene, so the gene is not essential. However, it is not easy to conclude much from the mutant as other genes are also missing, so it would be interesting to construct a mutant lacking only this gene.

(c) dacA

The evidence discussed in Chapter 1 showed that PBP5 is one of the cell's major D-alanine carboxypeptidases. The experiments of Mirelman's group suggested that DD-carboxypeptidase activity may be important in vivo. This protein appears to regulate the level of cross-linking in the cell wall, and may also stabilize the structure of the mature sacculus by removing terminal D-alanine residues from pentapeptide sidechains.

I also discussed in Chapter 1 the correlation found by Mirelman and his colleagues between the level of available DD-carboxypeptidase in the cell wall, and cell division. Essentially, they found that the carboxypeptidase activity was at its maximum just before division. Furthermore, cells in which division was inhibited in a variety of ways all had reduced DD-carboxypeptidase levels. However, it is not possible to conclude whether this is a primary factor in septation, as it could be just a side effect of the cell division process.

De Pedro and Schwarz (1981) provided evidence that PBP4, which acts as a DD-carboxypeptidase and an endopeptidase in vitro, is a transpeptidase in vivo. They pulse-labelled cells with [^{3}H]DAP, and isolated the SDS-insoluble sacculi. They found that peptidoglycan cross-links formed very quickly (in less than 20 seconds). This is consistent with the work of Mirelman et al. (1976) and Mett et al. (1980), who showed that nascent peptidoglycan was partially cross-linked before attachment to the SDS-insoluble sacculus. The level of cross-linking found by de Pedro and Schwarz in peptidoglycan newly attached to the sacculus corresponded closely to the levels of 19-22% cross-linking in TCA-insoluble, SDS-soluble peptidoglycan, described by both Mirelman et al. and Mett et al.

The cross-linking remained at this low level for 10 minutes, and then gradually increased to the average level found in sacculi of 30%. The loosely cross-linked peptidoglycan, comprising 29% of the total, had a higher proportion of pentapeptide side-chains, which decreased as cross-links were formed. Analysis of the peptidoglycan synthesized in a dacB mutant showed that the peptidoglycan was incorporated in the same, loosely cross-linked form, but that the cross-linking remained at the initial level. They concluded that transpeptidation takes place in two discrete steps. The primary transpeptidation, is, presumably, catalysed mainly by PBPs1A and 1B, and the secondary transpeptidation is catalysed by PBP4.

Markiewicz et al. (1982) showed that a 6-fold overproduction of PBP5 caused cells to grow as spheres, similar to those caused by inactivation of PBP2 or by treatment of cells with mecillinam. They analysed the degree of cross-linking in the peptidoglycan of these cells, and found that the new peptidoglycan was inserted already highly cross-linked. No secondary maturation was detected. pbpA mutants, and cells treated with mecillinam, were analysed and showed the same kinetic pattern of cross-linking. Since there is no evidence that PBP2 modulates the expression of PBP5, or vice versa, Markiewicz et al. suggested that the excessive removal of terminal D-alanine residues from nascent peptidoglycan caused by the overproduced PBP5 might convert it to a form which is the preferred substrate for enzymes specifically involved in the division process. This agrees well with the results of Mirelman et al. (1976, 1977, 1978), who proposed that the nascent peptidoglycan generally cross-links preferentially with the acceptor molecules present at high concentration in the pre-existing sacculus. At division (or artificially, by increasing the DD-carboxypeptidase present) there is an accumulation at

the cell equator of an increasing amount of acceptor substrate, so that the equilibrium is shifted and the nascent peptidoglycan crosslinks preferentially with nascent acceptor. This might automatically lead to the synthesis of hemispherical peptidoglycan. Presumably the balance is shifted the other way in filamenting mutants.

One prediction of this model is that cells which totally lack DD-carboxypeptidase activity might not be able to divide. dacA dacB mutants lacking carboxypeptidase 1B and 95% of carboxypeptidase 1A activity nevertheless appear to divide quite normally. De Pedro et al. (1980) found that peptidoglycan in these cells was incorporated already highly cross-linked, and of course did not mature since PBP4 was absent. This conflicted slightly with the results of de Pedro and Schwarz (1981), who reported the incorporation of loosely cross-linked peptidoglycan in this double mutant, and again, no maturation. The authors did not comment on this discrepancy.

No conclusions about the role of DD-carboxypeptidases in surface synthesis can be drawn until mutants also lacking PBP6 have been constructed. Until recently, no PBP6 mutant had been isolated, although the structural gene, dacC, had been cloned (Spratt, 1980b). A strain deleted for dacC has now been constructed (Broome-Smith and Spratt, 1982), which grows without serious defects. Attempts are in progress to construct a dacA dacC double mutant.

III. THE ATTACHMENT OF LIPOPROTEIN TO PEPTIDOGLYCAN

The growth of lpp, ompA mutants as spherical cells (Sonntag et al., 1978) suggests that the interaction between the outer membrane and the

peptidoglycan might be important in cell-shape determination. The lipoprotein in the cell exists in two forms - that which is covalently linked to the peptidoglycan (the bound form), and that which is not (the free form). Mett *et al.* (1980) found that nascent, water-soluble peptidoglycan was already covalently linked to lipoprotein molecules. 4.6% of the side-chains were joined in this way, compared to approximately 10% in the mature sacculus of exponentially growing cells. Wensink *et al.* (1982) showed that the percentage of bound lipoprotein molecules gradually increased as the peptidoglycan became TCA-insoluble, and finally SDS-insoluble. De Pedro and Schwarz (1981) demonstrated that peptidoglycan newly attached to the sacculus carried about 70% of the amount of lipoprotein found in uniformly labelled cells. The kinetics of attachment of lipoprotein to peptidoglycan showed a remarkable similarity to that of the degree of peptidoglycan cross-linking.

Mett *et al.* (1980) and de Pedro and Schwarz (1981) analysed the incorporation of new peptidoglycan into sacculi in a lipoprotein-deficient mutant. They found that it was incorporated in a loosely cross-linked way as normal, but there was no secondary transpeptidation. Conversely, new peptidoglycan in a *dacB* mutant contained less bound lipoprotein, and showed less of a secondary maturation, than wild-type cells. The level of bound lipoprotein was even lower in a *dacA dacB* mutant, and no secondary increase at all was seen. The experiment was unfortunately not performed with a $dacA^{-}$ $dacB^{+}$ strain. The authors concluded that peptidoglycan may be extensively redistributed after initial insertion into the sacculus. This would require the relocation of peptidoglycan fragments cut out by peptidoglycan hydrolases. They suggested that the displacement is accomplished with the fragments anchored by lipoprotein in the outer

membrane, so that they can be shifted around and reinserted without actual displacement from the sacculus. In the absence of lipoprotein this would be less feasible. The lowered levels of bound lipoprotein in dacB and dacA dacB mutants might be a consequence of the reduction in DD-carboxypeptidase levels. An increase in pentapeptide side chains automatically means a reduction in the level of tetrapeptide side chains; since these are presumably the donor molecules in the transpeptidation reaction with lipoprotein, this would result in reduced levels of bound lipoprotein.

It might therefore be expected that at division, when DD-carboxypeptidase activity is high, the proportion of bound:free lipoprotein would rise. In fact the reverse appears to be the case (see Chapter 1, Section IV(e)), possibly due to a concomitant increase in LD-carboxypeptidase activity, which would remove the substrate for the transpeptidation reaction.

Cell division in E.coli appears to commence by invagination of the cytoplasmic membrane and peptidoglycan (see Burdett and Murray, 1974a,b). The outer membrane remains excluded from the septum until the final cleavage. During septum formation, the outer membrane appears to form folds or blebs, presumably because growth of the membrane exceeds requirements. This is consistent with the reduction in the proportion of bound lipoprotein at division.

Braun and Wolff (1975) reported that mecillinam-treated cells contained twice as much bound lipoprotein as normal cells. This is difficult to interpret, but the results may be artifactual. Wensink et al. (1982) have shown that the proportion of bound lipoprotein doubles in cells in stationary phase. The mecillinam-treated cells discussed by

Braun and Wolff were analysed after the cessation of growth, and were compared with untreated, growing cells at the same optical density. It would be useful to repeat these experiments taking this criticism into account, and also to examine the kinetics of binding of lipoprotein to the peptidoglycan of spherical cells.

SUMMARY

The determination and maintenance of the shape of E.coli is a complex process. Future progress requires a better understanding of the biosynthesis of the cell wall and of the division cycle. The identification and analysis of the genes and gene products involved in these processes, combined with the increasingly sensitive chromatographic techniques used for analysing peptidoglycan structure, should provide a firm base which can be used for the construction of models and the planning of appropriate experiments. Further analysis of the pbpA-rodA-dacA gene cluster should prove fruitful in this field.

Chapter 9

THE CONSTRUCTION OF VERSATILE LOW-COPY-NUMBER *ESCHERICHIA COLI* PLASMID CLONING VECTORS

SUMMARY

Small versatile low-copy-number plasmid cloning vectors were constructed by *in vitro* and *in vivo* recombinant DNA techniques. pLG338 and pLG339 are derived from pSC105, have a copy number of six to eight per chromosome, and carry genes conferring resistance to tetracycline and kanamycin. pLG338 (7.3 kb) has unique restriction endonuclease sites for BamHI, SalI, HincII, SmaI, XhoI, EcoRI and KpnI, the first five lying within a drug resistance gene. pLG339 (6.2 kb) lacks the KpnI site, but has unique SphI and PvuII sites. These vectors should be useful for cloning many genes coding for membrane and regulatory proteins which cannot be cloned into high-copy-number plasmids.

I. INTRODUCTION

Multicopy plasmids are the most commonly used vectors for cloning in *E.coli* (for review see Bernard and Helinski, 1980). Most of these vectors are based on the replicons of ColE1, pMB1 or P15A, have copy numbers ranging from 18 to 60 per chromosome and can be amplified in the absence of protein synthesis. High-copy-number vectors aid the isolation of large amounts of plasmid DNA and also help in the purification of proteins coded by cloned genes.

There are, however, occasions when a high-copy-number is unsuitable. Several examples have been reported of genes which have not been successfully

cloned onto a high copy number vector, but which have been cloned into bacteriophage λ, or a plasmid with a low copy number. These include polA (Murray and Kelley, 1979), ompA (Beck and Bremer, 1980), lpp (Nakamura and Inouye, 1979) and dacA (Spratt et al., 1980; and unpublished results). It seems likely that multiple copies of many genes coding for regulatory or membrane proteins will be deleterious to the cell. Furthermore, attempting to clone these genes onto a multicopy plasmid may result in the cloning of a mutant gene.

Bacteriophage λ is an ideal low-copy-number vector in many respects, as it integrates into the host chromosome, and is replicated with it. However, for most techniques of in vitro gene manipulation, plasmid vectors are most suitable. Several low-copy-number plasmid vectors have been constructed (Kahn et al., 1980). These include derivatives of F and R6, which have a copy number of about one per chromosome, and of RK2, which have a copy number of about eight per chromosome. These vectors tend to be rather large, and in most cases have only one drug resistance gene and few unique restriction sites.

pSC101 (Cohen and Chang, 1973, 1977; Figure 9.1a), which has a copy number of about six per chromosome, is a good compromise between vectors based on F, which are rather hard to isolate in large amounts, and multicopy plasmids. Although pSC101 was the first plasmid to be used for in vitro cloning, it is now little used because it only carries a tetracycline-resistance gene and therefore insertional inactivation cannot be used to screen for recombinant clones. Cohen et al. (1973) cloned an EcoRI fragment conferring kanamycin resistance from R6-5 into the pSC101 EcoRI site, producing pSC105 (Figure 9.1b). This plasmid has two drug resistance genes, but only has two unique restriction enzyme sites, for BamHI and HpaI, and is approximately 16 kb.

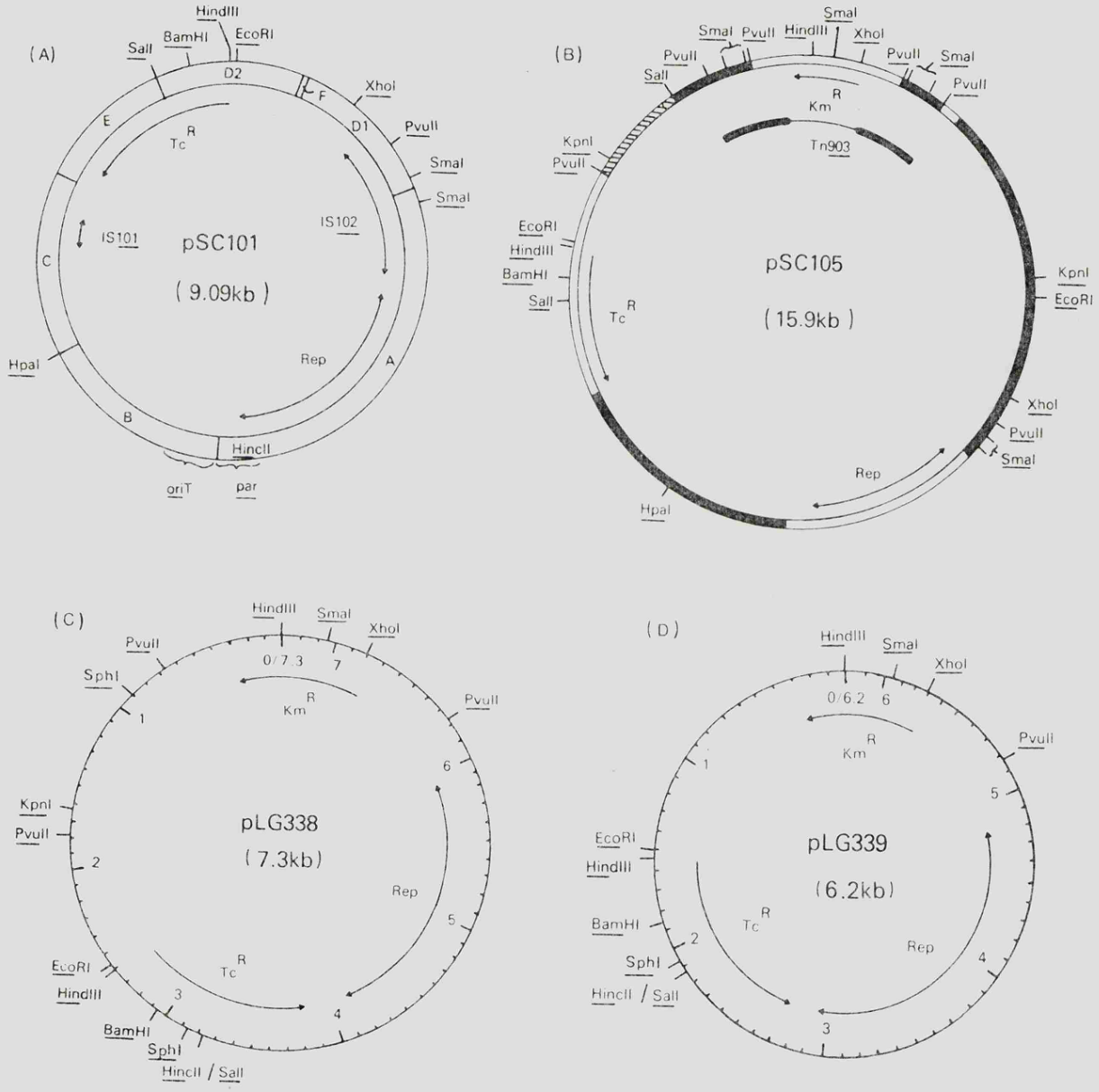
Figure 9.1

(A) Diagram of pSC101, compiled from information derived from Nordheim et al., 1980, Meacock and Cohen, 1980, Fischhoff et al., 1980, Bernardi and Bernardi, 1981 and P.A. Meacock, personal communication.

(B) Diagram of pSC105, compiled from information derived from Grindley and Joyce, 1980 and our unpublished data. The thick black line represents the DNA deleted in the construction of pLG338. The hatched region has also been deleted in pLG339.

(C) Restriction map of pLG338.

(D) Restriction map of pLG339.


(A)
HindIII
BamHI
EcoRI
SalI
D2
F
XhoI
E
D1
PvuII
Tc R
SmaI
SmaI
IS101
IS102
C
pSC101
(9.09kb)
HpaI
Rep
A
B
HincII
oriT
par
(B)
SmaI
SmaI
PvuII
HindIII
XhoI
PvuII
SmaI
PvuII
SalI
PvuII
Km R
Tn903
KpnI
PvuII
EcoRI
HindIII
BamHI
SalI
pSC105
(15.9kb)
Tc R
KpnI
EcoRI
XhoI
PvuII
SmaI
Rep
HpaI
(C)
HindIII
SmaI
XhoI
PvuII
0/7.3
7
SphI
Km R
PvuII
1
6
KpnI
PvuII
pLG338
(7.3kb)
2
Rep
5
EcoRI
HindIII
Tc R
3
4
BamHI
SphI
HincII / SalI
(D)
HindIII
SmaI
XhoI
0/6.2
6
Km R
PvuII
1
5
EcoRI
HindIII
pLG339
(6.2kb)
BamHI
Tc R
2
SphI
Rep
4
HincII / SalI
3

In the course of our work on the pbpA-rodA-dacA region we required a more convenient low-copy-number cloning vector, with unique EcoRI, SalI and KpnI sites, and we therefore deleted duplicated restriction sites and unwanted DNA from pSC105. pLG338 is a 7.3 kb derivative which has unique BamHI, SalI, HincII, SmaI, XhoI, EcoRI and KpnI sites, the first five of which lie within the Tc^R and Km^R genes. It can also be used for cloning PvuII or SphI generated fragments. pLG339 is a 6.2 kb derivative which has lost the KpnI site, but which has a unique SphI site within the Tc^R gene, as well as a unique PvuII site.

Part of this work, up to and including the construction of pLG318 was done in collaboration with Neil Fairweather.

II. MATERIALS AND METHODS

All materials and methods have been described in Chapter 2, with the exception of those specifically concerned with this chapter.

1. CONVERSION OF COHESIVE-ENDS TO FLUSH-ENDS USING DNA POLYMERASE I

5'-extensions produced by restriction endonucleases were "filled-in" by incubation of the DNA with DNA polymerase I Klenow fragment in 10 mM Tris, 10 mM $MgCl_2$, 50 mM NaCl, 0.5 mM DTT pH7.4 with 0.02 mM deoxynucleotides at 10°C for 90 minutes. The enzyme was inactivated by incubation at 65°C for ten minutes.

2. DIGESTION OF DNA WITH BAL31 NUCLEASE

Plasmid DNA was linearized with HpaI, and the enzyme inactivated by incubation at 65°C for ten minutes. The buffer was adjusted to 20 mM Tris-HCl pH8.0, 12 mM $MgCl_2$, 600 mM NaCl, 12 mM $CaCl_2$, 1 mM EDTA, and 5

units of BAL31 nuclease were added. This was incubated at 37°C, and aliquots were removed after 10, 20, 30 and 40 minutes and transferred to tubes containing 2.5 µl 0.25 M EDTA. Removal of Ca^{2+} irreversibly inhibits the nuclease (Gray et al., 1975). The activity of the nuclease was monitored by agarose gel electrophoresis.

3. DETERMINATION OF PLASMID STABILITY

An overnight culture of a plasmid-carrying strain was grown in nutrient broth containing an antibiotic to select for the presence of the plasmid. The culture was diluted 10^{-6} into nutrient broth with no antibiotic present. This was incubated for 12 hours, diluted 10^{-6} into fresh broth and grown for 12 hours. A factor of 10^{6} represents approximately 20 generations, and this was done a total of five times to make a total of 100 generations in non-selective media. At each stage, the culture was plated to single colonies onto nutrient agar. Fifty colonies were tested for the ability to grow on antibiotic, showing the presence of the plasmid.

4. DETERMINATION OF THE RATE OF [^{3}H]-THYMINE INCORPORATION

M9-medium containing casamino acids was prepared, and deoxyguanosine (200 µg/ml) added. The deoxyguanosine was prepared by dissolving 30 mg in 3 ml 0.15 M NaOH. 2 ml were added to 100 ml medium, and 0.1 ml 2.25 M HCl was added to neutralise the alkali.

0.1 ml of an overnight culture grown in M9-medium containing casamino acids was diluted into 10 ml medium containing added deoxyguanosine. Thymine was added to a concentration of 1 µg/ml, with [^{3}H]-thymine at a specific activity of 630 mCi/mmol. The culture was incubated with aeration at 37°C. At intervals, the A_{600} was measured, and 0.5 ml of culture was removed and added to a tube containing 1 ml ice-cold 10% TCA. After one

hour on ice, acid-precipitable material was collected on Sartorius membrane filters (0.45 μm pore size), and washed with two volumes of 5% TCA, followed by at least ten volumes of hot (90-95°C) water. Filters were dried and placed in tubes containing non-aqueous scintillation fluid consisting of 0.5% diphenyloxazole (PPO) and 0.0033% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) in toluene. These were counted in a Packard Tri-Carb 3255 liquid scintillation counter.

5. ESTIMATION OF PLASMID COPY NUMBER

0.1 ml of an overnight culture was diluted into 10 ml medium and labelled with [^{3}H]-thymine as described previously. The culture was incubated with aeration at 37°C to A_{600} 0.5, and the cells were chilled, harvested and washed twice in TES buffer. They were resuspended in 330 μl Tris-sucrose in an Eppendorf tube, and 67 μl lysozyme (5 mg/ml) containing RNase (250 μg/ml) were added, followed by five minutes incubation on ice. 133 μl 0.25 M EDTA pH8.0 were added, again followed by five minutes incubation on ice. 0.53 ml Brij lysis mix (see Table 2.3) was added and mixed until lysis was complete. Chromosomal DNA was sheared by vortexing the sample for one minute, followed by passage twice through a 23 gauge syringe needle.

A 5 μl sample was dropped onto filter paper, dried and counted as before. 300,000 cpm were loaded onto a CsCl-EtBr gradient. The lysate was made up to 1 ml with TES, and added to a Beckman 3" x 5/8" cellulose nitrate tube containing 6.55 g CsCl, 0.28 ml EtBr (5 mg/ml) and 5.52 ml TES buffer. The final refractive index was 1.390. The tube was centrifuged to equilibrium (Beckman 50 Ti rotor, 35,000 rpm, 40 hours, 15°C).

The gradient was collected from the bottom of the tube in 50 twelve drop fractions. 50 µl of each fraction were spotted onto filter paper. The filters were dried and added to 200 ml ice-cold 5% TCA containing thymine (100 µg/ml). After 15 minutes, the TCA was discarded and the papers were washed twice for 15 minutes in TCA containing thymine. The filters were then washed for 10 minutes in cold IMS, and for 10 minutes in ice-cold acetone. They were dried, and scintillation counted as before.

6. CONSTRUCTION OF PLASMIDS

(a) pLG314

pSC105 was digested with SalI and religated at a low DNA concentration. Km^R transformants were selected and screened for Tc^S. Plasmid size was then screened.

(b) pLG316

pLG314 was digested with EcoRI and KpnI, and the linear DNA used to transform C600. Transformation with linearized plasmids has been shown to give a greatly reduced number of transformants, most or all of which contain plasmids carrying deletions of varying size (Thompson and Achtman, 1979). Km^R transformants were selected and screened for plasmid size.

(c) pLG318

pLG316 and pSC105 were digested with SalI, and ligated together. Tc^RKm^R transformants were selected and screened for size.

(d) pLG336

pLG318 was digested with PvuII and religated at a low DNA concentration. Tc^RKm^R transformants were selected and plasmids screened for size. Several of the smallest class of plasmid were prepared and the orientation of the PvuII fragment carrying the Km^R gene relative to

that carrying the Tc^R gene was determined by comparing HindIII and HindIII XhoI digests. pLG336 was chosen as having the same orientation as pSC105. It was found on restriction with PvuII that one PvuII site had been lost, presumably due to mild exonuclease activity in the restriction or ligation reactions.

(e) pLG331

pBR328 (Soberon et al., 1980) was digested with BamHI and SalI, and the linear DNA used to transform C600. Three Cm^R transformants were obtained, all of which had lost their BamHI and SalI sites. pLG331 was chosen as still having a HindIII site.

(f) pLG333

pLG331 was digested with HindIII and treated with alkaline phosphatase. This DNA was ligated with a HindIII digest of pSC105. Cm^R transformants were selected and plasmid size screened. Most plasmids fell into two size classes, depending on which HindIII fragment from pSC105 they carried. pLG333 was chosen as containing the small HindIII fragment of pSC105.

(g) pLG334

pLG333 was digested with SalI and SmaI. The 5'-extension generated by SalI was made flush using DNA polymerase I. The DNA was religated at low DNA concentration and used to transform C600 to Cm^R. pLG334 was, like the majority of transformants, slightly smaller than pLG333, and as expected, had lost its SalI and SmaI sites.

(h) pLG337

pLG336 was digested with HindIII and the larger fragment purified. This was ligated with a HindIII digest of pLG334. $Km^R Tc^R$ transformants were selected.

(i) pLG338

pLG337 was linearized with HpaI and 7.5 μg were incubated with 5 units BAL31 nuclease for 20 minutes. The DNA was ligated at low DNA concentration and $Tc^R Km^R$ transformants selected. Plasmid size was screened, and the smallest chosen.

(j) pLG339

The small HindIII fragment from pLG336 and the large HindIII fragment from pLG338 were purified and ligated. $Km^R Tc^R$ transformants were selected.

III. RESULTS

(a) Removal of duplicate EcoRI, KpnI and XhoI sites from pSC105

We wished to retain the EcoRI and KpnI sites near the promoter of the Tc^R gene in pSC105 (Figure 9.1b). These sites lie conveniently within the 2.5 kb SalI fragment. This fragment was therefore removed (pLG314) before deleting the remaining EcoRI and KpnI sites by transforming C600 with DNA digested with these enzymes. Transformants appeared at a low frequency, and these carried deletions of varying sizes. pLG316, which carried the largest deletion, was 4.3 kb smaller than pLG314. The deletion extends towards the replication region, also removing the duplicate XhoI site, as well as a PvuII site and two SmaI sites. The 2.5 kb SalI fragment from pSC105 was then restored, producing pLG318.

(b) Removal of duplicate SalI and SmaI sites

pLG318 has five SmaI sites. One lies within the Km^R gene, and the other four lie within the inverted repeats of Tn903, which lie on either side of the Km^R gene (Figure 9.1b). These four sites were removed by digestion of pLG318 with PvuII, followed by religation, producing pLG336.

pLG336 has a single SmaI site, in the Km^R gene. It also has a single SalI site, but has lost its KpnI site.

The KpnI site was now restored. For this we required a cloning vector which had a HindIII site, but no SalI or SmaI sites. We constructed such a vector (pLG331) from pBR328, removing the SalI site by *in vivo* deletion. The small HindIII fragment from pSC105 was cloned into pLG331, producing pLG333. The region between the SalI and SmaI sites was removed and the resulting plasmid (pLG334) was not cut by either enzyme, but retained the KpnI site. The modified HindIII fragment was now recloned into pLG336, replacing its small HindIII fragment. pLG337 carries Km^R and Tc^R genes, and has unique KpnI, SalI and SmaI sites in addition to those already present in pLG318.

(c) Removal of excess DNA

Because pLG337 is still rather large, we made it smaller by deleting the DNA between the end of the Tc^R gene and the origin of replication. There is a unique HpaI site in the middle of this region. pLG337 was linearized with HpaI, incubated with BAL31 nuclease and blunt-end ligated. pLG338 is 2.8 kb smaller than pLG337. This deletion removed three of the four HincII sites in pLG337, leaving only the HincII (SalI) site within the Tc^R gene.

A smaller vector, pLG339, was made by replacing the small HindIII fragment in pLG338 with that from pLG336. pLG339 has no KpnI site, but has the advantage of a unique SphI site within the Tc^R gene, as well as a unique PvuII site. This is summarized in Figure 9.2.

(d) Mobilization of pLG338

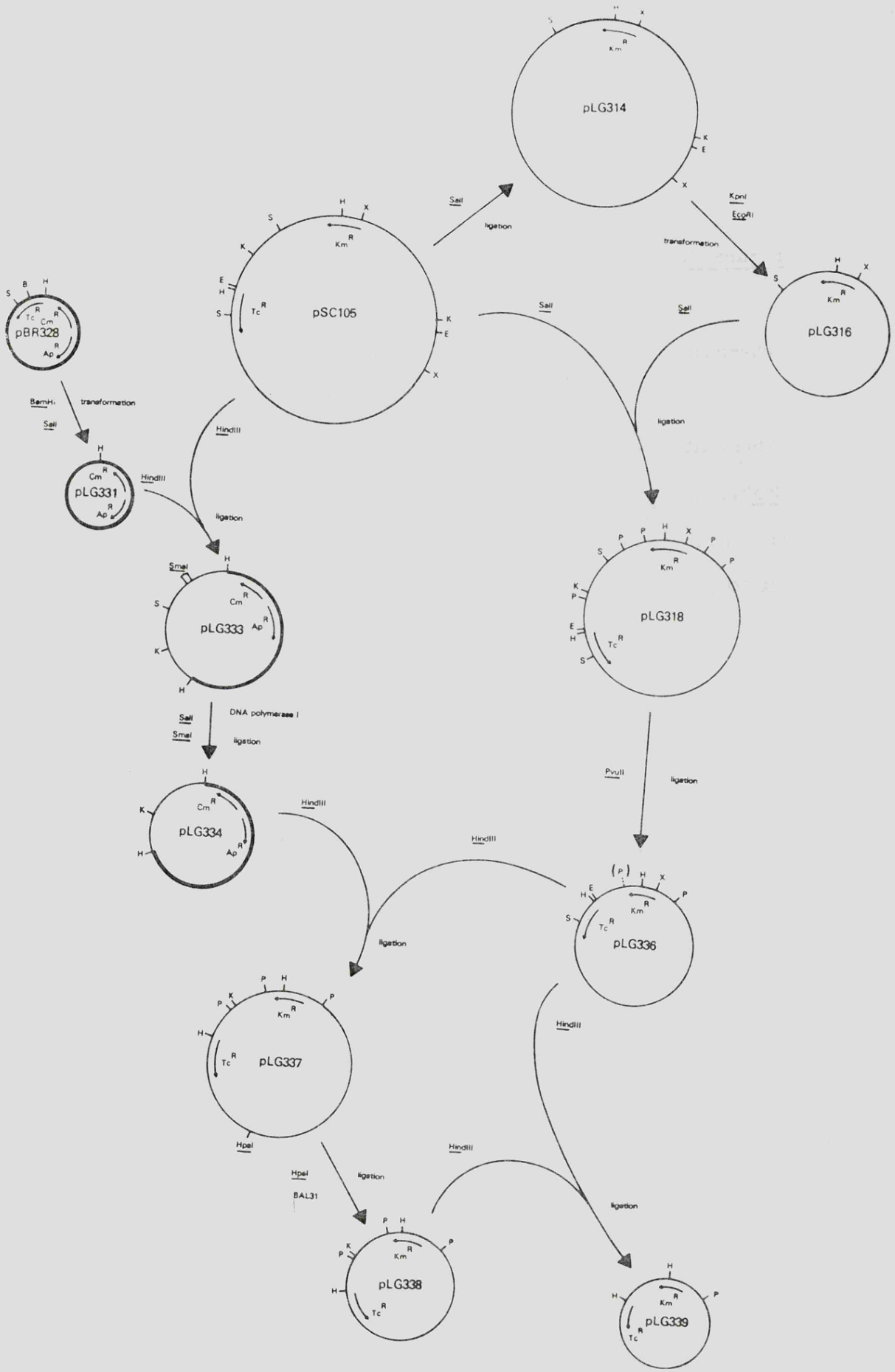
The origin of transfer of pSC101 has been mapped by Nordheim *et al.* (1980) to the HincII B fragment (Figure 9.1a). The deletion extending

Figure 9.2

Schematic diagram showing the construction of pLG338 and pLG339.

Abbreviations used are H, HindIII; X, XhoI; S, SalI; K, KpnI; E, EcoRI; B, BamHI; P, PvuII.

(P) indicates a PvuII site lost during the construction of pLG336.


pBR328
BamHI
SalI
transformation
pLG331
HindIII
ligation
pLG333
SmaI
SalI
SmaI
DNA polymerase I
ligation
pLG334
HindIII
pSC105
SalI
ligation
pLG314
KpnI
EcoRI
transformation
pLG316
SalI
ligation
pLG318
PvuII
ligation
HindIII
ligation
pLG336
HindIII
pLG337
HpaI
HpaI
BAL31
ligation
pLG338
HindIII
ligation
pLG339

from the HpaI site of pLG337 completely removes this fragment, including, presumably, oriT. pSC101 is mobilized by the conjugative plasmid R64drd11 (Cohen and Chang, 1973), and loss of oriT should drastically reduce this mobilization. We compared the efficiency of mobilization (e.o.m.) of pSC105, pLG337 and pLG338 by R64drd11. The plasmids were transformed into C600 [R64drd11] and these strains were mated with MC4100 rpsL. Dilutions were plated onto nutrient agar containing streptomycin alone or with tetracycline or kanamycin. The efficiency of mobilization was calculated as the ratio of kanamycin-resistant to tetracycline-resistant transconjugants. pSC105 and pLG337 had an e.o.m. of 2.0×10^{-2}, while pLG338 had an e.o.m. of 4.4×10^{-5}.

(e) Stability of pLG338

The deletion which removed the oriT region of pSC101 extends into the pSC101 HincII A fragment, which carries all of the essential replication functions of pSC101 (Meacock and Cohen, 1979). Meacock and Cohen (1980) described a small region of DNA, designated par, close to the end of the HincII A fragment, which is required for stable partitioning of the plasmid (Figure 9.1a). Plasmids lacking this region are not stably inherited when grown in non-selective medium. To see whether we had inadvertantly deleted the par region, we grew C600 [pLG338] for one hundred generations in non-selective medium and plated onto nutrient agar. Fifty colonies were tested, and all still carried pLG338. According to Meacock and Cohen (1980), with pSC101 derivatives lacking par, over seventy per cent of cells were plasmid free after this time. We therefore presume the deletion does not extend into par.

(f) Estimation of the copy number of pLG338

The copy number of pLG338 was estimated and compared with that of pSC101. This was done by labelling DNA with [^{3}H]-thymine, lysing the cells and separating plasmid and chromosomal DNA on a CsCl-EtBr density equilibrium gradient. The radioactivity in each peak was then calculated.

For efficient labelling of DNA, a thyA mutant should be used, but the plasmids were in C600, a thyA$^+$ strain. The rate of incorporation of [^{3}H]-thymine into C600 was measured, to check that it was constant, and to check that sufficient incorporation could be obtained for the experiment. The results demonstrated that [^{3}H]-thymine was incorporated at a constant rate over the course of the experiment, and that incorporation was high enough (Figure 9.3).

The copy numbers of pSC101 and pLG338 were then measured (Figure 9.4). By counting the radioactivity in the peaks, the plasmid copy number per E.coli chromosome was calculated. 3.8×10^3 kb was used as the value for the length of the E.coli genome (Twigg and Sherratt, 1980). pSC101 had an estimated copy number of 5 copies per genome, while pLG338 had a copy number of 7.5 copies per genome.

IV. DISCUSSION

We have constructed versatile low copy number plasmid cloning vectors by deleting various regions of pSC105 in order to remove duplicate restriction sites and to make the plasmids as small as possible. These plasmids are extremely versatile as cloning vectors.

pLG338 has unique sites for SalI, HincII, BamHI, XhoI, SmaI, EcoRI and KpnI. The first five sites lie within drug-resistance genes. It can also be used to clone PvuII- and SphI-generated fragments. pLG339

Figure 9.3

Incorporation of [^{3}H) thymine into C600 DNA.

- ● [^{3}H] incorporated into TCA-precipitable material;
- ▲ cell mass.

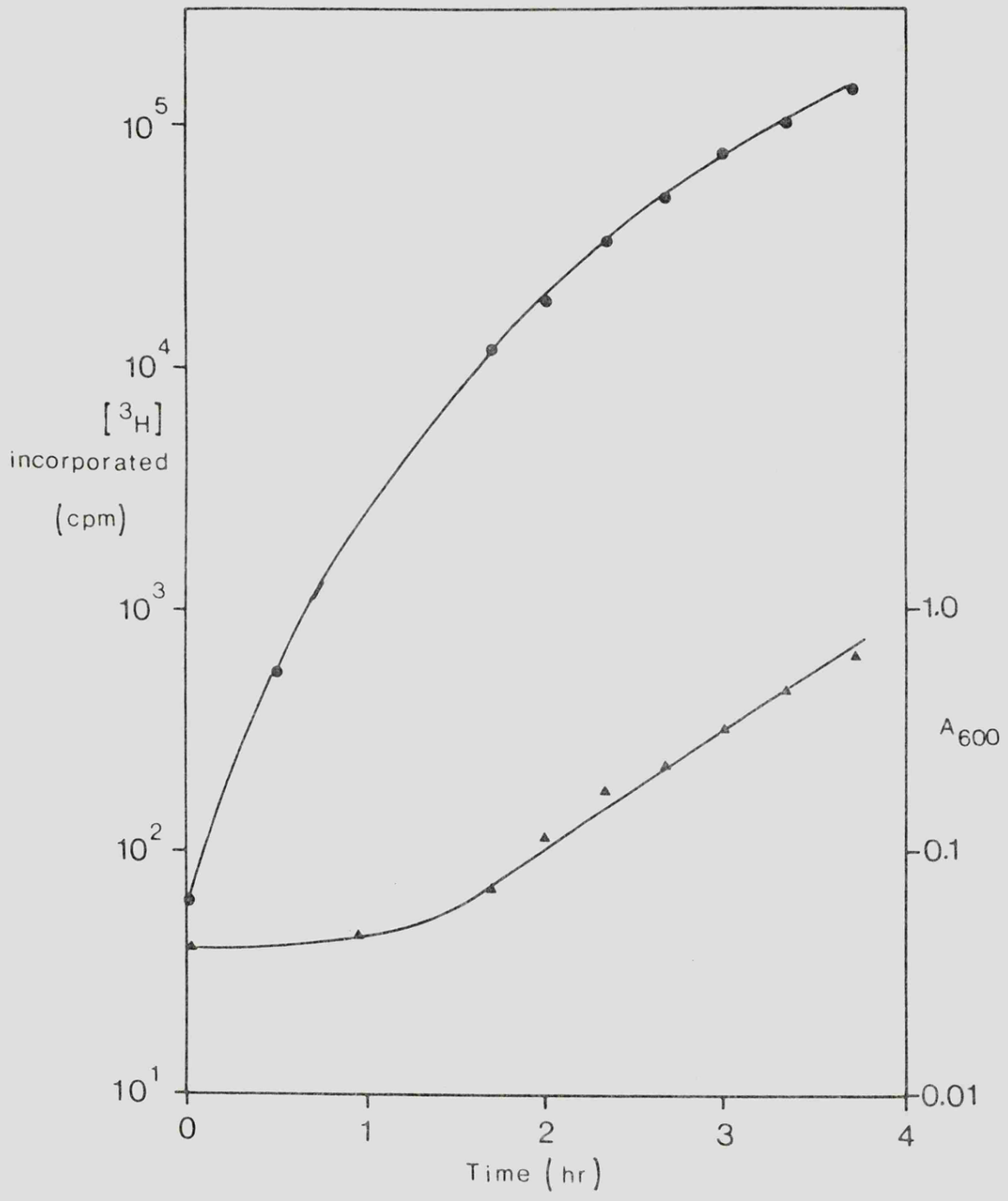
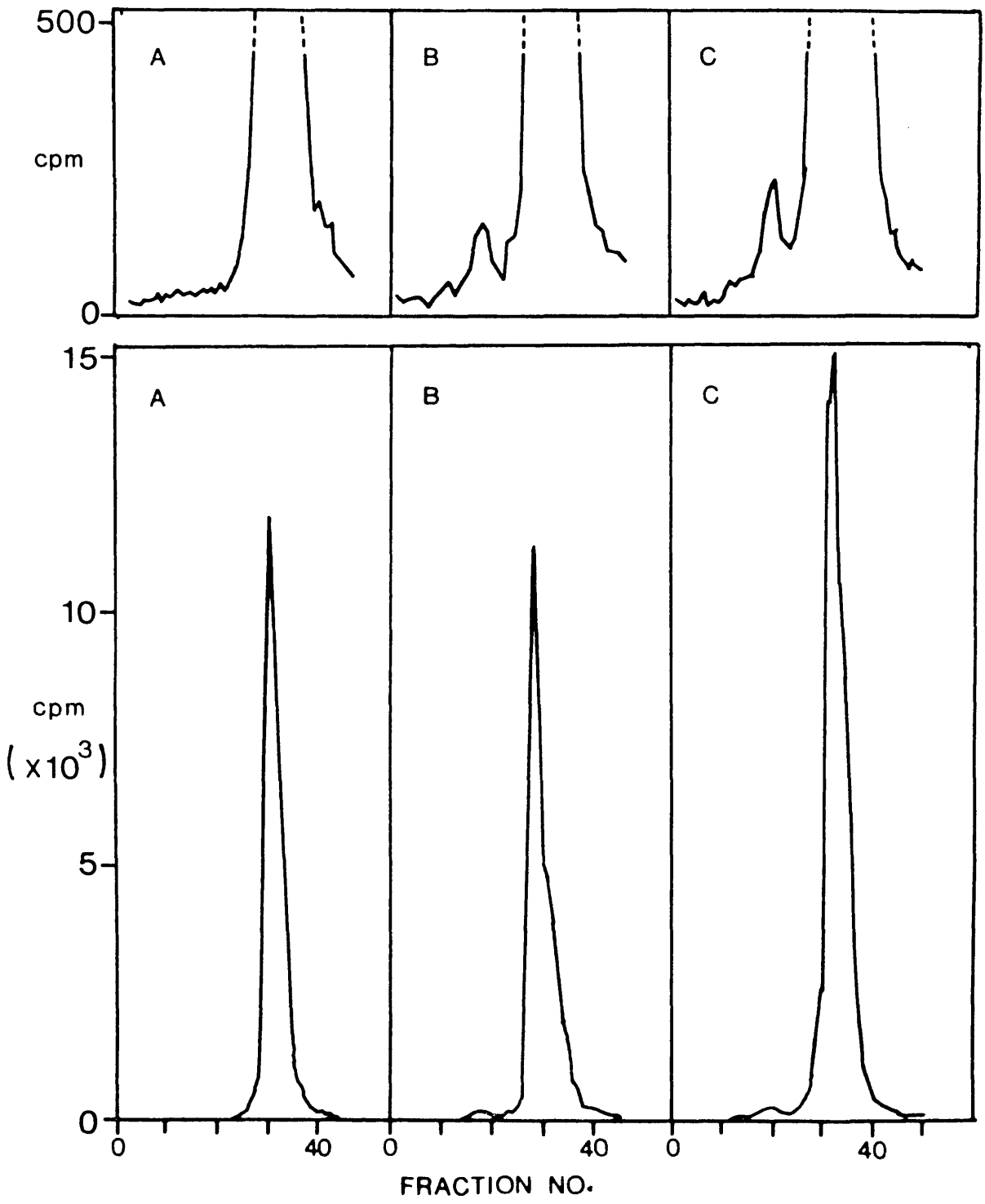

10^5
10^4
[^{3}H]
incorporated
(cpm)
10^3
10^2
10^1
1.0
0.1
0.01
A_{600}
0
1
2
3
4
Time (hr)

Figure 9.4

Estimation of copy number of pLG338.

Cultures of (A) C600, (B) C600[pSC101] and (C) C600[pLG338] were labelled with [^{3}H] thymine and the DNA was centrifuged to equilibrium in CsCl-EtBr. The gradients were collected and the fractions scintillation counted. The areas under plasmid and chromosomal peaks were calculated and the plasmid copy number was determined.


500
cpm
0
A
B
C
15
10
cpm
(x10³)
5
0
0
40
0
40
0
40
FRACTION NO.

is a smaller derivative which has lost the KpnI site, but has in addition a unique SphI site within the Tc^R gene and also a unique PvuII site.

The plasmids are suitable for the cloning of many fragments resulting from double-digests. There are three unique sites for enzymes which produce flush-ended cuts, two of which lie within drug resistance genes. The BamHI site is particularly useful, as it may also be used for cloning fragments generated by BglII, BclI, XhoII and SauIIIA. The KpnI and SphI sites provide 3'-extensions suitable for cloning by 3'-tailing with terminal transferase.

It is preferable to remove any transposable elements from potential cloning vectors, in order to avoid the possibility of unpredictable recombinational events with other genomes as well as DNA rearrangements (e.g. deletions) within the vectors themselves. pSC105 contains several of these elements. IS101 and IS102 are IS elements that lie within pSC101 (Fischhoff et al., 1980; Bernardi and Bernardi, 1981; Figure 9.1a), and the Km^R gene, which is originally derived from R6, is part of the transposon Tn903 (Young et al., 1980; Figure 9.1b). All of these IS elements have been deleted. IS102 was removed in the construction of pLG316. The inverted repeats of Tn903 were deleted in the construction of pLG336 and pLG337. IS101 was deleted in the construction of pLG338.

pLG338 is suitable for mutagenesis with the transposable element $\gamma\delta$ (Guyer, 1978; J. Broome-Smith and B.G. Spratt, unpublished results). Identification of polypeptides coded for by genes cloned in pLG338 is helped by the very clean background obtained using the minicell system (unpublished results).

The deletion made in pLG337, producing pLG338, extends from the Tc^R gene towards the replication region. The origins of transfer and replication are known to lie within about a kilobase of each other, on either side of a HincII site (Figure 9.1a). The origin of transfer lies in the HincII B fragment, while the origin of replication lies in the A fragment. The entire B fragment was deleted. We presumed the origin of transfer had been removed, and this is supported by the fact that pLG338 has a much lower efficiency of mobilization by R64drd11 than pLG337. We assume that the low level of mobilization which remains with pLG338 is due to the formation of R64drd11-pLG338 co-integrate plasmids, allowing passive transfer of pLG338.

There is a region (par) near the origin of replication of pSC101 (Figure 9.1a) which is involved in the stable inheritance of the plasmid. We found pLG338 to be stably maintained, and conclude that the deletion did not remove par.

The copy number of pLG338 was estimated at 7.5 molecules per genome. This compared with a figure of 5 molecules pSC101 per genome. The slightly lower figure for pSC101 can be accounted for by the fact that, unlike pLG338, it forms relaxation complexes, which may become relaxed in the lysis procedure or during the formation of the density equilibrium gradient. These relaxed molecules will band with the chromosomal DNA, causing an underestimate of the true copy number. We therefore conclude that the copy number of pLG388 has not been significantly altered from that of pSC101.

Derivatives of pSC105 have been successfully used to subclone the dacA and pbpA genes from the λ transducing phage λdlip5 (see Figure 3.1; B.G. Spratt, unpublished results) which cannot be stably cloned into pBR322. pLG338 and pLG339 will be useful for cloning other genes coding for membrane proteins or those involved in key regulatory events in the

cell cycle, where multiple copies may be toxic to the cell. Since these vectors are as versatile as pBR322, it may be wise to clone initially into a low-copy-number plasmid when working with genes of this type.

pLG338 and pLG339 provide a measure of containment, since they lack an origin of transfer, and have a narrow host range. This might be useful for the cloning of any genes from eukaryotic organisms or prokaryote pathogens which cannot be cloned into multicopy vectors. A versatile plasmid cloning vector, pHSG415, has been developed by Hashimoto-Gotoh et al. (1981) specifically with biological containment in mind. This plasmid replicates at 30°C, but fails to replicate at temperatures of 37°C or above, and is therefore rapidly lost from host cells grown at 37°C. However, this vector is seriously handicapped in the cloning of prokaryote genes, in that it cannot be used to complement temperature-sensitive mutations, since mutations in essential genes generally have to be isolated as temperature-sensitive or amber mutations.

Conditional expression derivatives of pLG338 and pLG339 have been developed by cloning in the λ p_L promoter, together with the thermolabile repressor gene, cI857. These plasmids allow overproduction of proteins from genes which must be kept at a low copy number (P. Holt and B.G. Spratt, unpublished results).

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ABSTRACT

Title THE MOLECULAR GENETICS OF CELL SHAPE IN *ESCHERICHIA COLI*

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The organization of a cluster of genes involved in peptidoglycan synthesis and cell-shape determination in *Escherichia coli* was studied. The gene cluster, which includes *pbpA* and *dacA*, the structural genes for penicillin-binding proteins 2 and 5 respectively, and the *rodA* gene, had been previously cloned into a λ vector. Inactivation of *pbpA* or *rodA* results in the growth of cells as osmotically-stable spheres, and *dacA* encodes one of the major D-alanine carboxypeptidases of the cell.

The order and location of genes in this cloned region was determined by the isolation of deletion derivatives of the phage. The *rodA* gene product was not initially identified, and consequently, *rodA* was subcloned separately into a plasmid vector. The gene product was then identified as a minor cytoplasmic membrane protein (M_R 31,000). It had previously escaped detection because it apparently aggregates when boiled in SDS. The protein did not appear to be synthesized as a larger precursor. The direction of transcription of *rodA* was determined.

The role of these genes in peptidoglycan synthesis and in the determination of cell-shape is discussed.

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Defective and Plaque-Forming Lambda Transducing Bacteriophage Carrying Penicillin-Binding Protein-Cell Shape Genes: Genetic and Physical Mapping and Identification of Gene Products from the *lip-dacA-rodA-pbpA-leuS* Region of the *Escherichia coli* Chromosome

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A series of defective lambda transducing phage carrying genes from the *lip-leuS* region of the *Escherichia coli* chromosome (min 14 on the current linkage map) has been isolated. The phage defined the gene order as *lac---lip-dacA-rodA-pbpA-leuS---gal*. These included the structural genes for penicillin-binding protein 2 (*pbpA*) and penicillin-binding protein 5 (*dacA*) as well as a previously unidentified cell shape gene that we have called *rodA*. *rodA* mutants were spherical and very similar to *pbpA* mutants but were distinguishable from them in that they had no defects in the activity of penicillin-binding protein 2. The separation into two groups of spherical mutants with mutations that mapped close to *lip* was confirmed by complementation analysis. The genes *dacA*, *rodA*, and *pbpA* lie within a 12-kilobase region, and represent a cluster of genes involved in cell shape determination and peptidoglycan synthesis. A restriction map of the *lip-leuS* region was established, and restriction fragments were cloned from defective transducing phage into appropriate lambda vectors to generate plaque-forming phage that carried genes from this region. Analysis of the proteins synthesized from lambda transducing phage in ultraviolet light-irradiated cells of *E. coli* resulted in the identification of the *leuS*, *pbpA*, *dacA*, and *lip* gene products, but the product of the *rodA* gene was not identified. The nine proteins that were synthesized from the *lip-leuS* region accounted for 57% of its coding capacity. Phage derivatives were constructed that allowed about 50-fold amplification of the levels of penicillin-binding proteins 2 and 5 in the cytoplasmic membrane.

The characteristic shape of bacterial cells is maintained by the structural rigidity of the peptidoglycan layer of the cell wall (30). Little is known about the processes that determine cell shape, but presumably the properties of the enzymes that incorporate peptidoglycan precursors into the preexisting cell wall have a major role in determining the shape of the peptidoglycan, and therefore the shape of the bacterial cell. The enzymes that catalyze this final cross-linking reaction in peptidoglycan synthesis have been identified as the peptidoglycan transpeptidases, and these enzymes are the targets that penicillin and other β-lactam antibiotics inhibit to produce their lethal effects on bacteria (2, 7).

Bacteria contain multiple forms of these penicillin-sensitive enzymes in their cytoplasmic membrane, and they can be detected on sodium dodecyl sulfate (SDS)-polyacrylamide gels as penicillin-binding proteins (PBPs) (32, 34). One of the seven PBPs of *Escherichia coli* has been shown to have a crucial role in cell shape determination since inactivation of the protein (PBP2), either by the atypical β-lactam antibiotic mecillinam or by mutation, results in the growth of the bacteria as large spherical cells (32, 33, 36, 37). PBP2 has a molecular weight of 66,000 and is the least abundant of the *E. coli* PBPs.

Several mutants that have defects in PBP2 have been described (32, 36, 37). Some of these lack detectable activity of PBP2 and grow under all conditions as spherical cells. Other mutants have highly thermolabile forms of PBP2 and grow as rod-shaped cells at 30°C and as spherical cells at 42°C. Genetic studies with these cell shape mutants have established clearly that the spherical shape of the cells is a direct consequence of the defect in PBP2 activity (36, 37).

All mutations that produce defects in PBP2 map close to *lip* at 14 min on the current linkage

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map (1, 36, 37, 41), and this cell shape gene has been called *pbpA* (35). Other mutations that cause spherical growth of *E. coli* map close to *aroE* at 71 min; these have no effect on PBP2 activity, and have been designated *envB* (43) or *rodY* (12).

Recently we isolated a temperature-sensitive cell shape mutant, SP52, which had a cell shape mutation that mapped very close to *lip*, but was distinguishable from *pbpA* mutants since it had normal PBP2 activity. We show here that this mutant defines a previously unidentified cell shape gene, which we call *rodA*, that maps very close to *pbpA*.

A further gene, *dacA*, which is involved in peptidoglycan metabolism, maps close to the *pbpA* and *rodA* genes; *dacA* has been reported to be 87% cotransducible with *leuS* (41), which is 42% cotransducible with *lip* (16). Inactivation of *dacA* results in loss of the activity of the penicillin-sensitive enzyme D-alanine carboxypeptidase 1a (19). This enzyme corresponds to PBP5 and PBP6 (39), and recently it has been shown that *dacA* is the structural gene for PBP5 (molecular weight of 42,000), since an altered form of this enzyme was found in a *dacA* mutant (20).

We show here that *dacA*, *pbpA*, and *rodA* are a cluster of genes involved in peptidoglycan metabolism and cell shape determination which maps between *lip* and *leuS* and describe the isolation of a series of lambda transducing phage which carry these genes. The transducing phage have been used to derive a restriction map, to analyze the products of the genes in the *lip-leuS* region, and to construct strains of *E. coli* that produce greatly elevated levels of PBP2 and PBP5.

MATERIALS AND METHODS

Bacterial strains and bacteriophage. The bacterial strains used in this work are listed in Table 1. P1 *vir* was used for generalized transduction. λ^+, λ *imm*^{434}, λ cI857 *S*am7, λ cI857 *Q*am73, λ cI857 *Q*am73 *S*am7, λ *imm*^{21} *Q*am73, and λ h^{82} cI$^-$ were kindly provided by Bill Brammar. The lambda phage used as cloning vectors had the following genotypes: λL41, *chiA*131 Δ(*srI*λ1-2) *imm*^{434} cI$^-$ *srI*λ4° *nin*5 *shn*λ6° *srI*λ5°; and λNM627, Δ(*srI*λ1-2) cI857 *srI*λ4° *nin*5 *srI*λ5° *S*am7. The phage were kindly provided by Wilhelmine Loenen and Noreen Murray (via Neil Fairweather), respectively. The nomenclature for restriction endonuclease targets is that used in reference 26, where *srI*λ1° and *shn*λ1° refer, respectively, to the mutational loss of the *Eco*RI and *Hin*dIII sites 1 in the lambda genome.

Growth media and buffers. Luria broth (15) was used for routine growth of cells; it was supplemented with 0.2% maltose when optimal lambda phage absorption was required. Phage titers were made on Trypticase agar (BBL Microbiology Systems, Cockeysville, Md.) (28). Phage lysates were prepared on Luria broth solidified with 1% agar (Difco Laboratories, Detroit, Mich.) for plates or 0.65% for top agars.

Penassay broth (Difco antibiotic medium no. 3) and M9 minimal medium (23), supplemented as required with L-amino acids (20 μg/ml), adenine (10 μg/ml), thiamine (10 μg/ml), biotin (10 μg/ml), and lipoic acid (1 μg/ml), were used in some experiments.

Selection of cells containing the Tn*10* transposon used nutrient agar (Oxoid Ltd., London) containing tetracycline (20 μg/ml).

TE buffer contained 10 mM Tris-hydrochloride and 1 mM EDTA, pH 7.4; lambda buffer was 6 mM Tris-hydrochloride, 10 mM $MgSO_4$, and 50 μg of gelatin per ml, pH 7.4.

Preparation of cell envelopes and assay of PBPs. Cell envelopes were prepared and PBPs were assayed as described previously (34) except that the separating gel used in the fractionation of the PBPs was modified to 10% acrylamide-0.13% methylenebisacrylamide.

Genetic techniques. Transduction with phage P1 *vir* and conjugation were performed by standard methods (23). Cells of AB1325 *lip-9* that were to be transduced to *lip*$^+$ were prestarved by growing at 37°C for 4 h in minimal medium lacking lipoic acid (10).

Genetic manipulation of the cell shape mutants (*rod* mutants) was simplified by constructing a strain that had the tetracycline resistance transposon, Tn*10*, inserted into the chromosome close to the *rod* gene(s). This was achieved by mating SP299 with SP13711 and selecting for tetracycline resistance (Tet^r), *lac*$^+$, and *rod*$^+$. Colonies arising from this mating result from the transposition of Tn*10* from its position in the chromosome of SP299 onto F′254 (which carries the genes *lac*$^+$ *rod*$^+$; see Results) and its transfer into SP13711 (Tet^s *lac rod*). Some of the exconjugants should carry an F′254 which has Tn*10* inserted sufficiently close to the *rod* gene(s) on the F-prime to be cotransducible with it by P1 phage. About 1,000 exconjugants were therefore pooled; P1 *vir* phage were grown on these cells, and the lysate was used to transduce SP45 to Tet^r and *rod*$^+$ (i.e., growth at 42°C on Penassay agar containing tetracycline). Fourteen transductants of this type were obtained, and the linkage of Tn*10* to *rod* was determined by P1 transduction. One strain in which Tn*10* was about 50% cotransducible with the *rod* gene(s) was used for further work. P1 *vir* grown on this strain was used to transduce Tn*10* into the same position in the chromosome of each of the *rod* mutants, TMRL12 and AB1325 *lip-9*, and P1 lysates grown on the resulting strains were used to transduce the *rod*, *leuS*, or *lip* mutations into other strains by cotransduction with Tn*10* (see reference 13).

Transposon genetics (13) was also used to put the *rod* mutations onto F′254, to allow complementation analysis. For example, strain SP62 (*rod-6*/F′254 *rod*$^+$) was transduced to Tet^r with P1 *vir* grown on Tn*10 rod* strains. Integration of the Tn*10-rod* fragment into F′254 results in spherical cells because of diploidy for *rod*.

In practice, when SP62 was transduced to Tet^r with P1 *vir* grown on SP60, 12% of the transductants were

TABLE 1. *E. coli strains used*

Strain	Genotype[a]	Source or reference
KN126	*trp*(Am) *tyr*(Am) *ilv supD126*(Ts)	34
SP6	KN126 *pbpA6 ponA6*	32, 39
SP45	KN126 *pbpA45*	36
SP137	KN126 *pbpA137 pur*E	37
SP52	KN126 *rodA52*	This paper
AB1325*lip-9*	*thi his purB proA lacY lip-9 rpsL*	J. R. Guest (10)
SP600	AB1325 *lip*$^+$ *pbpA6*	This paper
SP4500	AB1325 *lip*$^+$ *pbpA45*	36
SP13711	AB1325 *lip*$^+$ *pbpA137*	This paper
SP5211	AB1325 *lip*$^+$ *rodA52*	This paper
CGSC 4282	*metE trpE purE proC leu lacZ rpsL recA*/F'254 (*lac*$^+$ *purE*$^+$ *lip*$^+$)	B. J. Bachmann
SP62	SP6/F'254	38
SP454	SP45/F'254	This paper
SP1376	SP137/F'254	This paper
SP521	SP52/F'254	This paper
SP60	SP6 *zbe*::Tn*10*[b]	This paper
NK5304	Hfr; *ilv thi thr srl*::Tn*10 recA56*	N. Kleckner (13)
SP522	SP52*srl*::Tn*10 recA56*	SP52 transduced to *srl*::Tn*10 recA56* with P1 phage grown on NK5304 (13)
W3110 *lip-9*	*sup*^{0} *zbe*::Tn*10 lip-9*	This paper
TMRL12	*arg pro purB his thi leuS lacY sup*^{0}	M. Matsuhashi (20)
DU3099	*his trp lys lac* recA56 *zie*::Tn*10*	T. Foster (6)
SP299	DU3099/F'254	CGSC 4282 × DU3099
AB2463	*leu thr his proA argE thi lacY recA13*	B. M. Wilkins
833	Δ(*gal-att*λ-*bio*) *his trp tonA*	W. J. Brammar
159	*gal uvrA*	G. Plastow
C600	*thr leu thi supE tonA*	W. J. Brammar
ED3184	*his lac tsx supF*	W. J. Brammar

[a] Genetic symbols are those used in reference 1. For definition of *pbpA* and *rodA*, see text; *ponA*, penicillin-binding protein 1A gene (41).

[b] Transposon nomenclature is that used in reference 13; e.g., *zbe*::Tn*10* denotes the insertion of Tn*10* into an unknown gene in the 14-min region of the chromosome.

spherical. One of these strains was used to transfer F'254::Tn*10 rod-6* into the *recA* strain AB2463 selecting for *lac*$^+$ Tet^r, and the F-prime was transferred from there into SP6, SP45, SP52, and SP137 by selection for Tet^r at 30°C. The morphology of the resulting partial diploids was examined at 30 and 42°C, and complementation was indicated by the recovery of the normal rod shape at 42°C.

Isolation of specialized λ transducing phage. A mixed low-frequency transducing lysate was prepared from strain 833 Δ(*gal-uvr*B) lysogenized with λ *c*I857 *S*am7 as described by Schrenk and Weisberg (31) and was used to transduce SP5211(λ$^+$), SP13711(λ$^+$), and TMRL12(λ$^+$) to temperature resistance by using the selective conditions described below.

Transduction of *leuS*$^+$ was measured by selecting for the growth of TMRL12(λ$^+$) on minimal agar at 42°C (16). Selection for *lip*$^+$ was by the growth of lipoic acid-starved cells of AB1325 *lip-9* on minimal agar at 30°C (10). Testing for the transduction of the cell shape genes *pbpA*$^+$ and *rodA*$^+$ (see Results) used strains SP13711(λ$^+$) and SP5211(λ$^+$), respectively. Cells were plated on Penassay agar containing Sarkosyl NL97 (0.1%), incubated at 30°C for 3 h, and then transferred to 42°C. Addition of Sarkosyl eliminated the background growth of the temperature-sensitive *pbpA* and *rodA* mutants at 42°C since the spherical cells formed under these conditions were supersensitive to the detergent.

Preparation of phage DNA and digestion with restriction endonucleases. High-frequency transducing stocks of defective transducing phage were prepared by induction from double lysogens of the type AB1325 *lip-9* (or SP600) (λ *c*I857 *S*am7) (λ d*lip*5 *c*I857 *S*am7), and were separated from helper phage by CsCl density gradient centrifugation (23). The purified phage were dialyzed against TE buffer and were extracted twice with phenol–chloroform–isoamyl alcohol–8-hydroxyquinoline (100:100:4:0.1). The phage DNA was dialyzed against TE buffer, ethanol precipitated, and redissolved in TE buffer at a concentration of 100 to 200 μg of DNA per ml.

Plaque-forming phage were prepared by induction from lysogens or by confluent lysis on plates, using *E. coli* C600 or ED3184 as host (23). The phage were concentrated by centrifugation into a CsCl block gradient, and DNA was prepared as described above.

Digestion of DNA with restriction endonucleases and analysis of DNA fragments on agarose gels were performed as described (22). The buffers used for

restriction enzymes were those recommended by the suppliers (Bethesda Research Laboratories, Rockville, Md.).

Construction of plaque-forming phage in vitro. Plaque-forming phage carrying genes from the *lip-leuS* region were obtained by cloning fragments of the defective transducing phage λ d*lip*5 into λ vectors.

*Hin*dIII fragments of λ d*lip*5 were cloned into the replacement vector λ *L*41 (see Fig. 2A) by using T4 DNA ligase (3) generously provided by Ron Wilson and Barry Ely. Portions of the ligation mixture were used to transfect $CaCl_2$-treated cells of C600(P2) (18), and the resulting clear-plaque, *spi*$^-$, recombinant phage were tested for their ability to transduce λ *imm*^{434} lysogens of TMRL12 (*leuS*), SP13711 (*pbpA*), SP5211 (*rodA*), and AB1325 *lip-9*.

*Sal*I fragments of λ d*lip*5 were cloned into the vector λNM627 (see Fig. 2C), and phage carrying genes from the *leuS-lip* region were detected by transduction of λ$^+$ lysogens of TMRL12, SP13711, SP5211, and AB1325 *lip-9*. The resulting dilysogens were induced, and the lysates were plated on ED3184. Single plaques of recombinant transducing phage were detected by their ability to transduce chromosomal genes and were purified.

Proteins synthesized from specialized transducing phage. The proteins synthesized from the chromosomal genes on λ transducing phage were analyzed by a method modified from Ptashne (29).

Cells of *E. coli* 159 (λ *ind*$^-$) were irradiated with UV light (12,000 ergs/mm^2) and infected with transducing phage (multiplicity of infection of 5 to 10), and the proteins synthesized after infection were labeled with [^{35}S]methionine, fractionated on 10 to 16% SDS-polyacrylamide slab gels (34), and detected by fluorography (14). Separation of cells into cell envelopes and cytoplasmic and outer membrane protein fractions was done as described previously (34).

Molecular weights of bands on fluorographs of SDS-polyacrylamide slab gels were estimated by comparison with those produced by a ^{14}C-methylated protein mixture (The Radiochemical Centre, Amersham, England) which provided molecular weight standards of 14,300, 30,000, 46,000, 69,000, 92,500, 100,000, and 200,000.

Construction of a λ d*lip*5 *c*I857 *Q*am73 *S*am7 lysogen. λ *c*I857 *Q*am73 *S*am7 was absorbed (multiplicity of infection of 1) to the defective single lysogen AB1325 *lip-9* (λ d*lip*5 *c*I857 *S*am7), and phage development was induced by a heat stock. The resulting lysate was used to transduce W3110 *lip-9* to *lip*$^+$ at 30°C, and the level of PBPs was measured in the transductants 2 h after phage development had been induced by a heat shock. Lysogens that showed the highest levels of overproduction of PBP2 and PBP5 were tested for the presence of a functional λ *Q* gene by spotting 10^5 λ *imm*^{21} *Q*am73 onto a lawn of the lysogen. Failure to produce plaques at 30°C showed that the lysogens contained λ d*lip*5 *c*I857 *Q*am73 *S*am7.

Chemicals. [^{14}C]benzylpenicillin (54–59 mCi/mmol) and [^{35}S]methionine (>600 Ci/mmol) were purchased from The Radiochemical Centre. Benzylpenicillin was kindly provided by Nigel Curtis of Glaxo Laboratories, Greenford, England. Sarkosyl NL97 was obtained from Geigy Industries, New York. Restriction endonucleases were purchased from Bethesda Research Laboratories; ampholytes were purchased from Bio-Rad Laboratories, Richmond, Calif.

RESULTS

Properties of cell shape mutants. The mutants SP6, SP45, and SP137 all have defects in the activity of PBP2 and have been described previously (32, 36, 37). SP6 grows under all conditions as spherical cells and lacks detectable levels of PBP2 (32). The mutants SP45 and SP137 are temperature-sensitive cell shape mutants which grow at 30°C or below as rod-shaped cells, but at 42°C as spherical cells (36, 37). The temperature sensitivity of cell shape in these latter two mutants has been shown to be due to the production of highly thermolabile forms of PBP2 (36, 37).

The temperature-sensitive cell shape mutant SP52 was isolated from strain KN126 after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described previously (35). SP52 grew as rod-shaped cells at 30°C or below and as spherical cells at 42°C, and was morphologically indistinguishable from the mutants SP45 and SP137. However, it differed from the latter mutants in that SP52 produced a thermostable PBP2 (data not shown). SP52 contains the temperature-sensitive amber suppressor *supD126*, and the temperature sensitivity of cell shape could have been due to an amber mutation in the PBP2 gene, such that PBP2 was synthesized at 30 but not at 42°C. This was not the situation, since the level of PBP2 activity in cells of SP52 that had been grown for three mass doublings at 42°C was the same as that in cells of the parent strain, KN126, grown under the same conditions (data not shown). Furthermore, the cell shape mutation of SP52 could be readily transferred into strain TMRL12 by cotransduction with *leuS*, and the resulting strains showed the same temperature sensitivity of cell shape as SP52, although the TMRL12 strain was free of amber suppressors as judged by its inability to support the growth of λ *c*I857 *Q*am73 or λ *c*I857 *S*am7 (data not shown).

Genetic mapping and complementation studies on cell shape mutants. In this section, the cell shape mutations of SP6, SP45, SP52, and SP137 will be described as *rod-6*, *rod-45*, *rod-52*, and *rod-137*, respectively.

The *rod-6* mutation, which results in the loss of PBP2 activity, maps close to *lip* (37), and the cell shape of SP6 was corrected by introduction of F'254 which carries chromosomal genes between approximately 7 and 15 min on the genetic map (38). F'254 was transferred by conjugation from strain CGSC 4282 into the cell shape mu-

tants SP45, SP52, and SP137 to obtain the partial diploids SP454, SP521, and SP1376, respectively. Both SP454 and SP1376 regained thermostable PBP2 and normal rod shape at 42°C, establishing that the *rod* mutations in these strains were recessive, and mapped within the region covered by F'254. The *rod-52* mutation also mapped within this region and was recessive, since the partial diploid SP521 was rod shaped at 42°C.

P1 *vir* phage was grown on strain SP6, SP45, SP52, and SP137, and the lysates were used to transduce AB1325 *lip-9* to *lip*$^+$ at 30°C. The frequency of cotransduction of the cell shape mutations *rod-6, rod-45, rod-52,* and *rod-137* were 86, 84, 92, and 89%, respectively (50 to 100 transductants tested in each cross).

Since the mutations *rod-6, rod-45,* and *rod-137* all mapped very close to *lip,* but differed in their effects on the activity of PBP2, complementation analysis was used to establish whether they were all mutations in the same *rod* gene. The *rod-6, rod-45,* and *rod-137* mutations were incorporated on F'254 and were transferred into strains SP6, SP45, SP52, SP137, and KN126 as described in Material and Methods. Complementation was indicated by the growth of the partial diploids as rod-shaped cells at 42°C. Partial diploids containing any combination of the *rod-6, rod-45,* and *rod-137* mutations failed to show complementation of cell shape, establishing that these three mutations, which all result in defects of PBP2, were allelic. However, introduction of F'254 carrying the *rod-6, rod-45,* or *rod-137* mutations into SP52, or into a *recA56* derivative of SP52 (SP522), resulted in complementation of cell shape, indicating that the *rod-52* mutation, which has no effect on PBP2 activity, was in a separate gene to the other *rod* mutations.

We have proposed (35) that the designation *pbpA* be used for mutations in the structural gene for PBP2, and the mutations of SP6, SP45, and SP137 will henceforth be called *pbpA6, pbpA45,* and *pbpA137,* respectively. The designation *rodA* has been used to describe spherical mutants that map close to *lip* (21), and we propose that this designation be now used for the second cell shape gene that maps in this region. The mutation of SP52 is therefore renamed *rodA52.*

Isolation of specialized λ transducing phage carrying the *lip-leuS* region. The cell shape mutants in their original genetic background (strain KN126) were found to be resistant to phage λ, and the cell shape mutations were transferred into the λ-sensitive strain AB1325 *lip-9* by cotransduction with *lip* to yield strains SP600 (*pbpA6*), SP4500 (*pbpA45*), SP13711 (*pbpA137*), and SP5211 (*rodA52*). Specialized transducing phage that could transduce SP13711(λ$^+$) to *pbpA*$^+$ were isolated, and high-frequency lysates were prepared as described in Materials and Methods. Nine transducing phage were obtained by using this selection, a further six were obtained by selecting for transduction of SP5211(λ$^+$) to *rodA*$^+$, and an additional five were obtained by selection for transduction of TMRL12(λ$^+$) to *leuS*$^+$.

The presence of the genes *leuS, pbpA, rodA,* and *lip* on these transducing phage was determined by spot tests on the appropriate mutants. No simple transductional test was available for the presence of the *dacA* gene on the phage. The presence of *dacA* was detected by the ability of the phage to program the synthesis of PBP5 on infection of UV-irradiated *E. coli* (see below). Table 2 shows the five classes of phage that could be distinguished, and these define unambiguously the gene order as *lac---lip-dacA-rodA-pbpA-leuS---gal.* (The orientation of *lip* and *leuS* is that established in the forthcoming genetic map; B. J. Bachmann, personal communication.) The *dacA* gene was mapped between *lip* and *pbpA* since phage that carried the complete *lip-rodA-pbpA-leuS* region synthesized PBP5 in infection experiments, whereas phage that only carried the *pbpA-leuS* region did not. Phage that carried *rodA-pbpA-leuS* fell into two classes, either producing (e.g., λ d*dacA*51) or not producing (e.g., λ d*rodA*1) PBP5. *dacA* must therefore map between *lip* and *rodA.*

High-frequency lysates of a representative of each of the five classes of transducing phage were diluted and plated on strain ED3184 to give single plaques. None of the phage from single plaques (20 plaques tested for each phage) was found to transduce TMRL12(λ$^+$) to *leuS*$^+$, indicating that each of the transducing phage had lost genes essential for plaque formation.

All of the phage carried the *leuS* gene, but they differed in the genes counterclockwise to *leuS* that were present. This suggested that all

TABLE 2. *Classes of* λ *transducing phage isolated*

Class	Chromosomal genes on phage[a]					No. of class isolated	Example of phage class
	lip	*dacA*	*rodA*	*pbpA*	*leuS*		
1	+	+	+	+	+	9	λ d*lip*5
2	−	+	+	+	+	5[b]	λ d*dacA*51
3	−	−	+	+	+		λ d*rodA*1
4	−	−	−	+	+	4	λ d*pbpA*108
5	−	−	−	−	+	2	λ d*leuS*27

[a] Presence of chromosomal genes was determined by transduction of *lip, rodA, pbpA,* and *leuS* and by programming of the synthesis of the *dacA* gene product.

[b] Presence of the *dacA* gene was not determined for all phage.

of the phage were formed by the aberrant excision of λ *c*I857 *S*am7 from the same secondary attachment site clockwise from *leuS*. The phage have been named according to the most distant gene from *leuS* that they are known to carry; e.g., λ d*rodA* has the chromosomal genes *leuS*, *pbpA, and rodA* but not *dacA* or *lip*.

The presence of the *pbpA* gene on the transducing phage was initially determined by the transduction of SP13711(λ$^+$) to *pbpA*$^+$, and 18 out of 20 of the phage carried this gene. All 18 of these phage also transduced the other *pbpA* mutants (SP600 and SP4500) to *pbpA*$^+$, whereas the two phage that failed to transduce SP13711(λ$^+$) also failed to transduce the other *pbpA* mutants to *pbpA*$^+$. This result confirms the assignment of the *pbpA6, pbpA45,* and *pbpA137* mutations into a single gene. Similarly, the isolation of the λ d*pbpA* phage that transduced all three of the *pbpA* mutants to *pbpA*$^+$, but did not transduce the *rodA* mutant (SP5211) to *rodA*$^+$, confirmed that *pbpA* and *rodA* are separate genes.

Restriction mapping of the *lip-leuS* region. DNA prepared from λ *c*I857 *S*am7, λ d*lip*5, λ d*dacA*51, λ d*rodA*1, and λ d*pbpA*108 was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Sal*I, *Sst*I, *Xba*I, and *Xho*I, and the resulting fragments were fractionated on agarose gels. In each transducing phage, the λ DNA fragments that derive from the right of the attachment site (*att*) were present, but some, or all, of those to the left of *att* were absent, indicating that the chromosomal DNA in the transducing phage had replaced DNA from *att* into the phage left arm (see Fig. 1).

Since all of the transducing phage appeared to have been formed by the aberrant excision of λ *c*I857 *S*am7 from the same secondary attachment site, but differed in the extent of the chromosomal replacement in the phage left arm, it was possible to order the cleavage sites for the restriction endonucleases in the chromosomal DNA of the phage by comparing the fragments produced by the digestion of each phage DNA. The order of cleavage sites was confirmed by digestion with combinations of the restriction enzymes, and the sites established for the enzymes *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Sal*I are shown in Fig. 1. No cleavage sites for the enzymes *Sst*I, *Xba*I, or *Xho*I were found in the *lip-leuS* region.

The distribution of targets for restriction endonucleases in λ d*lip*5 indicated that plaque-forming phage carrying genes from the *lip-leuS* region could be obtained by cloning *Sal*I fragments or the 15-kilobase (kb) *Hin*dIII fragment of the chromosomal DNA of the phage into appropriate vectors.

Construction of plaque-forming phage carrying genes from the *lip-leuS* region. The 15-kb *Hin*dIII fragment from the chromosomal DNA of λ d*lip*5 was cloned into the replacement vector λ*L*41 (Fig. 2A) as described in Materials and Methods. The resulting *att*$^-$ *int*$^-$ *spi*$^-$, *c*I$^-$ *imm*^{434} phage, λpBS10 (Fig. 2B), transduced the *rodA, pbpA,* and *leuS* genes but did not transduce the *lip* gene. λpBS10 also carried the *dacA* gene, since PBP5 (the *dacA* gene product) was synthesized after infection of UV-irradiated *E. coli* with this phage (see below). Digestion of λpBS10 DNA with *Hin*dIII established that the middle *Hin*dIII fragment of λ *L*41 (Fig. 2A) had been replaced with the 15-kb *Hin*dIII fragment from λ d*lip*5 (Fig. 2B and 3). The 15-kb chromosomal DNA fragment in λ pBS10 contained an asymmetric *Bam*HI site, and digestion of λpBS10 DNA with this enzyme was used to show that the orientation of the *dacA-leuS* genes in λpBS10 was the reverse of that in λ d*lip*5.

*Sal*I fragments of λ d*lip*5 DNA were cloned into the vector λNM627 (Fig. 2C). Plaque-forming phage that transduced both the *rodA* and *pbpA* genes were isolated, but we were not able to isolate phage that transduced *lip* or *leuS* with this method. One of the plaque-forming phages that transduced *rodA* and *pbpA* (λpBS99) was studied further. λpBS99 did not carry the *dacA* gene since PBP5 was not synthesized after infection of UV-irradiated *E. coli* with the phage (see below). Digestion of λpBS99 DNA with *Sal*I showed that the region between the *Sal*I targets in λNM627 had been replaced with the 7.1-kb *Sal*I fragment from the chromosomal DNA of λ d*lip*5 (Fig. 2D and 3). In addition, the right arm of λpBS99 was larger than that in the vector, λNM267, as the *nin*5 deletion in the vector had been lost by in vivo recombination with λ$^+$ during the isolation of this phage (see Materials and Methods).

The 7.1-kb *Sal*I fragment of chromosomal DNA in λpBS99 contained an asymmetric *Kpn*I site, and digestion of the phage DNA with a combination of *Kpn*I and *Eco*RI established that the orientation of the *rodA* and *pbpA* genes in λpBS99 was the same as that in λ p*BS*10 and the reverse of that in λ d*lip*5 (Fig. 2D).

λpBS99 was *red*$^-$ *gam*$^-$ owing to the loss of the 0.49-kb region between the *Sal*I targets of the vector phage (42), and since the phage lacked a *chi* site, it grew extremely poorly (40). A phage cross between λpBS10 and λpBS99 (Fig. 2B,D) allowed the construction of derivatives of λ pBS99 that had gained the *chiA*131 mutation

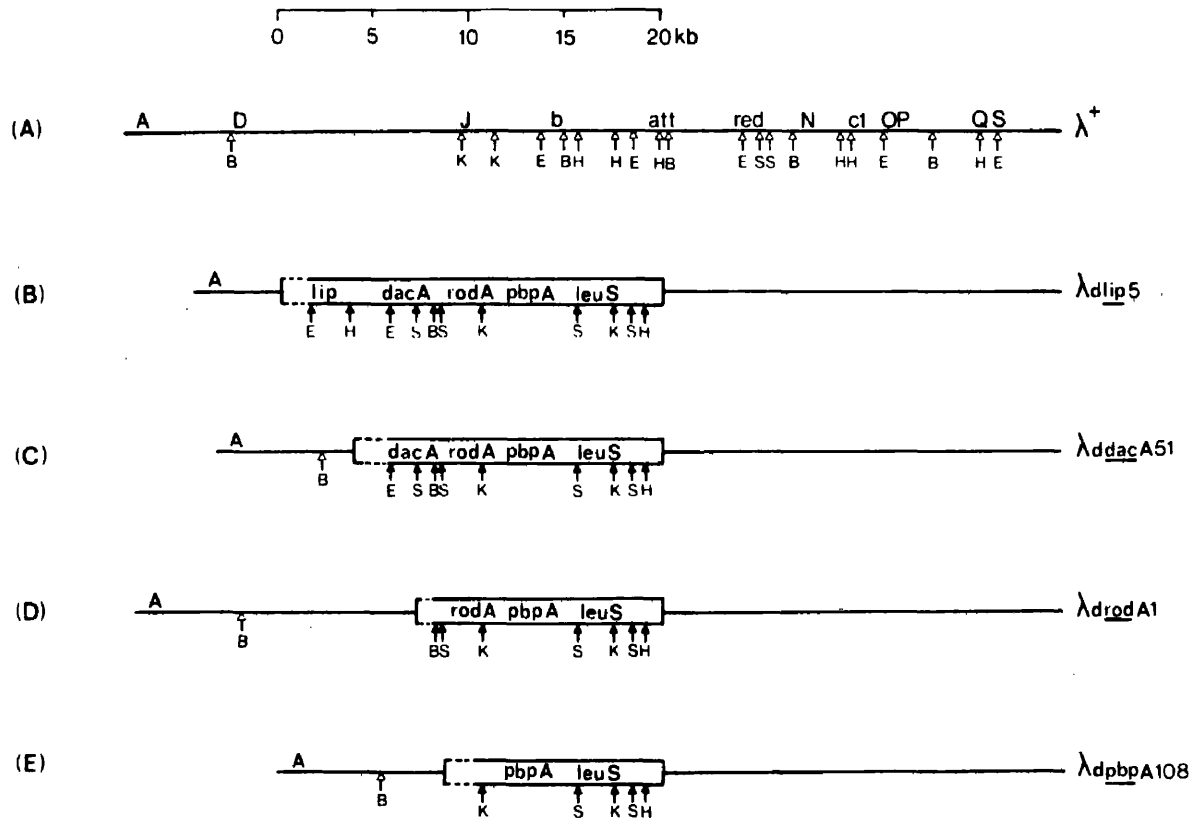

FIG. 1. *Structure of defective transducing phage carrying genes from the lip-leuS region. (A) λ+, (B) λ dlip5, (C) λ ddacA51, (D) λ drodA1, (E) λ dpbpA108. (△) Targets for restriction endonucleases in λ DNA. (▲) Targets in the chromosomal DNA: B, BamHI; E, EcoRI; H, HindIII; K, KpnI; S, SalI. Targets in the right arm of the transducing phage are not shown since they are identical to those in λ+. The phage right arm and the remaining parts of the left arm are shown by single lines. The chromosomal DNA extending from att into the phage left arm is shown within a box. The precise junction points between the left end of the chromosomal DNA and the remainder of the lambda left arm are unknown and are indicated approximately by the broken lines. The positioning of the known genes in relation to the restriction enzyme targets is approximate.*

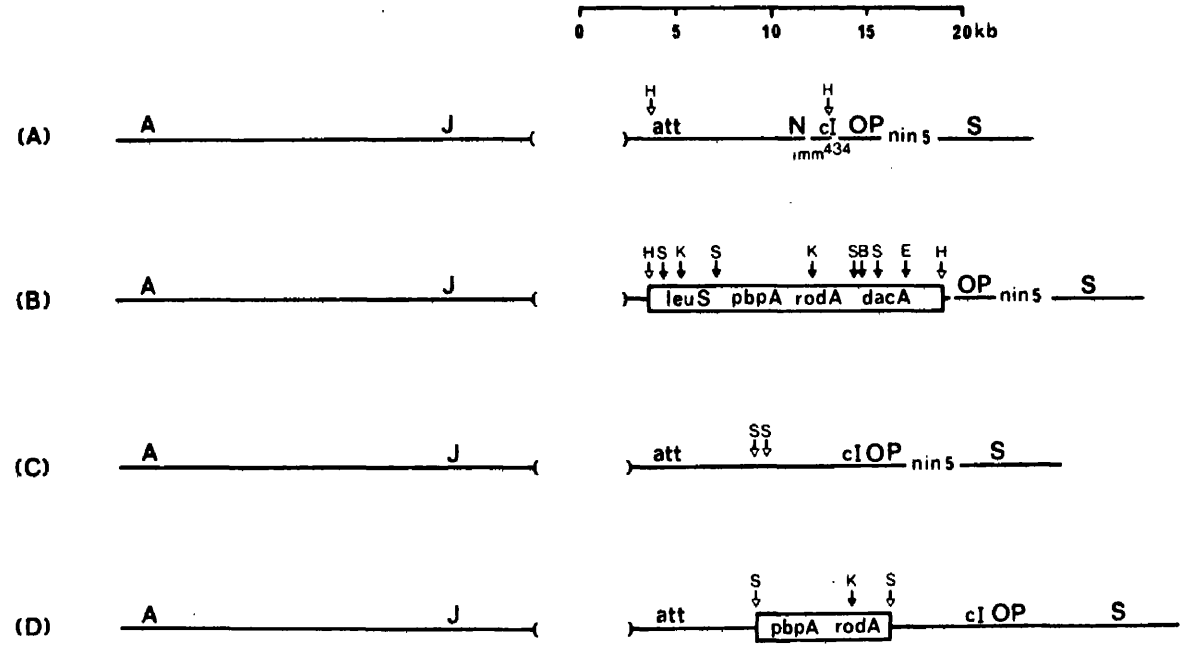

FIG. 2. *Structure of plaque-forming phage carrying genes from the lip-leuS region. (A) λ L41, (B) λ pBS10, (C) λ NM627, (D) λ pBS99. The targets for restriction endonucleases are represented as in Fig. 1. The targets in the lambda DNA of the transducing phage are not shown. The space between brackets in the left arm of the phage represents the deletion of DNA between EcoRI sites 1 and 2 of lambda. Full genotypes of λ L41 and λ NM627 are given in Materials and Methods.*

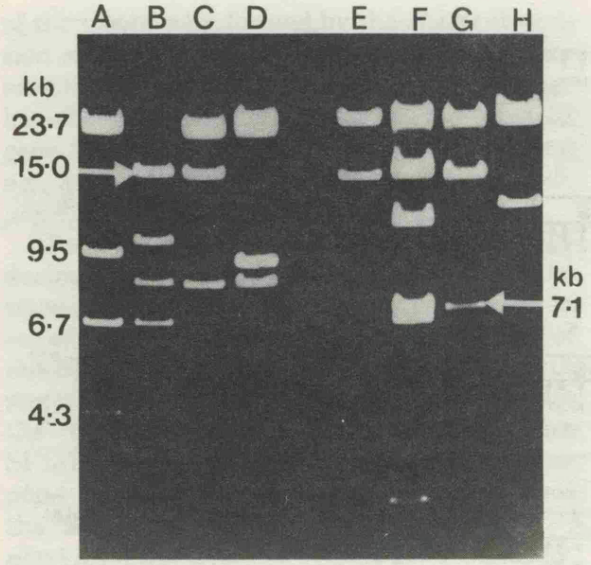

FIG. 3. *Electrophoretic analysis of restriction enzyme digests of DNA from plaque-forming λ transducing phage. DNA from λ cI857 Sam7 (A), λ dlip5 (B), λ pBS10 (C), and λ L41 (D) was digested with HindIII; DNA from λ cI857 Sam7 (E), λ dlip5 (F), λ pBS99 (G), and λ NM627 (H) was digested with SalI, and the fragments were separated on a 0.5% agarose gel. The arrows indicate the 15-kb HindIII and 7.1-kb SalI chromosomal DNA fragments inserted in the plaque-forming phage λ pBS10 and λ pBS99, respectively. DNA fragments below 4 kb are not visible in the photograph. The largest fragment in track (F) is from contaminating λ cI857 Sam7 helper phage present in this preparation of λ dlip5 DNA.*

from λ p*BS*10 (selection for *spi*⁻ *imm* λ *c*I857 recombinants). These derivatives (e.g., λ pBS104 = *chiA*131 Δ(*srl*λ1-2) *pbpA-rodA* Δ(*red-gam*) *c*I857 *S*am7) were fully *spi*⁻ and grew well. Since the chromosomal genes in λpBS10 and λpBS99 were in the same orientation, recombination between the chromosomal genes resulted in the formation of a new class of plaque-forming phage that transduced *leuS* in addition of *pbpA* and *rodA* (e.g., λpBS102 = *chiA*131 Δ(*srl*λ1-2) *leuS pbpA rodA* Δ(*att-int-red-gam*) *c*I857 *S*am7).

Proteins synthesized from the *lip-leuS* region. Infection of UV-irradiated *E. coli* 159 (λ *ind*⁻) with λ d*lip*5 resulted in the synthesis of 11 proteins (Fig. 4). Two of these proteins (molecular weights of 27,000 and 29,000) were also produced after infection with λ *c*I857 *S*am7 and were the products of the phage *c*I and *rex* genes, which are the only phage genes expressed in the presence of lambda repressor (42). The other nine proteins were not synthesized in uninfected control cells (Fig. 4A) or in cells infected with λ *c*I857 *S*am7, and were the products of the bacterial genes in λ d*lip*5.

Table 3 lists the proteins synthesized from λ d*lip*5 and from other phage carrying genes from the *lip-leuS* region of the chromosome. Infection experiments with each of the transducing phage were also carried out with a UV-irradiated nonlysogen of *E. coli* 159 since, in the absence of lambda repressor, chromosomal genes that have very weak promoters, or have lost their promoters on insertion into transducing phage, can be expressed from phage promoters. With all of the transducing phage, the only proteins synthesized in the nonlysogen that were not also synthesized in the lysogen were identified as lambda proteins by comparison with those produced in a parallel infection with λ *c*I857 *S*am7 (data not shown).

Comparison of the genes known to be on each of the transducing phage with the proteins synthesized in infection experiments, together with a knowledge of the molecular weights and properties of some of the gene products, has allowed the identification of some of the proteins synthesized by the transducing phage.

The protein of 105,000 molecular weight (105K protein) was identified as the *leuS* product (leucyl tRNA synthetase) since it was cytoplasmic, had the previously reported molecular weight (8), and was synthesized only in infection

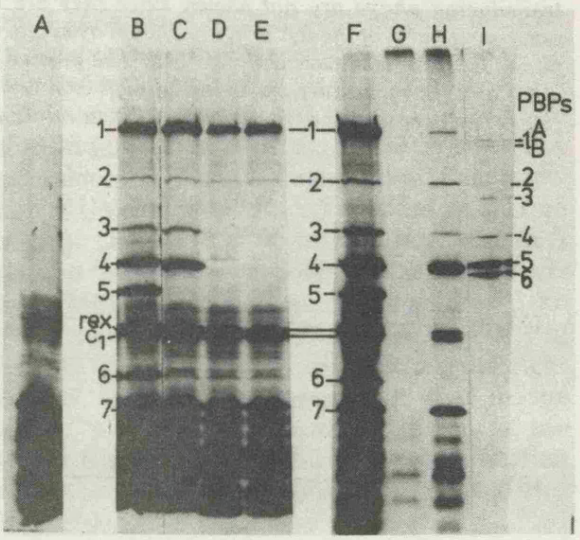

FIG. 4. *SDS-polyacrylamide gel electrophoresis of proteins synthesized from λ transducing phage. Cells of E. coli 159 (λ ind⁻) were UV irradiated, and the proteins synthesized after infection with λ transducing phage were labeled with [³⁵S]methionine. The total cell proteins (A–F) or cell envelope fractions (G, H) were fractionated on a 12% SDS-polyacrylamide gel and fluorographed. (A, G) controls (uninfected); (B, F, H) λ dlip5; (C) λ ddacA51; (D) λ drodA1; (E) λ dpbpA108. (I) PBPs of E. coli KN126 labeled with [¹⁴C]benzylpenicillin. The molecular weights of the seven proteins marked in this photograph and of two additional low-molecular-weight proteins, which are only detected on 16% SDS-polyacrylamide gels, are shown in Table 3.*

TABLE 3. *Proteins synthesized from λ transducing phage carrying genes from the lip-leuS region*

Protein	Mol wt	Synthesis of protein on infection of UV-irradiated *E. coli* with:						
		λ⁺	λ d*lip*5	λ d*dacA*51	λ d*rodA*1	λ d*pbpA*108	λ pBS10	λ pBS99
1	105,000	−	+	+	+	+	+	−
2	66,000	−	+	+	+	+	+	+
3	54,000	−	+	+	−	−	+	−
4	41,000	−	+	+	−	−	+	−
5	35,000	−	+	−	−	−	−	−
rex product	29,000	+	+	+	+	+	−[a]	+
cI product	27,000	+	+	+	+	+	−[a]	+
6	20,000	−	+	−	−	−	+	−
7	15,000	−	+	+	+	+	+	−
8	12,000	−	+	+	+	+	+	+
9	11,000	−	+	−	−	−	+	−

[a] *cI* and *rex* genes were deleted in λ pBS10.

experiments with phage that transduced the *leuS* gene. The *pbpA* gene product (PBP2) was identified as the 66K protein, since it was synthesized by all of the phage that transduced *pbpA*, was, as expected, located in the cell envelope (Fig. 4H), and had precisely the same mobility on SDS-polyacrylamide gels as PBP2 detected by the PBP assay using [^{14}C]benzylpenicillin (Fig. 4I).

The *dacA* gene, which is reported to be 87% cotransducible with *leuS* (41), was found to be in the *lip-leuS* region carried by λ d*lip*5, since induction of this phage from a lysogen resulted in massive overproduction of PBP5, the *dacA* gene product (see below), and because strains that overproduce PBP5 have been obtained by cloning fragments of λ d*lip*5 DNA into the multicopy plasmid pBR325 (B. G. Spratt, unpublished experiments).

The 41K protein synthesized from λ d*lip*5 had almost the same mobility on SDS-gels as PBP5 detected by the PBP assay and, as expected, was located in the cell envelope (Fig. 4H). The mobility of the 41K protein was, however, consistently slightly faster than that of PBP5. There is, however, little doubt that the 41K protein is identical with PBP5, and the slight difference in mobility on SDS-polyacrylamide gels may indicate that posttranslational modification of PBP5 occurs in vivo, but not in the UV-irradiated cell system.

No information about the *lip* gene product appears to have been published. The *lip* gene was transduced by λ d*lip*5 but not by any of the other phage, and the *lip* gene product should therefore be a protein synthesized after infection of UV-irradiated *E. coli* with the former but not with the latter phage. The 35K protein (Table 3) was the only protein synthesized exclusively by λ d*lip*5, and this cytoplasmic protein was tentatively assigned as the *lip* gene product. Further experiments will be necessary to confirm this assignment.

The *rodA* gene product should be a protein synthesized by λ d*rodA*1 but not by λ d*pbpA*108. However, no differences were found in the proteins synthesized by these phage.

The genes corresponding to the 54K, 20K, 15K, 12K, and 11K proteins have yet to be identified.

Figure 5 shows the approximate alignment of the proteins synthesized from the *lip-leuS* region with the known genes and restriction enzyme targets. The sizes of the genes required to code for the known proteins are indicated, based on the assumption that 1 kb of DNA can code for a 35K protein. The total molecular weight of the proteins synthesized from the *lip-leuS* region in λ d*lip*5 was 359,000, which would require a coding capacity of 10.2 kb. The size of the chromosomal DNA in λ d*lip*5 is about 18 kb, and the known proteins therefore account for 57% of the coding capacity.

Overproduction of PBP2 and PBP5 during λ d*lip*5 development. High levels of expression of genes cloned in λ transducing

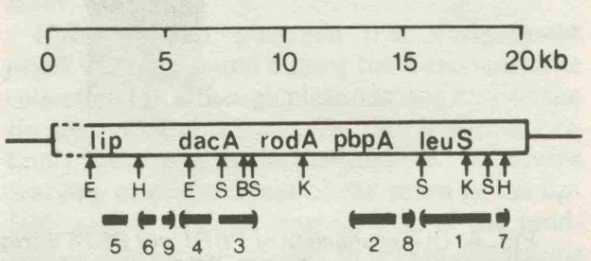

FIG. 5. *Alignment of the genes, gene products, and restriction enzyme targets in the lip-leuS region. The proteins synthesized from the lip-leuS region of the chromosome are numbered according to Table 3 and Fig. 4. The boxes above the numbers represent the estimated size of the genes required to code for each protein. The order of pairs of proteins between brackets is undetermined.*

phage have been achieved by allowing phage DNA replication to occur but preventing the packaging of the DNA into phage particles by an amber mutation in the λ *Q* gene (25).

Figure 6A shows the level of the PBPs in W3110 *lip-9* (λ d*lip*5 *c*I857 *Q*am73 *S*am7) grown at 30°C and in the same strain 2 h after induction of the phage from the lysogen (Fig. 6B). The overproduction of PBP2 and PBP5 was difficult to estimate accurately since their levels were so different in induced and uninduced cells, but the level was about 50-fold for both PBPs. (Exposure of the gel to X-ray film for a short period showed that the large amount of radioactivity at the position of PBP5/6 was due to overproduction of PBP5 rather than PBP6.) PBP4′ was prominent in induced cell envelopes; this PBP is a (proteolytic?) product of PBP2 (33). The level of some of the other PBPs decreased after phage induction (e.g., PBP1B and PBP4), and this probably reflects a decreased rate of transcription of their genes during λ development.

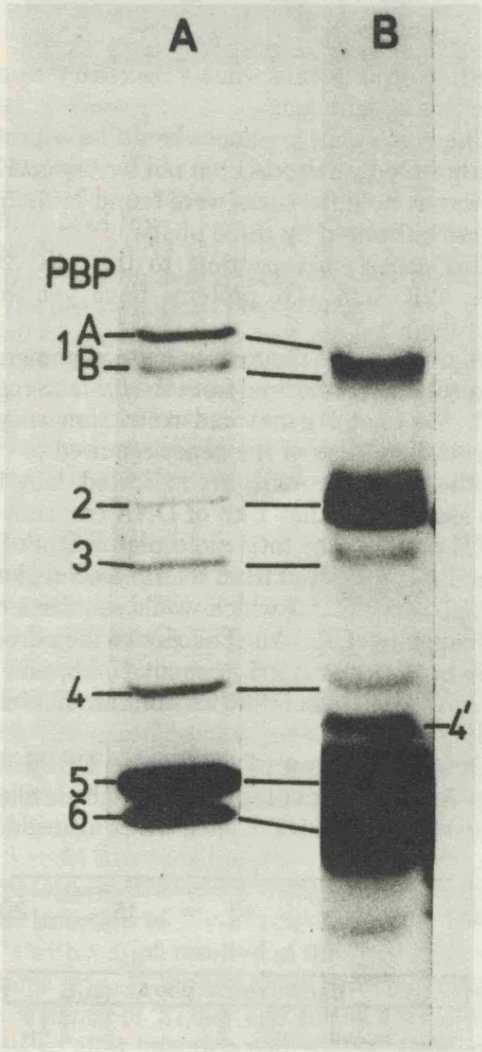

FIG. 6. *Overproduction of PBP2 and PBP5 during λ dlip5 development. E. coli W3110 lip-9 (λ dlip5 cI857 Qam73 Sam7) was grown at 30°C in Luria broth containing glucose (0.5%) to an absorbancy at 550 nm of 0.2; the culture was shifted to 42°C for 10 min, to induce phage development, and was grown for a further 2 h at 37°C. Cell envelopes were prepared, and the levels of PBPs were determined in cells grown at 30°C (A) or after phage induction (B). PBP4′ is believed to be proteolytic product of PBP2 (33).*

DISCUSSION

The mutations *pbpA6, pbpA45, pbpA137,* and *rodA52* all mapped very close to *lip* and resulted in the growth of the bacteria as large spherical cells under the restrictive conditions. Complementation analysis established that the *pbpA6, pbpA45,* and *pbpA137* mutations were in the same cell shape gene. This is consistent with the finding that all of these mutations caused alterations of PBP2. Since the latter two mutations resulted in a decreased thermostability of PBP2 (36, 37), the *pbpA* gene is the structural gene for this protein. The mutation *rodA52* had no detectable effect on the activity or synthesis of PBP2, and complementation analysis established that it defined a second cell shape gene close to *lip* and *pbpA*. The allocation of cell shape mutants that mapped near *lip* into two classes was supported by the isolation of specialized λ transducing phage that transduced all of the *pbpA* mutants, but not the *rodA52* mutant, to normal cell shape. S. Tamaki, H. Matsuzawa, and M. Matsuhashi (personal communication) have shown independently, by complementation analysis, the existence of two cell shape genes close to *lip*.

Several cell shape mutations that map close to *lip* have been reported previously (9, 11, 12, 21, 32). The temperature-sensitive mutant (*lss*-12) of Henning et al. (9) appears to be a *pbpA* mutant, since it produces a highly thermolabile PBP2 (37). The mutant of Matsuzawa et al. (21) appears to be a *rodA* mutant since it has a normal PBP2 and complements *pbpA* mutants (Tamaki et al., personal communication). Other cell shape mutations that map close to *lip* could be classified as *pbpA* or *rodA* on the basis of the activity of PBP2 or, preferably, by complementation analysis or transductional tests with λ d*rodA* and λ d*pbpA* phages. It is, of course, possible that further cell shape genes map close to *lip*.

The determination of the gene order as *lac---lip-dacA-rodA-pbpA-leuS---gal* raises the possibility that *dacA, rodA,* and *pbpA*, which are presumably all involved in the final stages of peptidoglycan synthesis, are contiguous genes. Certainly the three genes are within a 12-kb region, but further detailed physical studies are needed to settle this point. None of the other

PBP genes maps in, or close to, the *lip-leuS* region (41). The gene for PBP6 has not yet been located, but it is not in the *lip-leuS* region, since PBP6 was not synthesized by λ d*lip*5 phage. Furthermore, a 6.2-kb *Eco*RI fragment containing the PBP6 gene has been cloned into the plasmid pSF2124, and the restriction map around the PBP6 gene showed no similarities with that of the *lip-leuS* region (B. G. Spratt and R. Diaz, unpublished data).

The close linkage of the *dacA, rodA,* and *pbpA* genes is intriguing, and represents a cluster of genes involved in peptidoglycan synthesis and cell shape determination. Several of the unascribed proteins that were synthesized from the region between *lip* and *leuS* were located in the cytoplasmic membrane (unpublished experiments), and some of these may be the products of additional cell shape-peptidoglycan synthesis genes in the *dacA-rodA-pbpA* cluster. Although none of the other PBP genes has been shown to be clustered, those for the early steps in peptidoglycan synthesis are clustered in two groups at 2 and 89 min on the genetic map (24, 44). The group at 2 min appears to be a cell division-peptidoglycan synthesis cluster, in many ways analogous to the cell shape-peptidoglycan synthesis cluster described in this paper (5, 44). It includes the gene for PBP3 (a cell divison protein; 35), at least two further cell division genes, the genes *sulB* and *envA*, and genes for some of the soluble steps in peptidoglycan synthesis (5, 17, 44).

The products of the *leuS, pbpA,* and *dacA* genes, and the probable *lip* gene product, were identified among the nine proteins that were synthesized from the chromosomal genes in λ d*lip*5. The *rodA* gene product has not yet been identified. The 7.1-kb *Sal*I fragment, which contained the *rodA* and *pbpA* genes, programmed the synthesis of only two proteins in the UV-irradiated cell system. One of these was the *pbpA* gene product (PBP2) and the other, the 12K protein, was unlikely to be the *rodA* gene product, since it was synthesized by λ d*pbpA*108, which did not transduce *rodA*. The proteins synthesized from the 7.1-kb *Sal*I fragment accounted for only 32% of its coding capacity and there may be several proteins, including the *rodA* gene product, which for some reason are not expressed in the UV-irradiated cell system. Alternatively, the *rodA* gene product may lack methionine, or the gene may be part of a transcriptional unit that is expressed from a promoter some distance to the left of the *rodA* gene. In this case, the gene product could be the 54K protein or one of the proteins that appeared to be synthesized from a region to the left of *rodA*. These possibilities are being investigated.

The *dacA* gene product has been identified as PBP5, which is known to catalyze a D-alanine carboxypeptidase 1 reaction in vitro; its function in peptidoglycan synthesis in vivo is obscure, since mutants that lack the activity of the enzyme grow without obvious growth defects (19, 20). This appears to suggest that PBP5 has a minor role in peptidoglycan synthesis, but the possibility that these mutants have significant levels of enzyme activity in vivo cannot be eliminated. The availability of *E. coli* strains with *Tn10* inserted close to *dacA* should allow (13) the isolation of *E. coli* strains with complete deletions of the *dacA* gene if the gene product is nonessential for peptidoglycan synthesis. Derivatives of this kind are being sought.

The enzymatic, or regulatory, function of PBP2 is unknown. The number of molecules of this important protein per bacterial cell has been estimated to be about 10 to 20 (34), and its purification from the cytoplasmic membrane in substantial amounts is difficult. The considerable level of amplification of PBP2 that has been achieved after induction of λ d*lip*5 *c*I857 *Q*am73 *S*am7 from a lysogen will be extremely useful for purification purposes. This phage also greatly overproduce PBP5, but derivatives of λ p*BS*99, which overproduce PBP2, but lack the PBP5 (*dacA*) gene, have been constructed. The expression of PBP2 from these phage will be described elsewhere.

The purification of PBPs from the cytoplasmic membrane, where presumably they are intimately associated with the other enzymes catalyzing the membrane-bound steps of peptidoglycan synthesis, is likely to have drastic effects on their activity. Overproduction of PBP2 in mutants that lack the activity of the major *E. coli* penicillin-sensitive enzymes should allow the detection of any mecillinam-sensitive activities catalyzed by PBP2 without the need for purification from its normal membrane environment.

Hybrid ColE1 plasmids that complement *pbpA* were not found among the Carbon-Clarke collection (4), although plasmids that carried the *lip* gene were obtained (27). This is almost certainly because of the instability of *E. coli* strains carrying multiple copies of the genes in the *lip-leuS* region, since overproduction of the products of these genes results in severe distortions of bacterial cell shape (unpublished data).

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Versatile low-copy-number plasmid vectors for cloning in *Escherichia coli*

(Recombinant DNA; pSC101, pSC105; pLG338, pLG339; tetracycline, kanamycin resistance; phage λ p_L promoter)

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SUMMARY

Small low-copy-number plasmid vectors were constructed by in vitro and in vivo recombinant DNA techniques. pLG338 and pLG339 are derived from pSC105, have a copy number of six to eight per chromosome, and carry genes conferring resistance to tetracycline and kanamycin. pLG338 (7.3 kb) has unique restriction endonuclease sites for *Bam*HI, *Sal*I, *Hin*cII, *Sma*I, *Xho*I, *Eco*RI and *Kpn*I, the first five lying within a drug resistance gene. pLG339 (6.2 kb) lacks the *Kpn*I site, but has unique *Sph*I and *Pvu*II sites. These versatile vectors should be useful for cloning many genes coding for membrane and regulatory proteins which cannot be cloned into high-copy-number plasmids.

INTRODUCTION

Multicopy plasmids are the most commonly used vectors for cloning in *Escherichia coli*. Most of these vectors are based on the replicons of ColE1, pMB1 or P15A, have copy numbers ranging from 18 to 60 per chromosome and can be amplified in the absence of protein synthesis. High-copy-number vectors aid the isolation of large amounts of plasmid DNA and also help in the purification of proteins coded by cloned genes.

There are, however, occasions when a high copy number is unsuitable. Several examples have been reported of genes which have not been successfully cloned onto a high-copy-number vector, but which have been cloned into bacteriophage λ, or a plasmid with a low copy number. These include *polA* (Murray and Kelley, 1979), *dnaA* (Hansen and von Meyenburg, 1979), *ompA* (Beck and Bremer, 1980) and *dacA* (Spratt et al., 1980; and unpublished results). It seems likely that multiple copies of many genes coding for regulatory or membrane proteins will be deleterious to the cell. Furthermore, attempting to clone these genes onto a multicopy plasmid may result in the cloning of a mutant gene.

Abbreviations: Ap^R, resistance to ampicillin; Cm^R, resistance to chloramphenicol; DTT, dithiothreitol; e.o.m., efficiency of mobilization; kb, kilobase; Km^R, resistance to kanamycin; Tc^R, resistance to tetracycline; Tc^S, sensitivity to tetracycline; [ ], indicates plasmid-carrier state.

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Bacteriophage λ is an ideal low-copy-number vector in many respects, as it integrates into the host chromosome, and is replicated with it. However, for most techniques of in vitro gene manipulation, plasmid vectors are more suitable. Several low-copy-number plasmid vectors have been constructed (Kahn et al., 1980). These include derivatives of F and R6, which have a copy number of about one per chromosome, and of RK2, which have a copy number of about eight per chromosome. These vectors tend to be rather large, and in most cases have only one drug-resistance gene and few unique restriction sites.

pSC101 (Cohen et al., 1973; Fig. 1A), which has a copy number of about six per chromosome, is a good compromise between vectors based on F, which are rather hard to isolate in large amounts, and multicopy plasmids. Although pSC101 was the first plasmid to be used for in vitro cloning, it is now little used because it only carries a tetra-

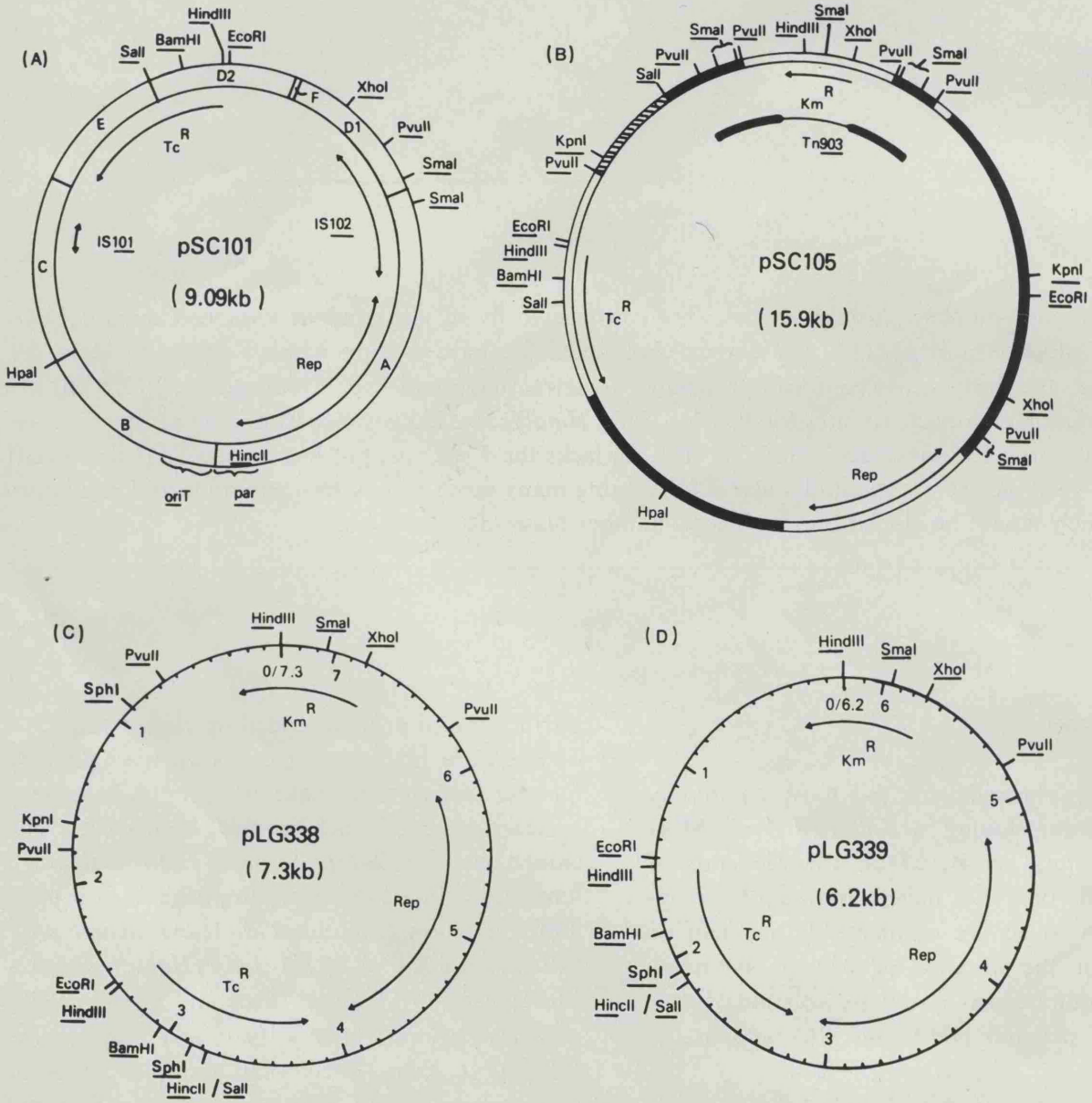

Fig. 1. Plasmids employed in the present studies. (A) Diagram of pSC101, compiled from information derived from Nordheim et al. (1980), Meacock and Cohen (1980), Fischhoff et al. (1980), Bernardi and Bernardi (1981) and P.A. Meacock, personal communication. (B) Diagram of pSC105, compiled from information derived from Grindley and Joyce (1980) and our unpublished data. The thick black line represents the DNA deleted in the construction of pLG338. The hatched region has also been deleted in pLG339. (C) Restriction map of pLG338. (D) Restriction map of pLG339.

cycline resistance gene and therefore insertional inactivation cannot be used to screen for recombinant clones. Cohen et al. (1973) cloned an *Eco*RI fragment conferring kanamycin resistance from R6-5 into the pSC101 *Eco*RI site, producing pSC105 (Fig. 1B). This plasmid has two drug resistance genes, but only has two unique restriction enzyme sites, for *Bam*HI and *Hpa*I, and is approx. 16 kb.

In the course of our work we required a more convenient low-copy-number cloning vector, with unique *Eco*RI, *Sal*I and *Kpn*I sites, and we therefore deleted duplicated restriction sites and unwanted DNA from pSC105. pLG338 (Fig. 1C) is a 7.3-kb derivative which has unique *Bam*HI, *Sal*I, *Hin*cII, *Sma*I, *Xho*I, *Eco*RI and *Kpn*I sites, the first five of which lie within the Tc^R and Km^R genes. It can also be used for cloning *Pvu*II- or *Sph*I-generated fragments. pLG339 (Fig. 1D) is a 6.2-kb derivative which has lost the *Kpn*I site, but which has a unique *Sph*I site within the Tc^R gene, as well as a unique *Pvu*II site.

MATERIALS AND METHODS

(a) Bacterial and plasmid strains

Plasmids used were pSC101, pSC105, pBR328 and R64*drd*11. C600 (*thr leu thi supE tonA*) was used as host strain.

(b) Enzymes, chemicals and media

All restriction endonucleases were purchased from Bethesda Research Laboratories, except for *Sph*I which was kindly donated by Martin Ryan and *Bam*HI which was kindly donated by Jenny Varley. BAL31 nuclease was purchased from New England Biolabs, and DNA polymerase I Klenow fragment and calf intestinal alkaline phosphatase were purchased from Boehringer Mannheim. T4 DNA ligase was a gift from Barry Ely and Ron Wilson. [Methyl-^{3}H]thymine was purchased from the Radiochemical Centre, Amersham.

Media used were as described previously (Spratt et al., 1980). Antibiotics were added when appropriate: chloramphenicol (Sigma) to 25 μg/ml; tetracycline (Sigma) to 10 μg/ml; kanamycin sulphate (Sigma) to 25 μg/ml; sodium ampicillin (Sigma) to 25 μg/ml and streptomycin sulphate (Glaxo) to 25 or 200 μg/ml.

(c) DNA preparation, manipulation and analysis

Plasmid DNA was prepared as described by Stougaard and Molin (1981). It was digested with restriction endonucleases and analysed on agarose gels as described by Broome-Smith (1980). The buffers used were those recommended by the suppliers. DNA fragments were purified by agarose gel electrophoresis followed by electroelution in dialysis tubing or onto Whatman DE81 paper (Dretzen et al., 1981). 5′-extensions produced by restriction endonucleases were made flush by incubation with DNA polymerase I Klenow fragment in 10 mM Tris, 10 mM $MgCl_2$, 50 mM NaCl, 0.5 mM DTT pH 7.4 with 0.02 mM deoxynucleotides at 10°C for 90 min. Ligations were performed in 100 mM Tris · HCl pH 7.4, 100 mM $MgCl_2$, 10 mM EDTA, 0.5 μg/ml gelatin, 10 mM DTT at 12°C overnight. Plasmid size was screened using SDS-cleared lysates, prepared and analysed on agarose gels as described by Broome-Smith (1980).

(d) Determination of plasmid copy number, stability and mobilization efficiency

Plasmid copy number was estimated as described by Bazaral and Helinski (1968), except that cells were lysed using Brij 58–sodium deoxycholate. Plasmid stability was measured as described by Meacock and Cohen (1980). Efficiency of mobilization of plasmids was calculated as described by Broome-Smith (1980).

(e) Construction of plasmids

Plasmids were constructed according to the scheme shown in Fig. 2.

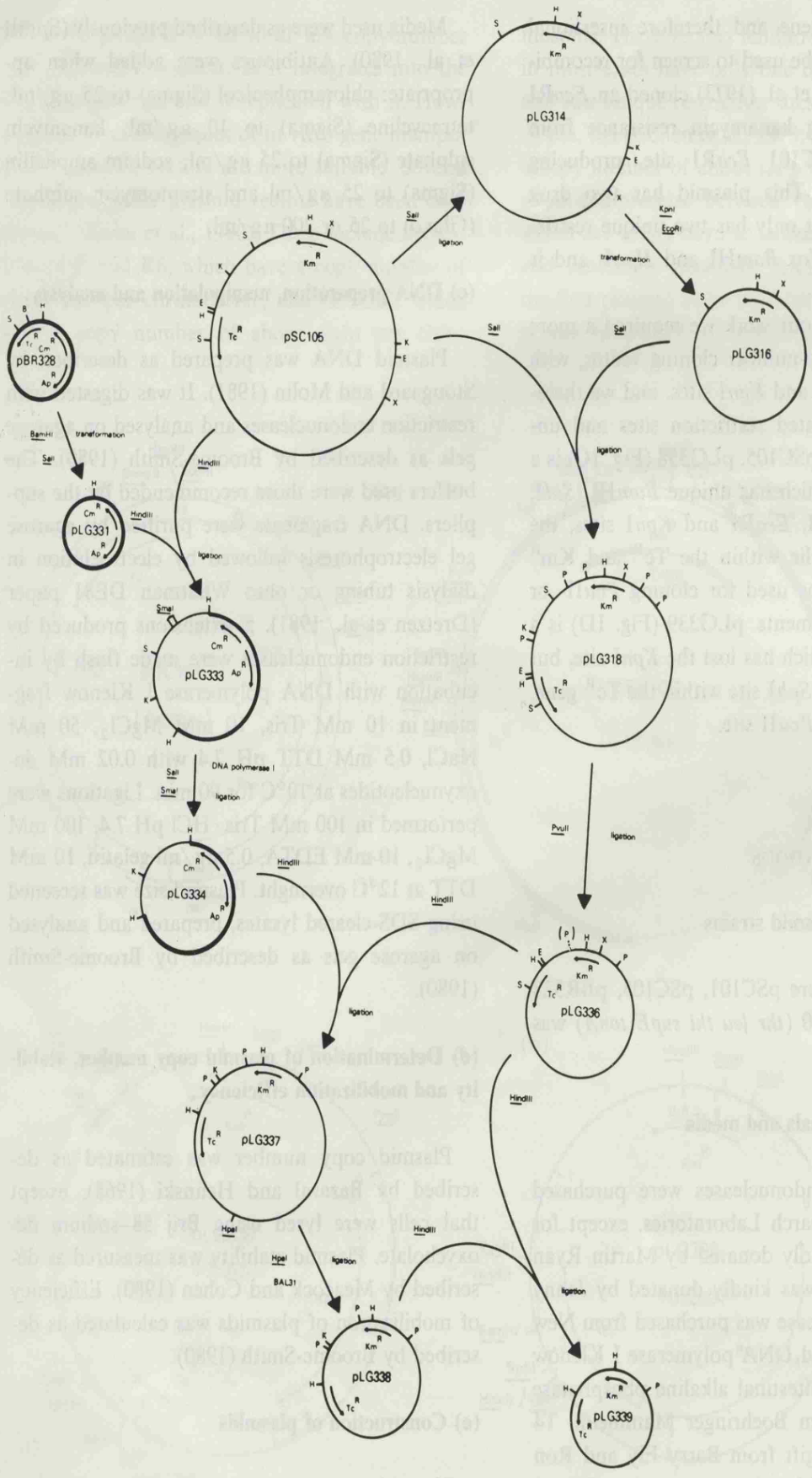

Fig. 2. Schematic diagram showing the construction of pLG338 and pLG339. Abbreviations used are H, *Hin*dIII; X, *Xho*I; S, *Sal*I; K, *Kpn*I; E, *Eco*RI; B, *Bam*HI; P, *Pvu*II. (P) indicates a *Pvu*II site lost during the construction of pLG336.

RESULTS

(a) Removal of duplicate *Eco*RI, *Kpn*I and *Xho*I sites from pSC105

We wished to retain the *Eco*RI and *Kpn*I sites near the promoter of the Tc^R gene in pSC105 (Fig. 1B). These sites lie conveniently within the 2.5-kb *Sal*I fragment. This fragment was therefore removed (pLG314) before deleting the remaining *Eco*RI and *Kpn*I sites by transforming C600 with DNA digested with these enzymes. Transformants appeared at a low frequency, and these carried deletions of varying sizes. pLG316, which carried the largest deletion, was 4.3 kb smaller than pLG314. The deletion extends towards the replication region, also removing the duplicate *Xho*I site, as well as a *Pvu*II site and two *Sma*I sites. The 2.5-kb *Sal*I fragment from pSC105 was then restored, producing pLG318.

(b) Removal of duplicate *Sal*I and *Sma*I sites

pLG318 has five *Sma*I sites. One lies within the Km^R gene, and the other four lie within the inverted repeats of Tn*903*, which lie on either side of the Km^R gene (Fig. 1B). These four sites were removed by digestion of pLG318 with *Pvu*II, followed by religation, producing pLG336. pLG336 has a single *Sma*I site, in the Km^R gene. It also has a single *Sal*I site, but has lost its *Kpn*I site. One of the *Pvu*II sites was lost during the construction of pLG336, presumably due to mild exonuclease activity in the restriction or ligation reactions.

The *Kpn*I site was now restored. For this we required a cloning vector which had a *Hin*dIII site, but no *Sal*I or *Sma*I sites. We constructed such a vector (pLG331) from pBR328, removing the *Sal*I site by in vivo deletion. The small *Hin*dIII fragment from pSC105 was cloned into pLG331, producing pLG333. The region between the *Sal*I and *Sma*I sites was removed and the resulting plasmid (pLG334) was not cut by either enzyme, but retained the *Kpn*I site. The modified *Hin*dIII fragment was now recloned into pLG336, replacing its small *Hin*dIII fragment. pLG337 carries Km^R and Tc^R genes, and has unique *Kpn*I, *Sal*I and *Sma*I sites in addition to those already present in pLG318.

(c) Removal of excess DNA

Because pLG337 is still rather large, we made it smaller by deleting the DNA between the end of the Tc^R gene and the origin of replication. There is a unique *Hpa*I site in the middle of this region. pLG337 was linearized with *Hpa*I, incubated with BAL31 nuclease and blunt-end ligated. pLG338 is 2.8 kb smaller than pLG337. This deletion removed three of the four *Hin*cII sites in pLG337, leaving only the *Hin*cII (*Sal*I) site within the Tc^R gene.

A smaller vector, pLG339, was made by replacing the small *Hin*dIII fragment in pLG338 with that from pLG336. pLG339 has no *Kpn*I site, but has the advantage of a unique *Sph*I site within the Tc^R gene, as well as a unique *Pvu*II site.

(d) Mobilization, stability and copy number of pLG338

The origin of transfer of pSC101 has been mapped by Nordheim et al. (1980) to the *Hin*cII B fragment (Fig. 1A). The deletion extending from the *Hpa*I site of pLG337 completely removes this fragment, including, presumably, *oriT*. pSC101 is mobilized by the conjugative plasmid R64*drd*11, and loss of *oriT* should drastically reduce this mobilization. We compared the e.o.m. of pSC105, pLG337, and pLG338 by R64*drd*11. pSC105 and pLG337 had an e.o.m. of 2.0×10^{-2}, while pLG338 had an e.o.m. of 4.4×10^{-5}.

Meacock and Cohen (1980) described a small region of DNA designated *par*, close to the end of the *Hin*cII A fragment which is required for stable partitioning of the plasmid (Fig. 1A). The growth of C600[pLG338] for 100 generations in non-selective medium did not lead to any loss of pLG338 in 50 colonies tested, which suggests that the *par* region is still present.

Finally, we compared the copy number of pLG338 with that of pSC101, obtaining an estimate of 7.5 copies of pLG338 per genome, and 5 copies of pSC101 per genome.

DISCUSSION

We have constructed versatile low-copy-number plasmids from pSC105. pLG338 has unique sites

for *Sal*I, *Hin*cII; *Bam*HI, *Xho*I, *Sma*I, *Eco*RI and *Kpn*I. The first five sites lie within drug-resistance genes. It can also be used to clone *Pvu*II- and *Sph*I-generated fragments. pLG339 is a smaller derivative which has lost the *Kpn*I site, but has in addition a unique *Sph*I site within the Tc^R gene and also a unique *Pvu*II site.

The plasmids are suitable for the cloning of many fragments resulting from double digests. There are three unique sites for enzymes which produce flush-ended cuts, two of which lie within drug-resistance genes. The *Bam*HI site is particularly useful, as it may also be used for cloning fragments generated by *Bgl*II, *Bcl*I, *Xho*II and *Sau*3A. The *Kpn*I and *Sph*I sites provide 3′-extensions suitable for cloning by 3′-tailing with terminal transferase.

pSC105 contains several transposable elements. IS*101* and IS*102* are IS elements that lie within pSC101 (Fischhoff et al., 1980; Bernardi and Bernardi, 1981; Fig. 1A), and the Km^R gene, which is originally derived from R6, is part of the transposon Tn*903* (Fig. 1B). All of these elements have been deleted in pLG338 and pLG339. pLG338 and pLG339 have a much lower efficiency of mobilization than pLG337 due, we presume, to the deletion of the origin of transfer. The stability and copy number of the plasmids do not differ significantly from pSC101. The slightly lower copy number estimate obtained for pSC101 can be attributed to the formation of relaxation complexes.

pLG338 is suitable for mutagenesis with the transposable element γδ (J. Broome-Smith and B.G. Spratt, unpublished results).

We have used derivatives of pSC105 to successfully subclone the *dacA* gene, which codes for penicillin-binding protein 5, from the λ transducing phage λd*lip*5 (Spratt et al., 1980 and unpublished results), but have not been able to clone this gene into pBR322. pLG338 and pLG339 will be useful for cloning other genes coding for membrane proteins or those involved in key regulatory events in the cell cycle, where multiple gene copies may be toxic to the cell. Since these vectors are as versatile as pBR322, it may be wise to clone initially into a low copy number plasmid when working with genes of this type.

pLG338 and pLG339 provide a measure of containment, since they lack an origin of transfer, and have a narrow host range. This might be useful for the cloning of any genes from eukaryotic organisms or prokaryote pathogens which cannot be cloned into multicopy vectors.

We have developed conditional expression derivatives of pLG338 and pLG339 by cloning in the λ p_L promoter, together with the thermolabile repressor gene, *c*I857. These plasmids allow overproduction of proteins from genes which must be kept at a low copy number (P. Holt and B.G. Spratt, unpublished results).

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