A GENETIC ANALYSIS OF THE SECRETION OF β-LACTAMASE

by

Douglas Elliott Koshland

B.A. Haverford College
(1976)

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January, 1982

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To my parents,

two very special people

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Douglas Elliott Koshland

Submitted to the Department of Biology
on January 6, 1982 in partial fulfillment of the
requirements for the Degree of Doctor of Philosophy in
Microbiology

ABSTRACT

TEM β-lactamase is a secreted protein of gram negative bacteria that confers upon these organisms resistance to high levels of ampicillin. Over 250 point mutations in the structural gene for this enzyme were isolated by *in vivo* and *in vitro* methods. Among these point mutations, chain terminating, frameshift and temperature sensitive alleles were identified. By crossing these point mutations with deletions of *bla* (five deletions previously existed and six deletions were isolated in this work) the gene was divided into 12 intervals. Fine structure genetic mapping was accomplished by two factor crosses between different *bla* alleles. We document that these crosses can resolve mutations that are separated by as few as two base pairs. The results from two factor crosses between mutations within the same deletion interval also indicate that *bla* has mutagenic hot spots for certain *in vivo* mutagens. The genetic map of *bla* was correlated with a physical map of the gene from the knowledge of the nucleotide sequences of certain point mutations and deletions. In addition the physical locations in *bla* of certain chain terminating alleles were identified from the knowledge of the nucleotide sequence of the gene, the specificity of the mutagen used to induce the mutations and the nature of the chain terminating allele (amber, ochre or frameshift).

The correlation between the physical and genetic maps revealed that only a few of the mutations isolated by classical methods of mutagenesis affect the signal sequence of β-lactamase. To isolate more mutations that affect the signal sequence, segment-directed mutagenesis was used. The virtues of using a combination of classical genetic methods and novel *in vitro* methods to mutagenize a specific region of the gene are discussed.

The synthesis and secretion of β-lactamase were studied in *Salmonella typhimurium* infected with P22 phage carrying the *bla* gene in mutant and wild-type form. Pulse-chase experiments show that the majority of β-lactamase molecules encoded by wild-type and chain terminating alleles of *bla* are synthesized initially in precursor forms with a larger apparent molecular weight than the relevant mature forms. Subsequently these precursor forms are converted to the mature forms. Two signal sequence mutations, *fs*(7,9) and pro_{20}->ser, reduce the rate of processing of the signal peptide from the precursor forms of these alleles while three other alleles, *fs*(18,21), *fs*(14,21) and pro_{20}->leu, eliminate the

processing of the signal peptide altogether.

The cellular locations of bla encoded proteins were determined by cell fractionation experiments and by comparing the accessibility of these proteins to trypsin in intact and lysed spheroplasts. By these criteria precursor forms that are encoded by wild-type, chain terminating and ts alleles of bla and that can be shown to chase into corresponding mature forms apparently are localized to the cytoplasm or the cytoplasmic side of the inner membrane. Two signal sequence alleles, fs(14,21) and fs(7,9), accumulate precursor forms that are also on the cytoplasmic side of the inner membrane. The other three signal sequence mutations, fs(18,21), pro_{20}->ser and pro_{20}->leu accumulate precursor that has traversed the inner membrane but is membrane bound. The mature forms of the chain terminating alleles appear to be weakly bound to the surface of a membrane in the periplasm while the mature forms of wild-type, tsH41, fs(7,9) and pro_{20}->ser are soluble periplasmic proteins. The ability of the different bla encoded proteins to migrate as doublets on SDS-polyacrylamide gels and their biological acitivity correlate with their cellular location. Finally the wild-type mature form and precursor form differ in conformation as evidenced by their different sensitivities to proteases in crude lysates. These data suggest that β-lactamase can traverse the inner membrane after its synthesis is complete. Neither processing of the signal peptide nor the presence of the carboxy terminus apparently are required for the successful execution of this step. However the removal of the signal peptide and the presence of the carboxy terminus are required in order that β-lactamase reach its final destination as a soluble periplasmic protein. A working model for the secretion of β-lactamase is presented.

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Shortle, D., Koshland, D., Weinstock, G.M., and Botstein, D. (1980). Segment-directed mutagenesis: construction *in vito* of point mutations limited to a predetermined region of a circular DNA molecule. Proc. Natl. Acad. Sci. USA 77, 5375-5379.

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TABLE OF CONTENTS

	page
DEDICATION	2
ABSTRACT	3
AUTOBIOGRAPHICAL NOTE	5
ACKNOWLEDGEMENTS	7
TABLE OF CONTENTS	9
LIST OF TABLES	14
LIST OF FIGURES	15
Chapter 1: Introduction	17
I. β-lactamase	17
II. Secretion in eukaryotes	19
A. Overview	20
B. Initial steps of secretion, the signal hypothesis	20
C. Alternative mechanisms for traversing membranes	23
III. Secretion in prokaryotes	24
A. Structure of gram negative bacteria	24
B. Definition of secretion	25
C. Signal sequences	26
1. Identification and structure	26
2. Functional role of signal peptide in secretion	27
3. Processing of signal peptides	31
4. Functional role of processing in secretion	32

D. Translocation across the inner membrane 34

1. Role of ribosomes and protein synthesis 34

2. Role of cellular components 35

E. Physiological state of the membrane 37

F. Models for protein secretion 38

Chapter 2: Methods 42

I. Media and Enzymes 42

II. Genetics 43

A. Bacterial strains 43

B. Phage strains 46

C. Tests for the function of the bla gene 49

1. On P22bla 49

2. On pBR322 51

D. Crude assay for β-lactamase enzymatic activity 52

E. Mutagenesis of P22bla 52

F. Isolation of bla- mutations 54

G. Isolation of signal sequence mutations 54

H. Determination of the nucleotide sequence of signal peptide mutants 56

I. Screening bla- phages for condtional bla- alleles 57

J. Generation of deletions of bla from DB7606 58

K. Mapping of bla mutations 59

1. Spot deletion mapping 59

2. Two factor phage crosses 61

III. Biochemistry 62

A. Labeling of phage encoded proteins after infection 62

B. SDS polyacrylamide gel electrophoresis and autoradiography 63

C. Pulse-chase experiments 63

D. Cell fractionation procedures 64

1. Fractionation procedures for periplasmic proteins 64

2. Membrane isolation 65

3. Pulse-chase cell fractionation 66

E. Trypsin acessibility experiments 66

1. Pulse-chase trypsin accessibility experiments 66

2. Trypsin accessibility experiments of cells treated with phenethyl alcohol 69

F. Determination of the partial amino acid sequence of the amino terminus of bla encoded proteins 69

Chapter 3: Genetics of β-lactamase 72

I. Results 72

A. Tests for bla function 72

B. Isolation and characterization of mutations in bla 74

1. General mutations 74

2. Signal sequence mutations 76

3. Sensitivity of test for bla gene function 77

C. Generation of deletions in the bla from DB7606 82

D. Mapping of bla mutations 83

1. Deletion mapping 83

2. Two factor crosses 86

E. Correlation of genetic and physical map 94

1. Orientation of the bla gene on P22 in DB7606 and the orientation of the in vivo deletions of bla 94

2. Physical location of mutations in the bla gene 95

II. Discussion 101

A. A general strategy for isolating mutations in a particular region of a gene 101

B. Resolution of mapping methods 105

C. Hot spots 107

D. Distribution of point mutations among conditional and non-conditional mutations 109

E. Correlation of genetic and physical map of bla 110

Chapter 4: Secretion of β-lactamase 112

I. Results 112

A. Analysis of the protein products of wild-type and mutant β-lactamase genes by SDS-polyacrylamide gel electrophoresis 112

1. Identification of the protein products of the wild-type bla gene 113

2. Identification of the protein products of bla harboring mutations in the mature protein 114

3. Identification of protein products of the signal sequence alleles 117

B. Partial amino acid sequence of the amino termini of bla encoded proteins 123

C. Precursor product relationships of bla encoded proteins 126

1. Products with wild-type signal sequences 126

2. Products with altered signal sequences 132

D. Cellular localization of bla encoded proteins by cell fractionation 136

1. bla$^+$ (mature species) 137

2. Chain terminating alleles of bla 137

3. Localization of the precursor products of wild-type and mutant signal sequence alleles of bla 148

E. Trypsin accessibility experiments 162

1. The bla$^+$ allele 164

2. The alleles tsH41, tsH1, fsI7 and amH46 164

3. The signal sequence alleles 168

F. Protease sensitivities of bla+ precursor and mature forms in crude lysate 176

G. The doublet phenomenon 177

H. Phenethyl alcohol and β-lactamase secretion 183

II. Discussion 189

A. Translocation of β-lactamase across the inner membrane 190

B. Role of the removal of the signal sequence in secretion 196

C. Role of carboxy terminus in secretion 199

D. Intrinsic properties of bla encoded proteins 200

1. Resistance to proteases in crude lysates 200

2. The doublet phenomenon 201

3. Ability to confer ampicillin resistance 203

E. Cell fraction and cellular location 203

Chapter 5: Conclusion, a working model for β-lactamase 205

References 211

LIST OF TABLES

		page
Table 1.	Bacteria and phage strains	44
Table 2.	Distribution of *bla* alleles into groups with conditional and non-conditional phenotypes	75
Table 3.	Characterization of Bla phenotype of different *bla* alleles	78
Table 4.	Two factor crosses for mapping *bla* alleles	87
Table 5.	Molecular weights of β-lactamase-associated proteins in chain-terminating *bla* mutants	115
Table 6.	Partial amino acid sequences of precursor and mature forms of different *bla* alleles	124
Table 7.	Fractionation of β-lactamase-related peptides by the cold osmotic shock procedure	140
Table 8.	Fractionation of β-lactamase related peptides by membrane isolation	151
Table 9.	Pulse-chase membrane-fractionation of wild-type and signal sequence alleles of *bla*	158

FIGURES

		page
Figure 1.	Prokaryotic signal sequences	29
Figure 2.	Heteroduplex between Tc10 and P22*bla*	47
Figure 3.	Nucleotide sequences of the signal sequence alleles of *bla*	80
Figure 4.	Genetic map of the *bla* gene	84
Figure 5.	The corrrelation between recombination frequency and physical distance in *bla*	90
Figure 6.	The physical map of *bla*	102
Figure 7.	^{35}S-methionine labeled cells infected with *bla*-B501, *bla*-B510 and *bla*$^+$ phage.	119
Figure 8.	^{35}S-methionine labeled cells infected with P22*bla* phage containing signal sequence alleles	121
Figure 9.	Autoradiogram of ^{35}S-methionine-labeled extracts from pulse-chase analysis of *bla*$^+$ gene products	128
Figure 10.	Quantitation of pulse-chase analysis of *bla*$^+$ protein products	130
Figure 11.	Pulse-chase analysis of the gene products of signal sequence alleles of *bla*	134
Figure 12.	Osmotic shock analysis of ^{14}C-labeled cells infected with *bla*-*fs*I7 and *bla*$^+$ phage	138
Figure 13.	Membrane isolation of cells infected with *bla*$^+$ and *bla*$^-$ phage	149
Figure 14.	Pulse-chase cell-fractionation experiments of cells infected with *bla*$^+$ phage	156
Figure 15.	Pulse-chase trypsin accessibility experiment with cells infected with P22*bla*-*ts*H41	167
Figure 16.	Pulse-chase trypsin accessibility experiment with cells infected with P22*bla*-*am*H46	169

Figure 17. Pulse-chase trypsin accessibility analysis of *bla* encoded proteins 171

Figure 18. Sensitivity of *bla*$^+$ encoded proteins to proteases in crude lysates 178

Figure 19. Analysis of the doublet formation of *bla*$^+$ encoded proteins 181

Figure 20. Trypsin accessibility analysis of proteins produced by cells treated with phenethyl alcohol after infection with P22*bla*$^+$ phage 185

Figure 21. Trypsin accessibility analysis of proteins produced by cells treated with phenethyl alcohol after infection with P22*bla*-pro$_{20}$→leu phage 187

Figure 22. A working model for the secretion of β-lactamase 206

Chapter 1:

I. TEM β-lactamase

The bla gene codes for the secreted enzyme, TEM β-lactamase, which is responsible for conferring upon gram negative bacteria resistance to high levels of penicillin and related antibiotics. Studies of the bla gene and β-lactamase were initiated for several compelling reasons. The use of penicillin (or its analogs) is the most effective method for curing individuals of infections with gram negative bacteria. Gram negative pathogens that produce β-lactamase are resistant to penicillin. Therefore, infections caused by these bacteria present physicians with a major medical problem (Abraham, 1981). It is hoped that a better understanding of both the synthesis and mode of action of β-lactamase will allow one to improve the treatment of infections caused by pencillin resistant pathogens.

Additional reasons for studying the bla gene and its product are: 1) The ampicillin resistance phenotype of strains harboring the bla gene has provided biologists with an extremely powerful selection for detecting the introduction of this gene (and any genes linked to it) into a naive bacterium. This property of bla has been used extensively in recombinant DNA experiments and in the study of transposition by the TnA elements (Heffron et al., 1975, 1977, 1979). 2) The existence of bla on the cloning vector, pBR322, and the ability to score easily the absence or presence of bla function (by using the ampicillin resistance phenotype)

make bla an excellent model gene for developing new in vitro methods to manipulate cloned genes (Shortle et al., 1980). 3) Since β-lactamase is secreted to the periplasmic space in many different gram negative bacteria, it can be considered a model for understanding how a protein traverses the inner membrane of these organisms.

Studies of the bla gene have revealed several important features of this gene. The bla gene is expressed constitutively (Richmond and Sykes, 1973). It is common to many different R factors from a variety of different gram negative bacteria. The presence of this gene on different R factors in different bacteria is due apparently to its presence on a class of transposable elements called TnA, which includes the elements Tn1, Tn2 and Tn3 (Heffron et al., 1975). A comparison of the the bla genes from these different elements suggests that variants of the bla gene exist in nature (Sutcliffe, 1978; Ambler and Scott, 1978).

The bla gene has been moved from these R factors to numerous other genetic vehicles by transposition (Weinstock et al., 1979; Winston and Botstein, 1981; Davis et al., 1980) and by recombinant DNA technology. Among the many genetic vehicles which harbor the bla gene, a derivative of phage P22, a temperate phage of Salmonella typhimurium, is particularly useful for classical fine structure genetic analysis. This phage, P22Ap31pfr1 (refered to as P22bla in this work) contains all the essential functions for its normal lytic and lysogenic growth as well as the bla gene (Winston and Botstein, 1981); therefore all the techniques developed for classical phage genetics are available to study the bla gene. A

natural variant of the structural gene for this enzyme also exists on the common cloning vector pBR322 (Sutcliffe, 1979; Bolvia et al., 1977). As a result this gene is easily amenable to genetic anaysis by more recent recombinant DNA technology. In this study the properties of both of these vehicles are used to investigate the bla gene.

The product of the bla gene has also been investigated extensively (Hamilton-Miller and Smith, 1979). The mature enzyme catalyses the cleavage of the lactam ring in pencillin and as a result destroys the bactericidal effect of this antibiotic. Various penicillin-related substrates and inhibitors of β-lactamase have been synthesized (Richmond and Sykes, 1973; Abrahams, 1981). These substrates and inhibitors have proved useful in investigating the mechanism of this enzyme.

One aspect of β-lactamase that has not been extensively studied is its secretion by the gram negative bacteria. In this thesis the secretion of β-lactamase by the gram negative organism, Salmonella typhimurium, is examined. To make the discussion of the results from this work more meaningful, a review of secretion in eukaryotes and prokaryotes is presented below.

II. Secretion in eukaryotes

Cellular proteins encoded by nuclear DNA are found in sub-cellular and extra-cellular locations as well as in the cytoplasm. Most of these proteins (if not all) are localized in a particular cellular compartment apparently to the exclusion of all others. However, they are all

synthesized from free amino acids by complex cellular machinery localized in the cytoplamic matrix. These observations imply the existence of selective and efficient processes that direct specific proteins from their birthplace in the cytoplasm to their proper non-cytoplasmic location. The process which allows specific proteins to leave the cell and become free in the cell's external milieu is called secretion.

A. Overview

The pathway of secretion in eukaryotes was outlined by the pioneering work of George Palade (Palade, 1975). In summary, proteins destined for secretion are synthesized on ribosomes bound to the membrane of the endoplasmic reticulum (ER). The proteins enter into the lumen of this organelle and then are transferred from the ER to the Golgi complex, and from the Golgi complex to secretory vesicles. Secreted proteins are discharged from the cell when the secretory vesicles fuse with the plasma membrane. A consequence of this pathway is that once a protein destined for secretion passes into lumen of the ER, it need not pass through any other membrane to reach its final destination.

B. Initial steps of secretion, the signal hypothesis

The passage of proteins destined for secretion from the cytoplasm into the lumen of the ER has been studied primarily by *in vitro* biochemical experiments. Milstein *et al.* (1972) discovered that an immunoglobulin

light chain (a secreted protein) when synthesized *in vitro* contains an additional small peptide at its amino terminus. This precursor form of light chain can be converted to the mature form if homologous membranes are present during its synthesis (Cowan *et al.*, 1973). Blobel and Dobberstein (1975a,b) showed that the efficient localization of another immunoglobulin light chain into the lumen of the ER and the efficient removal of the extra peptide found at its amino terminus requires the presence of vesicles of the ER during the *in vitro* synthesis of these proteins. They also showed that the extra amino terminal peptide is cleaved from the amino terminus while the nascent chain is still growing. When the vesicles of the ER are added after the translation of the precursor molecules is completed, the precursors are neither transported into the lumen of the ER nor processed at their amino termini. These findings suggest that some step required for the efficient transport of a protein destined to be secreted into the lumen of the ER occurs during the synthesis of that protein. If the processing enzyme responsible for the removal of the amino terminal peptide were located on the lumenal side of the ER membrane then these results would also indicate that the protein traverses the membrane during its synthesis.

The results described above led Blobel and Dobberstein (1975a) to propose the "signal hypothesis." They postulated that the extra amino terminal peptide, "the signal peptide", is responsible for initiating the transport of proteins across the membrane of the ER. As soon as the signal peptide emerges from the ribosome, it binds to the membrane of the ER and induces the formation of a transient pore through which the nascent

chain passes. Once the signal peptide reaches the lumen of the ER, it is cleaved from the nascent chain of the protein. The result of this process is a cotranslational vectorial transport of the protein across the membrane of the ER.

Results from many independent studies of secreted proteins support the basic tenets of the signal hypothesis. First, almost all secreted proteins are initially synthesized with signal peptides at their amino termini which are subsequently removed during the secretion process. Just a few examples are lysozyme (Suchanek et at., 1978), ovomucoid (Thibodeau et al., 1978), and prolactin (McKean and Muarer, 1978). Second, a portion of the glycoprotein of VSV, the G protein, traverses the membrane of the ER on its journey to the plasma membrane. Rothman and Lodish (1977) were able to demonstrate that glycosylation of this protein occurs during its synthesis. Since glycosylation is believed to occur on the lumenal side of the rough ER (Rothman and Lenard, 1977), this experiment provides additional evidence for the cotranslational transport of a protein into the lumen of the ER. Rothman and Lodish also provided the first experimental evidence that the signal peptide is responsible for bringing free ribosomes translating VSV G mRNA to the membrane of the ER. Third, the signal hypothesis predicts the existence of proteins in the membrane of the ER that function as pores and as receptors for ribosomes. Several laboratories have identified proteins associated with the membrane of the ER that may have these fuctions (Sabatini and Kriebich, 1976; Kriebich et al., 1978a,b; Meyer and Dobberstien, 1980a,b).

C. Alternative mechanisms for traversing membranes

The secretion of chicken ovalbumin is not consistant with the signal hypothesis as originally proposed. First, chicken ovalbumin is not synthesized initially with a signal peptide at its amino terminus (Palmiter et al., 1978). Second, when vesicles of the ER are added after two thirds of the ovalbumin molecule is synthesized in vitro, the molecule is nevertheless efficiently sequestered into these vesicles (Lingappa et al., 1979). Therefore, the first two thirds of this molecule apparently need not be transported into the ER as they are synthesized.

The processes which allow proteins to leave the cytoplasm and to enter the stroma of chloroplast or the matrix of the mitochondria are topologically analogous to the process of proteins entering the lumen of the ER from the cytoplasm. In vitro studies of proteins encoded by nuclear DNA but destined to enter these two organelles show that several of these proteins are synthesized initially in a precursor form. Unlike proteins destined to be secreted these precursors are transported post-translationally into the appropriate organelle and are converted post-translationally to the mature forms as well (Chua and Schmidt, 1978; Highfield and Ellis, 1978; Maccecchini et al., 1979; Nelson and Shatz, 1979). To account for the results from these studies and from the studies of ovalbumin requires either radical amendment of several aspects of the signal hypothesis, or postulation of alternative mechanisms, which allow a protein to pass through a membrane.

III. Secretion and protein localization in prokaryotes

Non-cytoplasmic proteins play an important role in the biology of prokaryotes as well as eukaryotes. These proteins are involved in cellular functions such as the biosynthesis of membranes and the transport of small molecules into and out of the cell. Probably the most important single advantage to studying protein localization in prokaryotes is that the genetic manipulation of these organisms is simple and rapid. Genetics has been a particularily powerful tool in dissecting complex biological processes in bacteria, and already limited application of genetic analysis to the problem of protein localization has provided several important insights. Mutational analysis of the secretion process can and should provide a much needed framework to determine the relevance of *in vitro* biochemical studies to the *in vivo* process.

A. Structure of gram negative bacteria

The cellular compartments of gram negative bacteria such as *Salmonella typhimurium* are defined by three macro molecular structures, the inner membrane, the peptidoglycan and the outer membrane. The inner membrane consists of a phospholipid bilayer and proteins. The lipids and the proteins are maintained in specific asymmetric orientations. Only small neutral molecules diffuse passively through the bilayer of the inner membrane. Nutrients traverse this membrane and accumulate in the cell as the result of special systems for their transport. The inner membrane

also serves to maintain an electro-chemical gradient, which is used to generate energy. Certain steps in the biosynthesis of lipids, peptidoglycan and lipo-polysaccharide are associated with this membrane.

The inner membrane is surrounded by a single layer of peptidoglycan which is extensively cross linked. The rigid nature of the peptidoglycan is largely responsible for the shape of these organisms.

Surrounding the peptidoglycan is yet another membrane, called the outer membrane. Though this membrane exhibits some of the properties of a unit membrane, it differs substantially from the inner membrane. the outer membrane contains unique lipo-polysaccharides (Funahara and Nikaido, 1980). Small molecules that fail to diffuse across the inner membrane diffuse across the outer membrane because the outer membrane contains many pores. The properties of the inner membrane, peptidoglycan and the outer membrane have been extensively reviewed DiRienzo et al. (1978) and Osborn and Wu (1980).

B. Definition of secretion

Using these structures, we can subdivide the cell into four compartments, the cytoplasm, the inner membrane, the outer membrane and the region between the inner and outer membrane called the periplasm. The soluble proteins of the periplasm are most analogous to secreted proteins in higher organisms, since they are soluble proteins that reside on the opposite side of the plasma membrane from the cytoplasm. For this

reason proteins which are free and soluble in the periplasm are said to be secreted proteins.

The review of secretion in gram negative bacteria presented below includes studies of the localization of outer and inner membrane proteins as well as periplasmic proteins. The study of outer and inner membrane proteins are included in this review because the apparent absence of any internal organelles in gram negative bacteria suggests that proteins localized in the inner membrane, the periplasm or the outer membrane must have interacted directly with the inner membrane. Therefore, it seems possible that the processes which localize proteins to these three cellular locations may share common steps.

C. Signal sequences

1. Identification and structure

Proteins with non-cytoplasmic locations have been examined for the presence of signal peptides. Initial attempts in which bacterial cells were pulse labeled with radioactive amino acids failed to uncover precursor forms of most of these proteins. However, when the cells were perturbed by chemical and genetic methods, precursor forms of periplasmic and outer membrane proteins were discovered (for more detail see below). The existence of precursor forms of other non-cytoplasmic proteins was demonstrated by examining the products of these genes in a coupled *in vitro* transcription translation system (Marchal *et al.*, 1980; Inouye and

Beckwith, 1977) or by *in vitro* completion of nascent chains found on membrane-bound ribosomes formed *in vivo* (Randall *et al.*, 1978; Randall and Hardy, 1977). The existence of precursor forms of various non-cytoplasmic proteins has also been inferred from the nucleotide sequence of their structural genes (Figure 1). The fact that so many of the outer membrane and periplasmic proteins have signal peptides and that precursor forms for most of these proteins were not detected in wild-type cells under standard growth conditions suggests either that the half-lives of these precursors are very short, or that the signal peptides are removed prior to the completion of these proteins.

The precise amino acid sequences of several signal sequences have been determined (Figure 1). Though the primary sequences of these signal peptides share no obvious sequence homology, these peptides do share several general features. The amino terminal portion of the peptide usually includes a positively charged amino acid residue. This residue is followed by a core of 10-15 hydrophobic amino acid residues. Finally the amino acid residue of the signal peptide that is adjacent to the cleavage site contains a small side chain.

2. Functional role of signal peptide in secretion

The role of the signal peptide in prokaryotic secretion has been studied by a combination of genetic and biochemical analysis. Fusions of the *malE* gene (the structural gene for the maltose binding protein, a secreted protein) with *lacZ* (the structural gene for β-galactosidase) were

PROKARYOTIC SIGNAL SEQUENCES

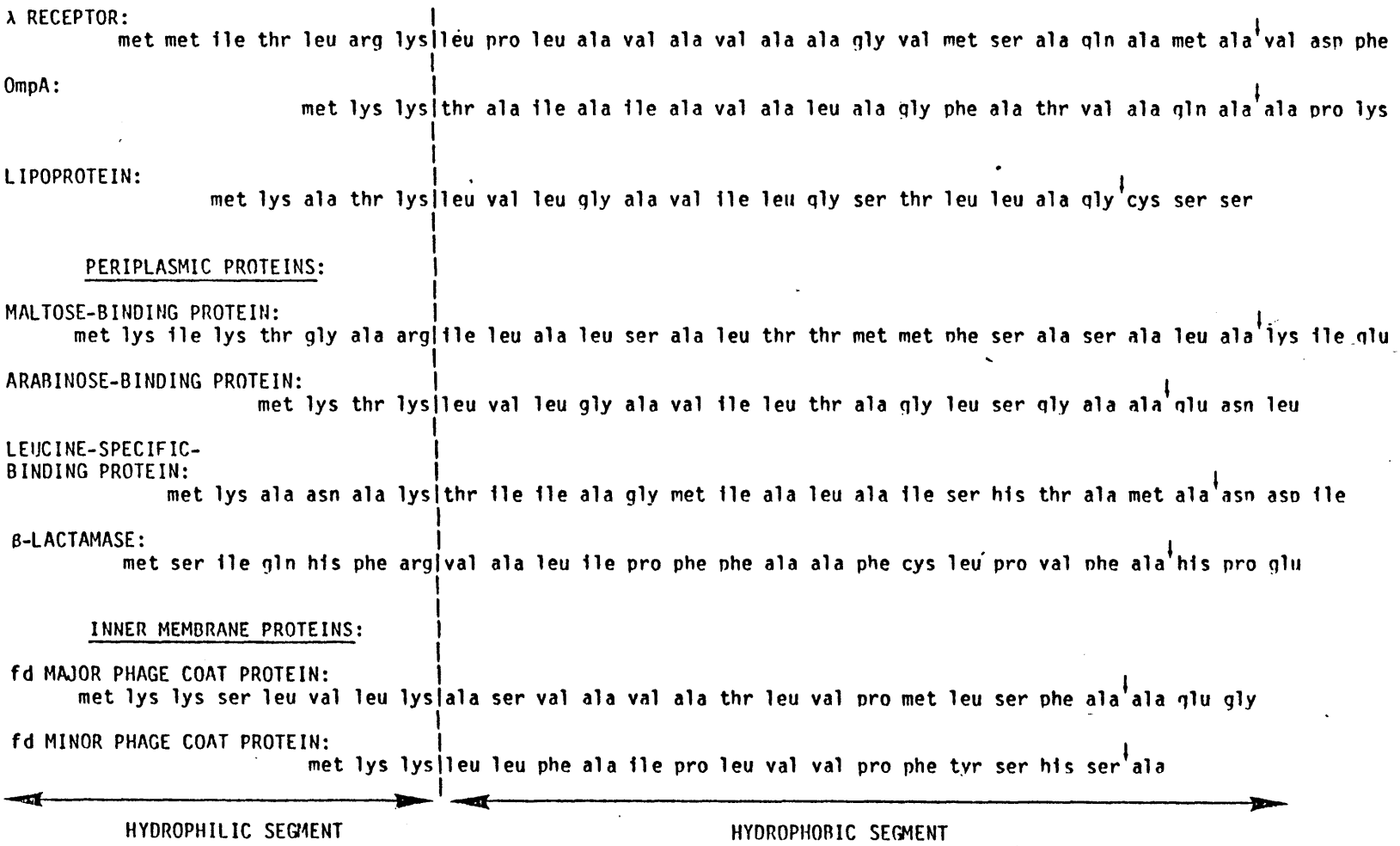


Figure 1: Prokaryotic signal peptides. The amino acid sequences of the signal peptides from nine different exported proteins in gram negative bacteria. This figure was taken from the thesis of Dr. S. Emr. The list of signal peptides includes the lipoprotein (Inouye et al., 1977; Nakamura and Inouye, 1979; Nakamura et al., 1980), fd major coat protein (Sugimoto et al., 1977), fd minor coat protein (Schaller et al., 1978), β-lactamase (Sutcliffe, 1978), maltose binding protein (Dedouelle et al., 1980), lambda receptor protein (Hedgpeth et al., 1980), arabinose binding protein (Wilson and Hogg, 1980), ompA protein (Beck and Bremmer, 1980; Movva et al., 1980) and the leucine specific binding protein (Oxender et al., 1980). The sequences are positioned relative to their hydrophobic segments and the processing sites are indicated with an arrow.

made (Bassford et al., 1979). The hybrid genes produce proteins in which varying amounts of the N-terminus of the maltose binding protein replace the first 10-30 amino acid residues of the β-galactosidase. All fusion proteins which contain a large segment of the maltose binding protein and therefore which are initially synthesized with a signal peptide at their amino terminal are localized to the inner membrane but not to the periplasm. These results suggest that a signal peptide alone is sufficient to change the cellular location of the β-galactosidase (from cytoplasmic to inner-membrane-bound) but is not sufficient to ensure the successful secretion of the protein to which it is attached (Bassford et al., 1979; Moreno et al., 1980).

The properties of the hybrid proteins were used to select mutations which alter the amino acid sequence of the signal peptide (Bassford and Beckwith, 1979). When cells are induced to make substantial amounts of a hybrid protein containing a large segment of the maltose binding protein, the majority of the cells die. Among the cells which survive the induction are cells harboring mutations that alter the signal peptide of the hybrid protein. These signal peptide mutations can be recombined from the gene fusions encoding for the hybrid protein into the wild-type malE gene. Using an identical approach, signal peptide mutations were isolated in the lambda receptor protein, an outer membrane protein (Silhavy et al., 1976; Silhavy et al., 1977; Emr and Silhavy, 1980). The products of these mutant genes have apparent molecular weights expected of the intact protein plus their signal sequences, and they generally reside in the cytoplasm. The phenotypes of these mutant proteins suggest that the

signal peptide functions during an initial step of protein export.

The nucleotide sequence of the signal sequence mutations described above revealed that in eight out of nine cases a charged amino acid is substituted for one of the hydrophobic amino acid residues (Emr et al., 1980; Bedouille et al., 1980). These results suggest that the integrity of the hydrophobic core in the signal peptide may have an important role in the localization process.

3. Processing of signal peptides

An endopeptidase activity that processed the signal peptide from the precursor form of the coat protein of coliphage f1 (an integral membrane protein) was first identified by Chang et al. (1978). Partial amino sequencing identified the products of the cleavage reaction as the mature protein and the signal peptide. This peptidase appears to be localized to the inner membrane. A signal peptidase activity that processes pre-alkaline phosphatase has also been identified. Apparently this activity is localized to the inner membrane (Chang et al., 1980).

Using the precursor form of the coat protein of the coliphage M13 (identical to f1 by several criteria) as substrate, Mandel and Wickner (1979) were also able to identify a signal peptidase activity. Date and Wickner (1981) used this assay to purify a signal peptidase and to isolate a recombinant DNA plasmid that apparently contains the gene that encodes for this peptidase. The purified peptidase also cleaves the signal peptides in an endoproteolytic fashion from precursors of several

periplasmic and outer membrane proteins. This peptidase has been localized to both the inner and outer membranes (Zwizinski and Wickner, 1981). At this time it is difficult to determine whether the difference in the localization of the peptidase activity identified by Chang *et al.*, (1978) and the one identified by Zwizinski and Wickner (1981) has any significance.

The temporal relationship *in vivo* between the synthesis of a protein destined to a non-cytoplasmic location and the processing of its signal peptide was examined for a variety of proteins (Josefsson and Randall, 1981). The nascent chains and completed polypeptides of several proteins were immune precipitated from cells and then examined for the presence or absence of the signal peptide by a method of tryptic mapping. From these studies it is apparent that molecules of certain periplasmic proteins (alkaline phosphatase, arabinose binding protein and maltose binding protein) and the outer membrane protein, the *ompA* protein, can be processed co-translationally or post-translationally. However, for each of these proteins, processing of the signal peptide does not occur until the nascent chains reach approximately 80% of their final length. Jofesson and Randall also showed that TEM β-lactamase (a periplasmic protein), and the lambda receptor protein (an outer membrane protein) are processed only post-tranlationally.

4. Functional role of processing in protein localization

The relationship between the processing of the signal peptide and

protein localization is unclear because conflicting results have been obtained. The lipoprotein of Escherichia coli is a modified protein of the outer membrane. A mutation in the signal sequence of the lipoprotein produces an altered form of the precursor of this protein that is no longer substrate for processing or for modification (Lin et al., 1977, 1980, 1980a). 57% of the precursor molecules reach their proper location.

The antibiotic globomycin also blocks the processing of the signal peptide from the lipoprotein but does not inhibit the modification (Hussain et al., 1980). This modified form of the precursor accumulates in the inner membrane only, that is virtually none of this protein reaches its proper location. The only difference between this precursor form and the mature form is the presence of the signal sequence. Therefore, the difference in localization between the modified precursor form and the modified mature form appears to be due to the failure to process the signal sequence from the modified precursor. The proper localization of the unmodified precursor form of lipoprotein is mysterious.

The effect of mutations in the M13 coat protein near the amino terminus of the mature protein on processing were examined (Boeke et al., 1980; Russel and Model, 1981). An amino acid substitution caused by one of these mutations results in a drastically reduced rate of processing of that protein. No precursor is recovered in free phage released into the medium from infected cells even though it accumulates in these cells. The authors suggest that removal of the signal peptide from this protein may be required in order that coat protein free itself from the membrane and become part of soluble phage particles. This result, like the result with

the modified precursor of the lipoprotein, suggests that the processing of the signal peptide from proteins destined to non-cytoplasmic locations may be required for their efficient localization.

D. Transocation across the inner membrane

1. Coupling with protein synthesis

The mechanism of localization of alkaline phosphatase to the periplasm shares several similarities with the process of transport of proteins from the cytoplasm into the lumen of the ER. Alkaline phosphatase is processed and sequestered inside inverted vesicles of the inner membrane only if these vesicles are present during its translation *in vitro* (Chang *et al.*, 1980; Smith, 1980). In addition, nascent chains of alkaline phosphatase can be labeled in intact spheroplasts by membrane-impermeable reagents suggesting that a portion of the nascent chain of alkaline phosphatase traverses the inner membrane prior to the completion of its synthesis (Smith *et al.*, 1977). These experiments are consistent with the model of co-translational vectorial transport as proposed by Blobel and Dobberstein (see above).

The localization process of the coat protein of the two apparently identical filamentous phages, M13 and f1, has been studied in detail. This protein is inserted into the inner membrane prior to its assembly into phage. When the localization of the coat protein was investigated different laboratories obtained conflicting results. Chang *et al.* (1979)

found that the insertion of this protein into inverted vesicles of the inner membrane is obligatorily cotranslational because the procoat protein (the precursor form of the coat protein) is inserted into inverted vesicles of the inner membrane and processed efficiently only if the vesicles are present during translation but not if they are added one hour after translation has begun. However, Wickner et al. (1978) found that this process could occur post-translationally if the time of protein synthesis was short and the vesicles were added immediately thereafter. These experiments were supported by experiments in vivo which also indicated the procoat protein is inserted and processed post-translationally into the cytoplasmic membrane (Ito et al., 1979, 1980).

Goodman et al. (1981) reported data which apparently resolves this conflict. The ability of the procoat protein to insert into the membrane post-translationally decays dramatically within minutes. Therefore, in experiments in which the vesicles are added post-translationally and the time of protein synthesis is long relative to the half-life of the insertion capacity of the procoat, the majority of the procoat fails to insert into the membrane post-translationally. These experiments suggest that post-translational insertion of this protein into the inner membrane can occur (though they do not rule out that co-translational insertion occurs as well). In addition they provide a warning against using experiments similar to design of Blobel and Dobberstein to prove obligatory co-translational vectorial transport.

2. Cellular components

An understanding of the mechanism of protein localization in gram negative bacteria requires the identification of the components necessary for the efficient execution of the step(s) of this process. Apparently M13 coat protein can assemble properly into liposomes reconstituted with lipids of Escherichia coli and purified signal peptidase (Silver et al., 1981; Watts et al., 1981). These experiments suggest that the number of components required for the proper insertion of the M13 coat protein into the inner membrane is two, the membrane lipids and the signal peptidase.

Contrary to the results above, the isolation of several different mutants in Escherichia coli with pleiotropic effects on the protein localization suggest that cellular components other than lipids and the signal peptidase may be involved in this process. Heat sensitive mutants in the secA gene, when incubated at the non-permissive temperature, cause the accumulation of some precursors (but not all) of periplasmic and outer membrane proteins in the cytoplasm of the cell (Oliver and Beckwith, 1981). Mutations that alter the signal peptide of several non-cytoplasmic proteins causes the accumulation of the precursor forms of these proteins in the cytoplasm (see above). The localization defect of these mutations can be suppressed by a mutation in an unlinked gene, prlA (Emr et al., 1981). Finally the presence of large quantities of the hybrid protein between the maltose binding protein and β-galactosidase causes the accumulation of some (but not all) periplasmic and outer membrane proteins in their precursor forms and impairs the proper localization of these proteins (Ito et al., 1981). The authors

suggest that the pleiotropic effect of the hybrid proteins is due to the saturation of the cellular machinery required for proper localization. At this time no evidence exists which addresses the question of whether the hybrid proteins, the secA gene product or the prlA gene product influence the localization process because they are componenets of a localization machinery or whether they influence the process by some indirect means such as changing the composition of the membrane.

E. Physiological state of the membrane

The presence of a membrane potential apparently is required for the proper localization of several periplasmic proteins and outer membrane proteins. The energy uncoupler, carbonyl cyanide m-chlorophenylhydrazone (CCCP), disrupts the normal processing and insertion into the inner membrane of the coat protein of M13 (Date et al., 1980a,b) and the processing and secretion of the leucine binding protein and β-lactamase (Daniels et al., 1981). These results suggest that the membrane potential might be required for the localization of these proteins, but this conclusion is tentative due to the pleiotropic effects of this drug.

If one uses valinomycin instead of CCCP to deplete the cell of its membrane potential, one can temporarily restore the membrane potential to these cells by removing potassium from the medium. When the cells are treated with valinomycin, precursor to leucine binding protein accumulates. When these cells are resuspended in medium lacking potassium, the precursor to leucine binding protein is processed and

secreted (Daniels et al., 1981). These results support more strongly that membrane potential plays a role in secretion.

The role of membrane potential in the process of protein localization was also addressed by examining the localization of proteins in cells harboring mutations in genes which uncouple energy metabolism and membrane potential (Enquist et al., 1981). When these cells are grown anaerobically, the precursor forms of several periplasmic (maltose and arabinose binding proteins) and several outer membrane proteins (the products of the ompF, ompA and lamB) accumulate. Whether the membrane potential is required for some step in the localization process before or after these proteins traverse the inner membrane has not yet been resolved.

The fluidity of the membrane also apparently influences protein localization in gram negative bacteria. Procaine alters membrane fluidity and inhibits the processing of alkaline phosphatase and glutamine binding protein (Lazdunski et al., 1979). Another molecule, phenethyl alcohol (PEA), also perturbs membrane fluidity and inhibits the processing and proper localization of the matrix protein (Sekizawa et al., 1977), the ompA protein (Halegoua and Inouye, 1979), the leucine binding protein and TEM β-lactamase (Daniels et al., 1981). Finally precursors of alkaline phosphatase (Pages et al., 1979) and the ompA (DiRienzo and Inouye, 1979) protein are accumulated in unsaturated fatty acid auxotrophs in the presence of elaidate or at low temperatures, respectively.

F. Models for protein secretion

The results from the studies descibed above characterize different aspects of protein localization in gram negative bacteria but provide little insight into the actual mechansim of how proteins traverse the inner membrane of these organisms. However, they do place constraints upon any mechanism(s) that one chooses to propose. Any model for the mechanism of protein localization will have to account for the effects of the particular perturbants on the process whether the perturbant is a *secA* mutation or CCCP. In every case so far examined the localization of a subset of the periplasmic and outer membrane proteins is affected by a particular perturbant. Th limited effect of each perturbant suggests either that a single mechanism of protein localization exists in gram negative bacteria and the ways in which specific proteins interact with that system varies greatly or that several mechanisms of protein localization exist in these organisms.

A variety of mechanisms have been proposed to explain how proteins traverse the inner membrane of gram negative bacteria. The two models which lie at opposite ends of the spectrum are the signal hypothesis as proposed by Blobel and Dobberstein (1975a) and the membrane trigger hypothesis as proposed by Wickner (Ito *et al.*, 1979). The signal hypothesis in gram negative bacteria is the same as that described for eukaryotes except the target membrane is the cytoplasmic membrane instead of the ER. The membrane trigger hypothesis was originally proposed to explain how the coat protein, an integral membrane protein, inserts post-translationally into the inner membrane. This model proposes that the signal sequence activates a protein for insertion into the inner membrane

by altering its conformation. When the protein interacts with the membrane, it undergoes a conformational change which allows it to insert into the membrane. Processing of the signal peptide might stabilize this configuration (an integral membrane protein) or drive the protein the rest of the way through the membrane into the periplasm (a secreted protein).

Two major differences between these two models stand out. The signal hypothesis suggests that the cell assists a protein in traversing a membrane by providing the energy (GTP hydrolysis during nascent chain elongation) required for this process and by providing a better envirnonment for the protein to pass through the membrane (a pore). The membrane trigger hypothesis suggests that a protein inserts (or traverses) the membrane independent of any cellular components except perhaps for the signal peptidase. As initially proposed the signal hypothesis implied that the secretion process must be co-translational. However, this model was modified subsequently to allow for both co-translational and post-translational traversal of the membrane (Blobel, 1980). In the latter case, post-translational traversal of the membrane can occur if some source of energy other than nascent chain elongation exists which drives the completed protein through the pore. The signal hypothesis also implies that a nascent chain passes through the membrane as an extended amino acid chain; therefore the information in the protein required for its proper cellular location must lie in the primary sequence of the protein. The membrane trigger hypothesis invokes a direct role for secondary and tertiary structure of the protein in the localization process.

Other models have been proposed which contribute potentially important ideas about the mechanism of protein localization. One model (DiRienzo et al., 1978) is very similar to the signal hypothesis except that the signal peptide interacts with the membrane as a loop. With such a structure the entire protein may pass through the membrane while the signal sequence remains attached to the membrane. This model requires processing as a terminal step in secretion. Another model proposes that the signal peptide assists the nascent chain across the membrane by interacting with the nascent chain to form a self contained pore (Engleman and Steitz, 1981) and therefore eliminates the need for a membrane imbedded machinery as proposed in the models of Blobel and Inouye. Daniels et al. (1981) have proposed a model similar to the membrane trigger hypothesis except that the protein is aided in folding into the membrane by the membrane potential.

Chapter II: Materials and methods

I. Media and enzymes

Liquid media used were LB (Levine, 1957), M9CAA (Smith and Levine, 1968) and M9 minimal (Miller, 1972) supplemented with 20ug/ml histidine and 1ug/ml biotin. LB agar, λ plates and buffered saline have been described (Davis et al., 1980). Red plates contained 10 g tryptone, 10 g Bacto-agar (both from Difco), 5 g NaCl, 1 mg ampicillin (just enough to inhibit growth of sensitive cells), 25 mg triphenyl tetrazoleum chloride and 33 ml of sterile 30% galactose per liter. Galactose, tetrazoleum, and ampicillin were added after autoclaving. Ampicillin was a gift of Bristol Laboratories. L-[2,3-^{3}H] alanine, L-[2,6-^{3}H] phenylalanine, L-[4,5-^{3}H] isoleucine, L-[3,4(n)-^{3}H] valine, 5'-[α-^{32}P] dCTP and ^{35}S-methionine was purchased from Amersham/Searle (Arlington Heights, Illinois). Trypsin was purchased from Worthington. Pronases, XI and XIV, and 2,3,5-triphenyl tetrazoleum chloride were purchased from Sigma. Restriction enzymes were purchased from New England Biolabs and used according to the manufactorer's instructions. Micrococcus luteus DNA polyermase 1 was purchased from Miles.

II. Genetics

A. Bacterial strains

Bacterial strains are derivatives of Salmonella typhimurium LT-2. The genotype and the source of these strains are listed in Table 1. DB7609 was isolated by mutagenizing DB7000 with nitrosoguanidine (Miller, 1972) and selecting for cells which could grow on LB agar plates containing 10 μg/ml ampicillin.

Construction of DB7606: DB7606 was constructed by crossing P22Ap30 (carrying Tn1; Weinstock et al., 1979) with P22Tc10 (carrying Tn10; Chan et al., 1972) by adding a multiciplicity of 5 particles (calculated from the titer on DB147). Infected cells were aerated at 37 C for 3 hours. Surviving cells were plated selecting for tetracycline resistance (25 μg/ml on LB agar) and scored for ampicillin resistance and P22 immunity. To check that the ampicillin resistant (ApR), tetracycline resistant (TcR) and P22 immune lysogens were indeed recombinant single lysogens, each purified candidate was induced and the simultaneous transduction of TcR and ApR at very low multiplicity (in the presence of helper) was checked. One of the lysogens which passed all these tests was purified and is called DB7602.

DB7602 is useful for generating Tn10-promoted deletions of the bla gene of Tn1. However these deletions are immune to P22bla, making marker-rescue tests of mutants cumbersome. For this reason the prophage

Table 1. Bacteria and phage strains.

Strain	Genotype	Source
Bacteria		
DB7000	*leuA-am414*	Botstein (unpublished)
DB7154	*leu-am4 hisC-am527 supD10*	Winston *et al.* (1979)
DB7155	" " *supE20*	"
DB7156	" " *supF30*	"
DB7157	" " *supJ60*	"
DB7302	" " *supG50*	"
DB7303	" " *supC80*	"
DB6322	*hisO1242 hisC-fs3736*	Riddle and Roth (1970,1972)
DB6323	" " *sufD41*	"
DB6325	*hisO1242 hisD-fs3749 sufA6*	"
DB4566	*hspL⁻ hspS⁻ proC90 his∇2253 dhuA1 purF145 glaE503*	Shortle *et al.* (1980)
DB7602	DB7000 P22 ($\frac{ImmC}{P22}$ Ap30 Tc10)	This work
DB7606	" " ($\frac{ImmC}{L}$ Ap30 Tc10)	This work
DB7609	DB7000 amp^{R}-$\frac{}{}$	This work
DB5290	*cysA1348 hisC-am527* r^- m^-	Botstein (unpublished)
DB4391	" " /pKT218	This work
DB4392	" " /pKT241	"
DB4393	" " /pKT280	"
DB4394	" " /pKT287	"
DB7618	DB7000/pKM101 (Tn5::amp^{R})	"
DB7572	DB7000 *cya408*	Winston and Botstein (1981)
DB5532	*cysA1348 hisC527* r^- m^-	Botstein (unpublished)
DB4381	*hisG46* ∇ (bio-uvrB)	Botstein *et al.* (1973)

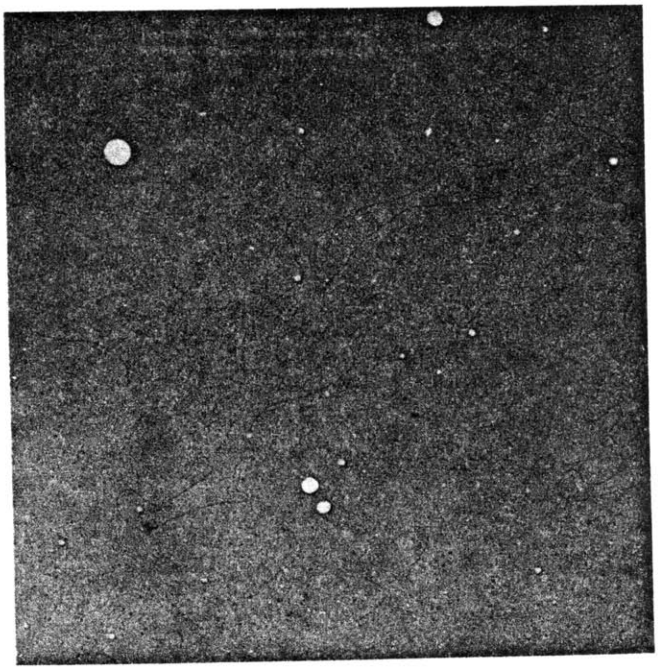
Phage	
P22Ap31 pfr1 (P22 *bla*)	Winston and Botstein
P22 *bla* H1 - P22 *bla* H146	This work
P22 *bla* fsI7 - P22 *bla* fsI40	"
P22 *bla* U1 - P22 *bla* U101	"
P22 *bla* V218	"
V241	"
V280	"
V287	"
V2	"
P22 *bla* *fs*(14,21)	"
fs(7,9)	"
fs(18,21)	"
$pro_{20} \rightarrow leu$	"
$pro_{20} \rightarrow ser$	"
$val \rightarrow ala$	"
B501	"
B507	"
B510	"
P22 *bla* B517	"
B808	"
P22 *sieA*44	Susskind *et al*. (1971)

in DB7602 was crossed as above with a P22$\underline{immC_L}$ phage and a P22$\underline{immC_L}$ Tn1 Tn10 recombinant lysogen was isolated as a TcR ApR lysogen with L-immunity. This lysogen was purified and checked as above; it is DB7606.

B. Phage strains

The phage strains used in this work are also listed in Table 1. The majority of them are derivatives of the phage, P22 Ap31 pfr1 (Winston and Botstein, 1981). This phage began as an insertion mutant (called P22Ap31) in which the translocatable ampicillin resistance elememt Tn1 was inserted into the anti-repressor gene of phage P22 (Weinstock, Susskind and Botstein, 1979). A spontaneous deletion which removes some Tn1 and some P22 DNA but which retains the ampicillin resistance determinant was selected. This phage, P22Ap31pfr1 (called P22$\underline{bla}$ for brevity) was extensively characterized and has normal P22 early and late functions, lysogenizes and integrates its prophage normally, and synthesizes β-lactamase both after infection and as a prophage (Winston and Botstein, 1971) β-lactamase synthesis is not under phage control. DNA heteroduplex analysis in the electron microsope (figure 2) indicates that the P22$\underline{bla}$ amounts to a simple substitution of the β-lactamase structural gene for the dispensable secondary immunity region (immI) of P22.

The following method was used to construct P22$\underline{bla}$ strains containing $\underline{bla}$ point mutations and deletions made in vitro. $\underline{bla}$ point mutations and deletions made in vitro on the plasmid, pBR322 were transferred from pBR322 to P22$\underline{bla}$ by recombination $\underline{in\ vivo}$. DNA of the pBR322 $\underline{bla}^-$ plasmid



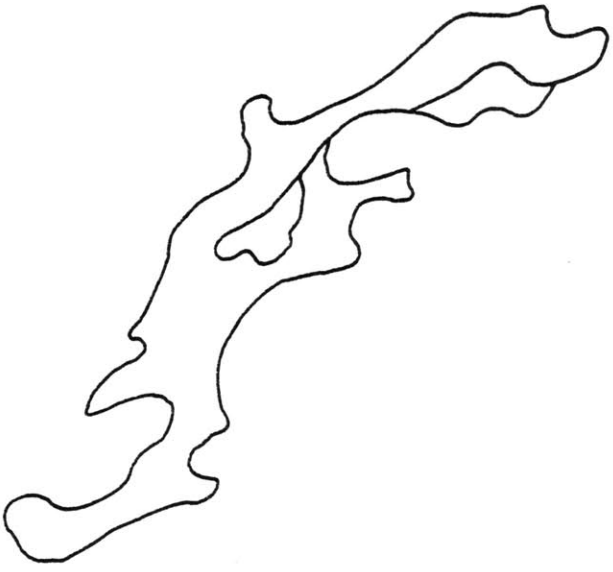


Figure 2: Heteroduplex between Tc10 and P22bla. Heteroduplex between Tc10 and P22bla was done according to the procedure of Tye et al. (1974). A cartoon of the heteroduplex is presented beneath the picture. In the picture one can see the stem and loop of the Tn10 element in Tc10 and a deletion substitution loop. One strand of this loop encodes for a portion of the Tn1 element including the bla gene and the other strand encodes for the dispensable ImmI region of P22.

was isolated (Rambach and Hogness, 1977) and used to transform either the Salmonella strain DB5290 or DB4566 (Lederberg and Cohen, 1974). Tetracycline resistant colonies were selected. P22 Ap32pfr1(bla+) was grown on the Salmonella strains harboring the plasmid and the progeny phage were screened by the red plate test for appearance of bla$^-$ phage (see below).

C. Tests for bla gene function in vivo

The following tests of bla gene function all work on the same principle. A functional bla+ gene encodes for β-lactamase which degrades ampicillin and related antibioties. Bacterial cells normally sensitive to ampicillin will grow in the presence of these antibiotics if β-lactamase is present in their immediate environment because this enzyme detoxifies these drugs by its degradative action.

1. Tests for bla gene function on P22bla

(i) Crude lysogen test: Because P22bla is competent for lysogenic as well as lytic growth, any bla allele carried by this phage can be introduced into any Salmonella typhimurium strain simply by lysogenization. A simple way to isolate a strain lysogenic for a particular P22bla phage is to make plaques of that phage on the desired strain. A large fraction of the cells which grow at the center of these plaques are the desired lysogens.

To test lysogens for their resistance to ampicillin we transferred them with a sterile toothpick from the center of a plaque directly to an LB plate containing ampicillin. Growth of these lysogens on the selective plates showed that the P22bla phage carried a functional bla gene. Since P22 resistant cells survived at the center of these plaques, non-lysogens were transferred to the selective plates as well. In general the presence of these non-lysogens did not damage the validity of this test since they neither had bla function nor inhibited the growth of the lysogens. In some cases it was desirable to have pure lysogens. To obtain pure lysogens the surviving cells from the center of a plaque were colony purified and tested for their immunity to P22bla (Susskind et al., 1971).

(ii) Quantitative lysogen test: Lysogens of P22bla could also be constructed in liquid. 0.1 ml of different dilutions of a lysate of P22bla phage were added to a tube containing 0.1 ml of P22sieA44 at 2 x 10^9/ml. 0.1 ml of DB7000 at 2 x 10^8/ml was added to this tube and the mix was allowed to sit at 30°C for 30 min without shaking. P22sieA44 was present at a high multiplicity of infection to insure a high efficiency of lysogeny for the P22bla phage. These lysogens were tested for their bla function by mixing them with 2.5 ml of soft agar and pouring the mix onto a LB plate containing ampicillin. The appearance of ampicillin resistant colonies indicated that at least some of the phage present in the lysate carried bla gene function. When lysogens of P22bla+ were made by this method, the ratio of ampicillin resistant lysogens (transductants) to plaque forming units of P22bla+ averaged approximately 0.3. This ratio did not change if 10^8-10^9 plaque forming units of a bla- phage were added to

the original mix indicating that with this method of lysogeny we could detect one bla+ phage among 10^8 to 10^9 bla$^-$ phage.

(iii) Red plate test: The preceding assays are extremely sensitive and versatile. However, they are tedious for screening large numbers of plaques for their Bla phenotype. To assay rapidly a large number of phage for their Bla phenotype, the red plate test was developed. The purpose of this assay is to allow the experimenter to identify visually phage which carry a functional β-lactamase gene. The assay takes advantage of the fact that when β-lactamase is released to medium, it destroys ampicillin, saving cells that make β-lactamase as well as sensitive cells in the vicinity. In the plaque assay we start with a phage which may or may not carry β-lactamase. We plate phage on a plate which contains galactose, tetrazolium dye and 1.0 μλ/ml (a very small amount) of ampicillin, using a mixture of two bacterial indicator strains. The galactose and tetrazolium act together as a color indicator which stains galactose-fermenting cells red, leaving galactose-negative cells colorless. The two indicators are both P22-sensitive; one is gal$^-$ and resistant to small amounts of ampicillin (but not by secretion of a β-lactamase). These cells do not save their neighbors. The other indicator is ampicillin-sensitive and gal$^+$. If it survives, it turns red. Thus phage plate whether or not they carry β-lactamase, but only the bla$^+$ phage save the gal$^+$ cells and therefore have a red halo around their plaques.

To perform a red plate test, DB7000 and DB7609 were grown to 2 x 10^8 cells/ml and then mixed in a ratio of 1:2 respectively. Then, 0.1 ml of this mixed indicator + 0.1 ml of the appropriate dilution of a phage stock

were added to a tube. The infected cells were spread onto a one-day old red plate with 2.5 ml of soft agar and 20 hrs later this plate was examined for the presence of plaques with red halos. When looking for a few bla^- phage among a large number of bla^+ phage (for example after a mutagenesis), no more than 150 phage could be put onto a single plate. If more than 150 phage were crowded onto a single plate, then the occasional colorless plaque (bla^- phage) became obscured by the large number of red plaques (bla^+ phage). On the other hand when looking for a rare bla^+ phage among a large number of bla^- phage, a maximum of 10^4 phage could be placed on a single plate. This upper limit is apparently due to the fact that at concentrations about 10^4 phage per plate a substantial fraction of the bla^+ indicator cells die during phage growth and can no longer serve as indicators of bla^+ function.

2. Test for bla function on pBR322

DNA from pBR322 or its putative bla- derivatives was introduced by standard transformation procedures into Escherichia coli (Dagert and Ehrlich, 1979) or Salmonella typimurium (Lederberg and Cohen, 1974). Transformants were selected as tetracycline resistant colonies on LB plates containing tetracycline at 10 μg/ml. These transformants were transferred with a sterile toothpick to LB plates containing ampicillin and growth of these colonies was scored after 24 hours.

D. Crude assay for β-lactamase enzymatic activity

Many assays of the enzymatic activity of β-lactamase exist. We modified the assay described by O'Callaghan et al., (1972) which uses the chromogenic lactam, nitrocefin. Nitrocefin is yellow, but when it is cleaved by β-lactamase, the cleavage product is red. To test plaques for the presence of β-lactamase activity a 20-50 µl sample of a solution of nitrocefin (50 µg nitrocefin/ml 0.1 M PO_4 pH 7) was dropped onto the desired plaque. The amount of β-lactamase present in a plaque from a phage carrying a bla^+ gene was sufficient to turn the nitrocefin from yellow to red within a minute after the drop had been applied to the plaque. When the plaques produced by different bla^- phage were tested, they varied in the time that they took to turn the drop of nitrocefin red. Some mutant phage plaques took only an additional 30 seconds longer than wild type to turn nitrocefin red, while others took as long as 2 hours. In general a direct correlation existed between the biological activity of β-lactamase as measured by the ability of lysogens to grow on plates containing different concentrations of ampicillin and the crude enzymatic activity of β-lactamase as measured by the nitrocefin test.

E. Mutagenesis of P22bla

To identify the mutagen used to isolate an allele, we included the following letters in the name of the allele; H=hydoxylamine, I=ICR-191, U=ultraviolet light and B=bisulfite. If the allele was suppressible we

included the following abbreviations in the allele name (am=amber, oc=ochre and fs=frameshift) to identify the nature of the suppressor.

Hydroxylamine mutagenesis of the phage P22bla was carried out in vitro according to the procedure of Davis et al. (1980). The phage were mutagenized until only 1% of the phage initially present survived. 1% of the viable phage gave clear plaque morphologies.

To mutagenize P22bla with the frameshift inducing mutagen ICR-191, we used the procedure of Uomini and Roth (1974). 0.3 ml of a fresh 0.5 mg/ml solution of ICR-191 was added to 5 ml of a culture of DB7000 growing exponentially in L broth. Immediately enough P22bla was added to give a final multiplicity of infection of 1. After 90 min, the culture was chloroformed. At this level of mutagenesis, less than 1% of the phage in this mutagenized lysate gave a clear plaque morphology.

P22bla was also mutagenized with ultraviolet light. A stock of P22bla was irradiated with ultraviolet light at 254 nm to 0.05% survival. 1×10^4 mutagenized phage were added to a culture of DB7618 growing exponentially at $37^{\circ}C$ in clarified casamino acids. A few minutes prior to the addition of the phage, the culture of DB7618 was irradiated with 14 joules/m^2 of 254 nm light. The presence of the plasmid PKM101 in the strain DB7618 was essential because it stimulates mutagenesis of UV-induced lesions (McCann et al., 1975; Mortelmans et al., 1976). 90 minutes after the infection was started, the culture was chloroformed. Between 1-2% of the phage from this mutagenized lysate gave clear plaque morphologies.

F. Isolation of bla- mutations

A mutagenized lysate of P22bla+ phage was screened by the red plate test (described above) for the presence of phage which lacked bla function. Putative P22bla- phage were purified and were retested for the absence of bla function by the crude lysogen test and by the spot nitocefin assay (see above). P22bla phage which failed these two tests were saved as bla- phage. To insure the independence of bla- mutations isolated by UV and by ICR-191 many independent mutagenized lysates were made and approximately 1,000 plaques from each lysate was screened for a bla^- phenotype. More than one bla- phage was saved from each lysate only if we could distinguish between these mutations by their ability to be suppressed or their map position (see below).

G. Isolation of signal sequence mutations

The amino terminal portion of the bla gene on pBR322 was subjected to localized mutagenesis by in vitro methods. A Taq 1 restriction fragment of pBR322 that includes the first 135 bp of bla was used to make D-loops with supercoiled pBR322. The formation of supercoils was catalyzed by the recA protein. The D-loops were nicked with S1 nuclease to produce relaxed circular molecules. The nicks in these molecules were enlarged to gaps with Micrococcus luteus DNA polymerase 1. The gapped molecules were mutagenized with the single stranded specific mutagen bisulfite, which causes C to U base changes. The gaps in the molecules were filled in and

the mutagenized DNA was used to transform a strain of Escherichia coli to tetracycline resistance. bla mutants were recovered on the plasmid which conferred upon the host strains resistance to less than 500 µg/ml of ampicillin and which mapped to the first 25 amino acids of the protein (23 of which make up the signal sequence). For the details of these procedures see Shortle et al. (1980).

These bla$^-$ mutations were transferred from the bla gene on pBR322 to the bla gene on P22bla. The plasmids harboring the signal sequence mutations were isolated and used to transform the Salmonella strain, DB4566, to tetracycline resistance. P22bla phage were grown on the DB4566 strains harboring the plasmids and the progeny phage were screened by the red plate test for the appearence of bla$^-$ phages. If the bla mutant on pBR322 conferred substantial ampicillin resitance to DB4566 then an alternate procedure was used. P22bla containing the bla deletion 218 were grown on DB4566 harboring the plasmid. The recombinant phage which carried the leaky mutations in place of the deletion 218 were selected from the progeny phage by their ability to transduce DB5532 to ampicillin resistance (25µg/ml) by the quantitative lysogen test.

Some of the in vitro induced mutations when transfered to the phage conferred minimal ampicillin resistance (less than 10µg/ml) to a strain lysogenic for that phage and subsequently were shown to harbor a base substitution and a frameshift mutation (Shortle and Botstein, 1981). P22bla phage that were phenotypically Bla$^+$ were selected from a stock of phage harboring the bla double mutants by using this stock to transduce DB7000 to ampicillin resistance. The transduction was done according to

the quantitative lysogen test which is described in detail above. The selection was done at varying concentrations of ampicillin (25, 200 and 1000 μg/ml). Ampicillin resistant transductants were purified and the P22bla prophage of each transductant was induced by ultraviolet light. Phage from individual plaques were purified and retested for their bla function by the crude lysogen test (see above). Phage stocks were made of P22bla phage that exhibited substantially greater bla activity by this test than the parental double mutant.

H. The determination of the nucleotide sequence of signal peptide mutants

Large stocks of P22bla were grown and concentrated by the following procedure. 20ml of a fresh saturated culture of DB7000 and 3 x 10^8 phage were added to one liter of LB broth in a 6 liter flask, and the flask was shaken vigorously for 16 hours. The cells were removed by centrifugation in the Sorvall and the phage were precipitated from the supernatant with polyethylene glycol. The phage were purified further by banding them on block density gradients in the ultracentrifuge (Davis et al., 1980). DNA was prepared from these purified phages by phenol extraction (Botstein, 1968). 120μg of phage DNA were digested with the restriction endonuclease, BamH1. The fragments were separated by gel electrophoresis on a 0.8% agarose gel run in E buffer (Davis et al., 1980). The 3.3 kb fragment was purified from the gel by the hydroxyappetite procedure(Davis et al., 1980). It was then digested with the restriction endonuclease,

Taq1, and the digested DNA was extracted with phenol and precipitated with ethanol. The 3' ends of the DNA produced by this digest were labeled specifically with ^{32}P-dCTP and Micrococcus luteus DNA polymerase 1 (Maxam and Gilbert, 1980). The labeled DNA was extracted with phenol and was percipitated with ethanol. Then it was digested with the restriction endonuclease, Msp1, and extracted with phenol. The labeled fragments were separated by gel electrophoresis on a 4% acrylamide gel (1:20 cross-linking). The fragment containing the bla gene was identified by its size (512 base pairs) which was determined from the nucleotide sequence of Tn3. This fragment was purified from the gel (Maxam and Gilbert, 1980). The nucleotide sequencing reactions and sequencing gels were performed on this fragment as described by Maxam and Gilbert (1980).

I. Screening bla- phage for conditional bla- alleles

P22bla- phage were screened for ts, amber, ochre and frameshift mutations by modifying the crude lysogen test (see above). The ts phenotype of each bla allele was investigated by testing the resistance of lysogens of DB7000 found at the center of plaques as described above except lysogens from each of these plaques were transferred to two plates containing ampicillin, one of these plates was incubated at 30°C and the other at 40°C. Phage which transduced DB7000 to ampicillin resistance at 30°C but not 40°C harbored heat sensitive alleles (ts) of bla.

We examined bla$^-$ mutations for a suppressible bla phenotype by plating the P22bla$^-$ phage onto a lawn of Su$^+$ bacteria as well as a lawn of

Su^- bacteria. Lysogens found at the center of these plques were tested for their drug resistance (see above). Those phage which transduced Su+ bacteria but not Su^- bacteria to ampicillin resistance contained suppressible bla- alleles. Lysogens of P22bla^- phages were made in the amber suppressing backgrounds, SupD, SupE, SupF, and SupJ, and the ochre suppressing backgrounds, SupG and SupC (Winston et al., 1979) for all bla^- mutations induced by ultraviolet light or hydroxylamine. Lysogens of P22bla^- phages were made in the frameshift suppressing backgrounds, SufA and SufD only for those bla^- mutations isolated by ICR191.

J. Generation of deletions of the bla gene from DB7606

DB7606 contains a P22 prophage which carries immmC of phage L, Tn10 (which specifies tetracycline-resistance, Tc10) and Tn1 (which specifies ampicillin resistance, Ap30). Like all P22 prophage carrying large transposons and no compensating deletion, the prophage genome in DB7606 is too large to be contained in a single particle. Phage particles are made after induction, but they contain incomplete permuted genomes lacking terminal repetition. However, a few phage particles will contain genomes with Tn10 generated deletions of the P22 late operon which simultaneously restore terminal redundancy and the ability of the phage to lysogenize upon single infection. These deletions can be selected from the phage population and propagated by using the induced lysate to transduce a sensitive strain to tetracycline resistance at a low multiplicity of infection (Chan et al., 1972; Weinstock et al., 1979).

To construct deletions of the bla gene, an exponential culture of the lysogenic strain, DB7606, was irradiated and the induced phage were harvested (Chan and Botstein 1972). These phages were added to an exponential culture of DB7000 at a multiplicity of infection of 10^{-2} to 10^{-3} and the mix was allowed to sit for 70 min at 37°C. The infected cells were spread on tetracycline plates (25 µg/ml). Tetracycline resistant colonies were replica plated to ampicillin plates (25 µg/ml). Ampicillin-sensitive tetracycline-resistant colonies were tested by cross-streaking (Susskind et al., 1971) for their ability to rescue markers flanking the Tn1 insertion in DB7606. The gene order in the prophage map of DB7606 is gene 8-Tn1-gene 1-Tc10. We used the markers amN18, the left-most marker in gene 8, and amN123, the right-most marker in gene 1. Tetracycline resistant and ampicillin sensitive colonies which rescue N18 but not N123 were deletions with endpoints most likely in the Tn1 element and potentially in the bla gene. All these candidates were saved and tested for their ability to reocmbine with bla^- mutations.

K. Mapping

1. Spot deletion mapping

A rapid method for detecting recombination between many point mutations and a deletion was developed. The method employed an aluminum block containing 32 wells and a 32 pronged device. 0.3 ml of a 10^9 phage per ml stock of each bla^- phage to be tested was delivered into a separate

well. With the pronged device liquid was transferred simultaneously from each one of these wells to a λ plate which had been overlayed with 2.5 ml soft agar. The soft agar contained 0.1 ml of an exponential culture of the bacterial strain which harbored the bla deletion to be tested. The pronged device was washed in ethanol and flamed twice. With the pronged device once again sterile, the same 32 phage suspensions could be transferred to another λ plate spread with a different bla deletion to be tested. The next day 32 phage spots, containing lysogens and phage, appeared on these plates. In this way bla point mutations were grown permissively on strains harboring bla deletions.

To score bla+ recombinants a portion of these spots was transferred with the 32 prong device to a LB agar which contained ampicillin at 250 µl/ml and which was overlayed with a mix of 0.1 ml of an exponential culture of DB7000 and 2.5 ml of soft agar. After each transfer the pronged device was washed in ethanol and flamed twice. The appearance of ampicillin resistant colonies in the spots after 20 hours at 30°C indicated the presence of bla+ recombinants. These ampicillin resistant colonies can arise in two ways. The recombinant event between the bla- point mutation on the phage and the bla deletion can result simultaneously in lysogeny and restoration of bla^+ function. These colonies should be represented among the lysogens at the center of the spot on the λ plate. An additional recombination event can result in restoration of bla^+ function on the progeny phage. These phage should transduce the cells of DB7000 present on the ampicillin plate to ampicillin resistance.

A more quantitative and sensitive method for measuring the bla$^+$ recombinantion frequency involved growing the phage carrying the bla$^-$ point mutations in the strain harboring the bla$^-$ deletion as described in the previous paragraph. Then in this test a plug is removed from the center of the spot with a sterile capillary tube and the phage are resuspended in 1ml of buffered saline and a drop of chloroform. Recombinant bla+ phage from this crude lysate are scored by the quantitative lysogen test (see above). A comparison of this method with the more crude method used to map the majority of bla$^-$ point mutations indicated that the crude method was capable of detecting 1 recombinant per 10^5 progeny phage.

2. Two factor phage crosses

When two bla$^-$ alleles are carried by P22bla, the most sensitive method for determining whether or not they recombine to give bla$^+$ progeny is a standard phage cross (Gough and Levine, 1966; Botstein and Matz, 1970). To enhance the sensitivity of detecting recombinants the standard procedure was modified by performing the crosses in a cya$^-$ strain, 7572. This mutation dramatically increased the burst size of P22 ImmC$^+$ phage. The number of P22bla+ recombinants in a cross was determined by the quantitative lysogen test (see above).

III. Biochemistry

A. Labeling of phage-encoded proteins after infection

All infections were with Salmonella typhimurium strain DB4381 [hisG46 del(bio-uvrB)], whose UvrB phenotype allows suppression of host specified protein syntheses at a relatively low dose (Botstein et al., 1973). DB4381 cells growing exponentially at a density of 2 X 10^8/ml in supplemented M9 minimal medium were concentrated 2.5 fold by centrifugation and resuspension in growth medium. The cells were then irradiated with 270joules/M^2 of 254 nm light. 2 ml aliquots of irradiated culture were added to a prewarmed (37°C) flask containing 3 ml of growth medium containg phage at the desired multiplicity of infection (usually 10 phage per cell). After incubation for 40 minutes, 1 ml aliquots were removed to microfuge tubes containing 50μCi uniformly labeled ^{14}C amino acids or 100μCi of ^{35}S-methionine. After a 3-5 minute period of incubation at 37°C, excess unlabeled amino acid (enzymatic casamino acids; Nutrirional Biochemicals) was added and the tubes were centrifuged for 2 minutes. The cell pellets were frozen in liquid nitrogen and stored frozen until analysis, when they were resuspended in sample buffer (Laemmli, 1970) and processed as described below.

B. SDS-polyacrylamide gel electrophoresis and autoradiography

Slab gels (Studier, 1973) were prepared using the stacking system and buffers of Laemmli (1970). Gels were fixed in 10% acetic acid (sometimes containing Coomassie blue) before drying in a heated apparatus (Hoefer) under vacuum. Dried gels were exposed to preflashed Kodak XR5 film at room temperature (Laskey and Mills, 1975). Autoradiograms were traced and the areas under the peaks were integrated using a Zeineh Soft Laser Scanning Densitometer (Biomed Instruments, Chicago). Several exposures of a gel were scanned to verify linearity of response.

C. Pulse-chase experiments

Strain DB4381 was grown and irradiated as before. Infected cells were labeled 30 or 40 minutes after infection by adding 0.5 ml to a centrifuge tube containing 250 µCi of ^{35}S-methionine. After 30 seconds, 50 µl of chase mixture (10% casaminoacids, 10mg/ml methionine) or 5µl of a concentrated solution of chloramphenicol (20 mg/ml solution in 50% ethanol) was added. Samples were taken immediately and every 30 seconds thereafter by transferring an aliquot to microfuge tubes containing an equal volume of 2 fold concentrated sample buffer preheated to 90^{o}C. In experiments with the signal peptide mutants of bla, the aliquots were removed and boiled in sample buffer at the indicated time points.

D. Cell fractionation procedures

1. Fractionation procedure for periplasmic proteins

(i) Osmotic shock: The method used was adapted for Neu and Heppel (1965). Labeled infected cells were centrifuged in an Epppendorf centrifuge for 2 minutes and the pellet was put on ice. All subsequent steps were carried out at 4°C. Pellets were resuspended in 0.15 ml cold solution containing 20% sucrose, 10 mM Tris-HCl (ph 7.5). Then 5 µl of 0.5M EDTA (pH 8) were added and incubation on ice was continued for 10 minutes. One third of the sample was removed and saved as the untreated control. The supernatant fluid was quickly removed and the pellet was rapidly resuspended by vigorous agitation 0.1 ml cold distilled water. The mixture was incubated for 10 minutes on ice and then centrifuged again for 5 minutes. The supernatant was removed and saved as the cytoplasmic/membrane-bound fraction. 2X sample buffer was added to each tube, and samples were heated to 90°C for 2.5 minutes and applied to an SDS-polyacrylamide gel as above.

(ii) Conversion of labeled infected cells to spheroplasts: The method used was adapted from Witholt et al. (1976). Labeled infected cells were centrifuged in an Eppendorf centrifuge for 2 minutes and the pellet was put on ice. All subsequent steps were carried out at 4°C. Pellets were resuspended in 75µl of cold H_2O. The sample was incubated for 25 minutes on ice. The success of spheroplasting was monitered by the roundness of cells and the extent of lysis after dilution as judged by phase contrast

microscopy. One third of the sample was saved as untreated control. To stabilize spheroplasts, 3µl of 1M $MgCl_2$ were added; cells were centrifuged in a microfuge centrifuge for 5 minutes. The supernatant was saved as the periplasmic fraction; the residual fluid was carefully drained off and the remaining pellet was resuspended in 0,1 ml water and saved as the cytoplasmic membrane fraction. 2X sample buffer was added to each tube, and samples were heated to 95°C for 5 minutes and applied to an SDS-polyacryamide gel as described above.

2. Membrane isolation

The method used was adapted from Silhavy et al. (1976). Infected and labeled cells were converted to spheroplasts as described above, except the $MgCl_2$ was omitted. After the spheroplasts had been centrifuged and the supernatant removed, they were rapidly resuspended in 100 µl H_2O. 250µl of a saturated culture of DB4381 cells lysed by the identical method were added to this mixture to serve as carrier. The procedure disrupted more than 90% of the cells as judged by examination in the microscope. The remaining intact cells were removed by centrifugation of the extracts at 1000 X g for 10 minutes, and the lysates were fractionated by centrifugation at 100,000 X g for 2 hours in a Beckman ultracentrifuge. The membrane pellet was not washed before resuspension in sample buffer to avoid disturbing any weak protein membrane interaction.

3. Pulse-chase cell fractionation

The purpose of this procedure is to examine the localization of bla encoded proteins by cell fractionation as a function of time. A culture of DB4381 was grown and irradiated as before. The culture was infected with P22bla$^+$ or a bla- derivative at a multiplicity of 20. 30 minutes after the infection began, 2ml of culture were labeled with 400µCi of ^{35}S-methionine for 30 seconds. 200µl of 10mg/ml of cold methionine were added. At the appropriate time intervals 500µl of labeled culture was transferred to Eppendorf tubes which were pre-chilled to 0°C in an ice-water bath. All subsequent steps were performed at 4°C.

Each 500µl aliquot was separated into three fractions (membrane, periplsmic and cytoplasmic) by the following procedure. The labeled cells were converted to spheroplasts as described above. The spheroplasts were pelleted in the Eppendorf centrifuge and the supernatant was saved as the periplasmic fraction. The spheroplasts were lysed, and the membrane and cytoplasmic fractions were separated from each other as desribed above.

E. Trypsin accessibilty experiments

1. Pulse chase trypsin accessibility experiments

This procedure allows one to follow, as a function of time, the appearance of bla encoded proteins on the periplasmic side of the inner membrane whether they remain associated with the inner membrane or become

soluble periplasmic proteins. A culture of DB4381 was grown and irradiated as before. The culture was infected with P22bla or a bla$^-$ derivative at a multiplicity of infection of 10. 30 minutes after infection 2ml of culture were labeled with 400µCi of ^{35}S-methionine for 30 seconds. 200µl of 10mg/ml cold methionine or 20µl of choloramphenicol (20mg/ml) were added. Approximatiely 20 seconds after the addition of the chase 1.0 ml of labeled culture was removed to an microfuge tube which was pre-chilled to 0°C in an ice-water bath. After 5 minutes the remaining 1.0 ml of labeled culture was transferred to a second tube prechilled to 0°C. In experiments in which the bla alleles, pro_{20}->ser or fs(7,9) were used, the second ml of labeled culture was not transferred to ice until 10 minutes after the chase had begun.

The cells in each aliquot were converted to spheroplasts as described. The final volume was 200µl. 25µl aliquots were transferred to four tubes. 2.5µl of 0.1M Tris pH8, 2.5µl of 2mg/ml trypsin in 0.1M Tris pH8, 2.5µl of 500µg/ml trypsin in 0.1M Tris pH8 and 2.5µl of 50µg/ml trypsin in 0.1M Tris pH8 were added to the four tubes respectively. Trypsin digestion continued for 10 minutes on ice. After 10 minutes the 27.5µl of digest were transferred to a tube containing 50µl of 1.5 fold concentrated sample buffer preheated to 95°C. The remaining 100µl of spheroplasts were centrifuged. The supernatant was removed and saved as the periplasmic fraction. The spheroplasts in the pellet were lysed by resuspending them in 100µl of H_2O. The 100µl of lysed spheroplast (minus the periplasmic fraction) were divided into 25µl aliquots and subjected to trypsin digestion exactly as desribed above. Note that the trypsin digestion for

lysed spheroplasts and intact spheroplasts are done in slightly different solutions. When these experiments were repeated such that the lysed spheroplasts were treated with trypsin in the same identical solutions the results were not affected.

The validity of the conclusions obtained from these experiments depended upon the success of two procedures, the converson of the labeled cells to intact spheroplasts and the lysis of these spheroplasts. In all the experiments reported below the conversion of cells to spheroplasts (greater than 90%), the intactness of those spheroplasts (80% to 95%) and their subsequent lysis (80% to 90%) were judged by examination of the cells by phase contrast microscopy. Consistent with these observations was the correlation between the cell fractionation of non bla encoded species and the extent of their digestion with trypsin in these experiments. Essentially all labeled proteins not encoded by bla fractionated as soluble in the cytoplasm. When labelled cells were converted to spheroplasts and treated with trypsin 80% or more of each labeled non bla encoded protein was recovered intact as measured by comparing the intensity of the labeled bands from spheroplasts treated with no trypsin and increasing amounts of trypsin (figure 15, lanes a, b, c, d, f, g, h and k; also see figures 16, 20 and 21). The majority of cellular proteins that were stained by Coomassie blue were not digested by trypsin when cells were converted to spheroplasts. The exception, a major staining band, may be the product of the Salmonella ompA gene because the product of this gene in Esherichia coli was shown to be sensitive to trypsin under these conditions (Inouye and Yee, 1972). In

contrast when the spheroplasts were lysed and then treated with trypsin less than 10% of the label in non bla encoded proteins was recovered (figure 15, lanes e and f). In addition many of the Coomassie blue stained proteins were now absent.

2. Trypsin accessibility experiments of cells treated with phenethyl alcohol (PEA)

These experiments were done exactly as described for the pulse chase trypsin accessibility experiments with the following modifications. At 20 minutes after the infection began, 0.5ml of the irradiated and infected cells were transferred to a tube containing 15µl of a solution which was 10% PEA and 20% ethanol. 15µl of 20% ethanol were added to .5ml of culture as a control. 30 minutes after infection the cells in the two cultures were labeled for 30 seconds then cold methionine was added as described above. 5 minutes after the addition of the chase the tubes were removed to ice and a standard trypsin accessibility experiment was performed.

F. Determination of the partial amino acid sequence of the amino terminus

Cells were irradiated and infected as described above. Thirty minutes after infection 1.0ml of these cells was labeled for 3 minutes with either 100 µCi of L-[3,4(n)-^{3}H] valine and 100 µCi of L-[2,3-^{3}H] alanine (to

determine the partial amino acid sequence of the mature species), 100 μCi of L-[2,6-^{3}H] phenylalanine and 100μCi of L-[4,5-^{3}H] isoleucine (to determine the partial amino acid sequence of the precursor species) or 100μCi of ^{35}S-methionine (to act as a marker, see below). They were centrifuged in a microfuge, and the supernatant was discarded. The pellet was resuspended in 50μl of sample buffer and heated to 95°C for five minutes.

To purify the ^{3}H-labeled bla encoded proteins the following steps were taken. 20μl of the sample that contained the ^{3}H-labeled proteins were loaded into a lane of a SDS polyacrylamide gel. The lanes flanking this lane were loaded with 10μl of cells treated identically to the ^{3}H-labeled cells but labeled with ^{35}S-methionine instead. The stacking gel and running gel were poured 24 hours in advance. After the electrophoresis step was completed, the gel was soaked in 10% acetic acid for 5 hours and then dried down under vacuum (see above). The dried gel was marked with ^{14}C-radioactive ink and then used to make a 5-10 hour exposure of an autoradiogram. The gel and the autoradiogram were aligned by the ink spots. ^{3}H-labeled bla encoded proteins were not visible in the autoradiogram but the ^{35}S-methionine labeled bla encoded proteins were. The bands from the latter proteins were used to determine the position of the ^{3}H-labeled bla encoded protein in the gel. A slice of the gel containing the ^{3}H-labeled bla encoded proteins was removed from the gel with a razor. The gel slice was soaked overnight at 37°C in 1.0 ml of a solution containing 1% triethylamine, 100μg BSA/ml and 0.1% SDS. The supernatant containing the eluted protein was frozen at -20°C.

To determine the partial amino acid sequence of the gel-purified proteins, the ^{3}H-labeled bla encoded proteins were subjected to automated Edman degradation in a Beckman 890C sequencer using the 0.1M Quadrol program as described by Brauer et al. (1975). Aliquots of the products of the first 10-20 cycles were resuspenced in aquasol and counted in an LS-230 Beckman liquid scintillation counter. In some cases the labeled phenylthiohydantoin amino acid derivatives were identified by high performance liquid chromatography using a Waters C_{18} u-Bondapek column (Zimmerman et al., 1979)

Chapter 3: Genetics of β-lactamase

The fine structure genetic analysis of a gene such as bla presents three problems. First, one must be able to direct mutagenesis to the gene or to a particular subregion of the gene in order to facilitate the isolation of the desired mutations. Second, one must be able to confirm that the mutagenesis has been successful, that is the mutagenesis has produced mutations at the expected positions. Third, one needs to be able to distinguish among mutations which lie in the same region of the gene but at separate sites from mutations that affect the same site. The solution of these problems becomes more difficult as the target size of the region of interest becomes smaller. In this chapter we present a fine structure genetic analysis of bla in which novel genetic methods and classical genetic methods were used. The combination of these methods provided us with an efficient means to solve the problems outlined above.

I. Results

A. Tests for bla gene function

Before one can begin the genetic analysis of any gene, one needs assays to detect the presence and absence of gene function; thereby distinguishing between the wild type and mutant phenotype of that gene. Several tests for bla gene function were available to us. The wild type

Salmonella typhimurium strain, DB7000 is resistant to less than 1µg/ml of ampicillin. The introduction of a single copy of the bla gene into a cell of this strain allows it to grow on LB agar plates containing ampicillin up to at least 1 mg/ml. To test a particular allele for bla gene function we asked if a cell which harbored that allele could grow on LB agar plates in the presence of ampicillin (see Methods).

A more direct test for the presence of functional TEM β-lactamase uses the chromogenic substrate nitrocefin (O'Callaghan et al., 1972). Nitrocefin turns from yellow to red when cleaved by TEM β-lactamase. This reaction can be followed qualitatively by eye (see Methods).

No selection exists which allows one to isolate mutations which reduce the amount of bla gene activity. Both of the assays described above can be used to screen for the loss of bla gene function. However, using these assays to screen a large population of cells for the absence of bla gene activity is tedious. To circumvent this problem we developed a new biological assay which permits one to distinguish visually between phages with and without a functional β-lactamase gene (see Methods). In this assay phages which carry a functional β-lactamase gene produce red plaques while those which carry a non-functional β-lactamse gene are colorless (see Methods).

B. Isolation and characterization of mutations in bla

1. General mutations

The β-lactamase structural gene was subjected to mutagenesis by a variety of methods. P22bla was mutagenized with hydroxylamine, ICR-191 or ultraviolet light. Individual phages in these mutagenized populations were screened for the absence of bla function (see methods). Approximately 1 in every 500 phage from the mutagenized phage population carried a Bla^- phenotype. The majority of these mutations revert at a frequency of 1 in 10^6 to 10^8. Alternatively mutations isolated in vitro on pBR322 as described by Shortle et al. (1980) and Talmadge et al. (1980) were transferred to P22bla (see Methods). We then screened the entire collection of P22bla- phage for conditional bla- alleles (see Methods).

Table 2 summarizes these results. Mutagenesis by ultraviolet light and hydroxylamine yielded non-conditional mutations and conditional mutations (temperature sensitives, ambers and ochres). The distribution of bla mutations among these two categories is similar for the two mutagens. The frequency of temperature sensitive mutations is 23% for UV induced mutations and 24% for hydroxylamine. The frequency of nonsense mutations is 12% for UV induced mutations and 9% for hydroylamine induced mutations. Both mutagens produced more ochre mutations than amber mutations.

Further examination of this table shows that the ICR-191 induced

Table 2. Distribution of bla alleles into conditional and non-conditional groups

Mutagen	Non-conditional bla⁻ mutations	Conditional bla⁻ mutations					
		suppressible				ts	Total
		nonsense		frameshift			
		amber	ochre	sufA	sufD		
Hydroxylamine	107	4	9	-	-	35	146
Ultraviolet light	66	1	11	-	-	23	101
ICR-191	11	-	-	2	27	0	40

(-) means not tested.

mutants differ from UV and hydroxylamine in several ways. ICR-191 mutagenesis produced no temperature sensitive mutations. 72% of the independently isolated ICR-191 induced mutations are suppressible by one or the other of two frameshift suppressors.

2. Isolation and Nucleotide Sequence of bla Mutations in the signal sequence

Very few of the 300 point mutations isolated by the classical genetic methods described above mapped to the region of the gene encoding the signal peptide (section E). The few mutations that did map to this region of the gene were either frameshift or nonsense mutations, which in comparison with missense mutations, are not as useful mutations for studying the function of the signal peptide in the secretion of β-lactamase.

To try to increase the fequency of missense mutations in this region of the gene without increasing the level of mutagenesis (one hit per gene), we mutagenized the bla gene by a novel method (Shortle et al., 1980). This method allowed us to direct the mutagenesis of the bla gene on pBR322 to the first 40 amino terminal codons.

Some of these bla^- mutations made in vitro affected bla gene function and mapped to the signal sequence (Section D and E). They were transferred from the bla gene of pBR322 to the bla gene, P22bla (Methods). At the same time the nucleotide sequences of some of these mutations on pBR322 were determined. The lesions fell into two classes; mutations

consisting of a single base pair substitution and double mutations consisting of a single base pair substitution and the addition or loss of a nearby base pair (Shortle and Botstein, 1981).

The double mutants were unable to confer upon the Salmonella strain, DB7000 (Leu^-, Ap^S), resistance to ampicillin when these alleles were introduced into DB7000 by lysogenization with the appropriate P22bla$^-$ phage. Pseudo revertants of these mutations were obtained by selecting for phage from a stock of the double mutants which transduced DB7000 to ampicillin resistance (Methods). Since the original mutants contained two mutations, the probability of restoring the wild-type nucleotide sequence was extremely small. As a result these revertants were expected to contain either just the original point mutation (in those cases where the original frameshift mutation was corrected by a true reversion event), or an additional frameshift mutation (in those cases where the original frameshift mutation was corrected by a compensating frameshift mutation at a second site in the bla gene). The nucleotide sequence of the lesions of these mutants were determined (Methods) and the results are presented in Figure 3.

3. Sensitivity of test for bla funtion

The amount of bla function from a single lysogen of P22bla carrying one of six mutations which alter the signal sequence or one of several ts alleles was measured by the crude lysogen test (Methods). The data are summarised in Table 3. Clearly this test can make phenotypic distinctions

Table 3. Characterization of Bla phenotype of different *bla* alleles.

bla allele	Temp °C	Concentration of ampicillin in plate (μg/ml)													
		0		5		10		25		100		250		500	
		26	40	26	40	26	40	26	40	26	40	26	40	26	40
ocU8		+	+	-	-	-	-	-	-	-	-	-	-	-	-
H3		+	+	-	-	-	-	-	-	-	-	-	-	-	-
tsH1		+	+	ND		+	+	+	+	+	-	+	-	+	-
tsH2		+	+	ND		+	-	+	-	+	-	+	-	-	-
tsH7		+	+	ND		+	±	+	-	+	-	+	-	-	-
tsH9		+	+	ND		+	+	+	+	+	+	+	+	+	-
fs(14,21)		+	+	+	-	+	-	+	-	-	-	-	-	-	-
fs(18,21)		+	+	+	ND	+	ND	+	ND	+	-	+	-	-	-
pro_{20}→leu		+	+	+	+	+	+	+	+	+	-	+	-	±	-
fs(7,9)		+	+	+	+	+	+	+	+	+	+	+	+	+	+
pro_{20}→ser		+	+	+	+	+	+	+	+	+	+	+	+	+	+
ala_g→val		+	+	+	+	+	+	+	+	+	+	+	+	+	+
bla$^+$		+	+	+	+	+	+	+	+	+	+	+	+	+	+

P22 bla lysogens of DB7000 were purified and streaked onto LB plates containing varying concentrations of ampicillin. The growth of the cells on these plates could be divided into two categories, confluent and single colonies. + means growth of confluent cells and single colonies. ± means growth only of confluent cells and - menas no growth. ND means not done. The ± phenotype of sone lysogens at particular ampicillin concentration is apparently due to sparing. A single cell apparently cannot produce enought beta-lactamase to detoxify the plate. It dies and therefore fials to give rise to a colony. However, a group of cells (the ancestors to the confluent area) can produce enought beta-lactamase to detoxify the media and give rise to a confluent patch.

Allele

	1								10								20			
wt	met	ser	ile	gln	his	phe	arg	val	ala	leu	ile	pro	phe	phe	ala	ala	phe	cys	leu	pro	val	phe	ala
	ATG	AGT	ATT	CAA	CTT	TTC	CGT	GTC	GCC	CTT	ATT	CCC	TTT	TTT	GCG	GCA	TTT	TGC	CTT	CCT	GTT	TTT	GCT
pro_{20} → ser																				ser			
																				Tct			
pro_{20} → leu																				leu			
																				cTt			
fs(18,21)																		ala	phe	leu	phe		
																	(-T)	gcc	ttT	tgt	ttt	(+T)	
fs(14,21)																leu	arg	his	phe	ala	phe	leu	phe
															(-T)	ttg	cgg	cat	ttt	gcc	ttT	ctg	ttt	(+T)
ala_9 → val									val														
									gTT														
fs(7,9)						pro	cys	arg															
					(+t)	Ccg	tgt	cgt	(-C)														

Figure 3: Nucleotide sequences of the signal seqence alleles of bla. Alleles of bla that alter the signal peptide were isolated and their nucleotide sequences were determined as described in Methods. The bla- mutation, B501 has a GC to AT base pair substitution at the first base pair in codon 20 of the signal sequence plus an addidtional AT base pair in the run of AT base pairs in codons 21 and 22. The revertants of B501, fs(14,21), fs(18,21) and pro_{20}->ser, contain the original base pair substitution in codon 20, but restore the gene to the proper reading frame in three different ways. The bla mutation, B510, has an AT base pair substitution for one of the two GC base pairs in codon 9 and the loss of the other GC base pair of this same codon. Two bla+ revertants of this mutation, fs(7,9) and ala_9->val, restore the gene to the proter reading frame by two different means. The other signal sequence mutation, pro_{20}->leu, was obtained directly from bisulfite mutagenesis.

between different bla$^-$ ts mutations. In addition by these criteria the signal peptide mutants fall into three distinguishable classes. The bla alleles, fs(7,9), pro_{20}->ser and ala_9->val all confer upon DB7000 resistance to greater than 500µg/ml of ampicillin. The alleles, fs(18,21) and pro_{20}->leu, confer upon DB7000 resistance to 200 µg/ml of ampicillin, but this resistance is heat sensitive. Finally by this test the bla allele, fs(14,21), was found to confer upon DB7000 resistance to 25µg/ml which is also heat sensitive. We conclude that this test of the ability of a bla allele to confer upon a cell resistance to different levels of ampicillin is a sensitive assay of the biological activity of that allele and is capable of revealing subtle differences in phenotype between two alleles.

C. Generation of deletions in the bla gene from DB7606

Chan and Botstein (1972) showed that deletions of P22 could be selected from a phage population by taking advantage of the requirements for normal P22 growth and of the properties of the transposable element Tn10. We used these principles to make deletions of the bla$^+$ gene (see Methods). A prophage (DB7606) was constructed which included a Tn10 in the a1 region and a Tn1 insertion in the middle of the P22 late operon between genes 8 and 1. This lysogen was irradiated with ultraviolet light and the induced phages were used to transduce a DB7000 strain to tetracycline resistance (see Materials and Methods). The majority of these tetracycline resistant colonies contained defective prophages which

carried the tetracycline resistance genes but had deletions of the P22 late genes. We screened these deletions for the subset of deletions with endpoints in the bla gene (see Methods). From a pool of 8,000 tetracycline resistant colonies, we found 17 colonies which harbor prophage deletions with enpoints in the bla gene. By genetic criteria 6 of these 17 deletions are different from each other (see below).

D. Mapping of bla mutations

1. Deletion Mapping

Using a spot deletion mapping method (see Methods) bla- mutations on P22bla were grown permissively in strains which harbored bla deletions either on the chromosome or on the plasmid, pBR322, and bla+ recombinants were scored. The results of these mapping experiments are shown in Figure 4. Each deletion interval is defined by several independently isolated mutations with the exception of the interval defined by deletions 218 and 241. In most intervals one can find at least one mutation induced by UV, ICR-191 and hydroxylamine. However the distribution of suppressible frameshift mutations and ochre mutations in the different deletion intervals is clearly clustered. 7 out of the 11 ochre mutations induced by UV map to the most amino terminal interval. The nine ochre mutations induced by hydroxylamine map to two intervals, four in the interval defined by deletions 48 and the right end of deletion 2 and five in the interval defined by deletions 60 and 74. Finally the intervals defined by

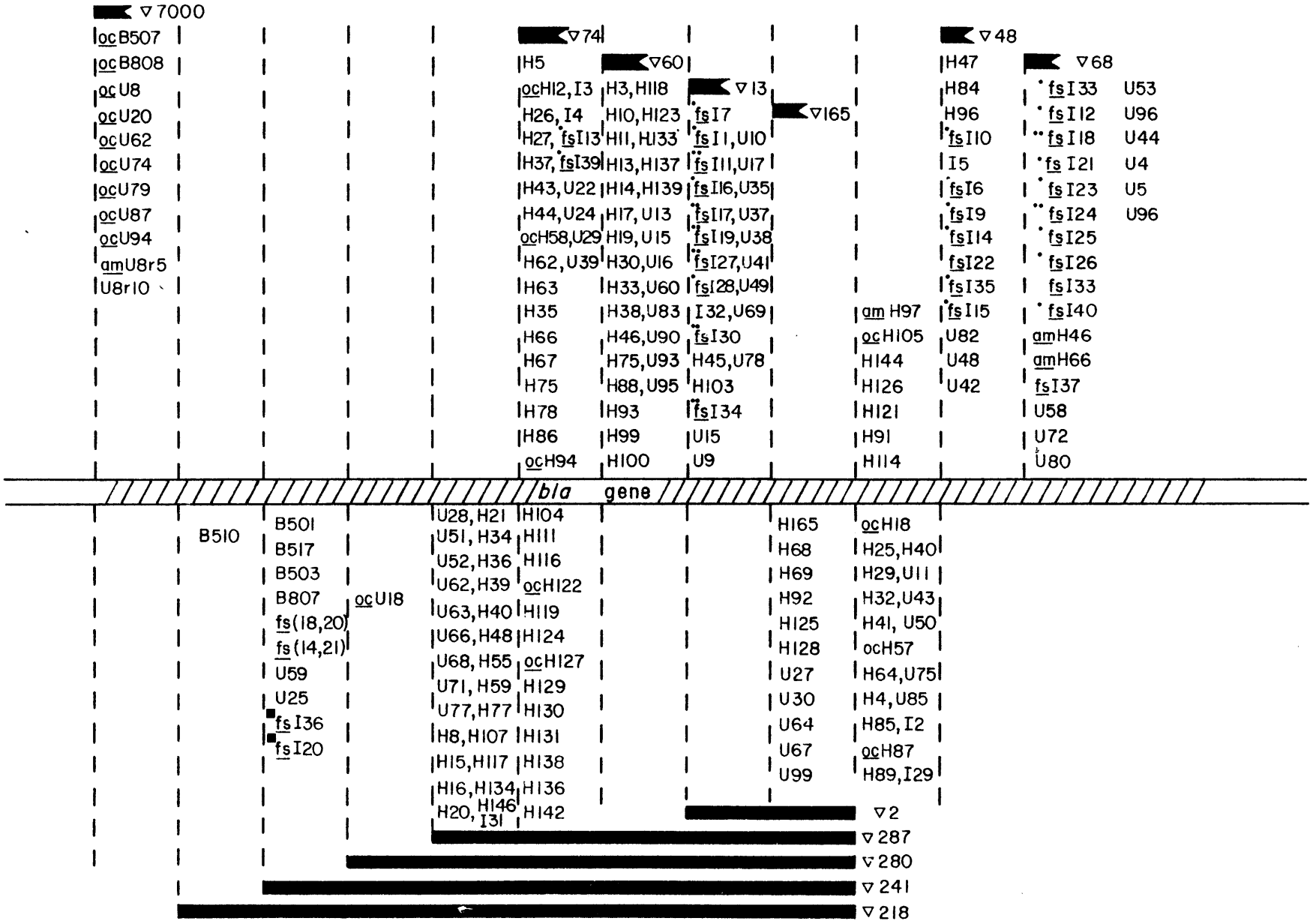

▽7000
ocB507
ocB808
ocU8
ocU20
ocU62
ocU74
ocU79
ocU87
ocU94
amU8r5
U8r10
▽74
H5
ocH12, I3
H26, I4
H27, fsI13
H37, fsI39
H43, U22
H44, U24
ocH58, U29
H62, U39
H63
H35
H66
H67
H75
H78
H86
ocH94
▽60
H3, H118
H10, H123
H11, H133
H13, H137
H14, H139
H17, U13
H19, U15
H30, U16
H33, U60
H38, U83
H46, U90
H75, U93
H88, U95
H93
H99
H100
▽13
fsI7
fsI1, U10
fsI11, U17
fsI16, U35
fsI17, U37
fsI19, U38
fsI27, U41
fsI28, U49
I32, U69
fsI30
H45, U78
H103
fsI34
U15
U9
▽165
amH97
ocH105
H144
H126
H121
H91
H114
▽48
H47
H84
H96
fsI10
I5
fsI6
fsI9
fsI14
fsI22
fsI35
fsI15
U82
U48
U42
▽68
fsI33
U53
fsI12
U96
fsI18
U44
fsI21
U4
fsI23
U5
fsI24
U96
fsI25
fsI26
fsI33
fsI40
amH46
amH66
fsI37
U58
U72
U80
bla gene
B510
B501
B517
B503
B807
fs(18,20)
fs(14,21)
U59
U25
fsI36
fsI20
ocU18
U28, H21
U51, H34
U52, H36
U62, H39
U63, H40
U66, H48
U68, H55
U71, H59
U77, H77
H8, H107
H15, H117
H16, H134
H20, H146
I31
H104
H111
H116
ocH122
H119
H124
ocH127
H129
H130
H131
H138
H136
H142
H165
H68
H69
H92
H125
H128
U27
U30
U64
U67
U99
ocH18
H25, H40
H29, U11
H32, U43
H41, U50
ocH57
H64, U75
H4, U85
H85, I2
ocH87
H89, I29
▽2
▽287
▽280
▽241
▽218

Figure 4: Genetic map of the bla gene. The phage P22bla+ was mutagenized with hydroxylamide (H), ultraviolet light (U), ICR-191 (I) or bisulfite (B). Phages which could not transduce wild-type S. typhimurium to ampicillin resistance were defined as having a Bla^- phenotype. These mutations were classified as amber (am), ochre (oc) or frameshift (fs) if a phage carrying a bla^- allele could stably transduce to ampicillin resistance Salmonella strains carrying the appropriate suppressors (Winston, Botstein and Millter, 1979). The frameshift suppressors (Roth, 1974) can only redirect the reading frame from the +1 frame to the proper reading frame. Therefore all suppressible frameshift mutations must alter the reading frame to the +1 frame. Mutant bla genes harboring reciprocal frameshift mutations were identified by determining their nucleotide sequence. The codons that these mutations affected are listed in parentheses following the letters fs, for example fs(14,21).

To generate deletions of the bla gene in vivo, we constructed a phage carrying both a Tn1 insertion and a Tn10 insertion at separate sites in the P22 phage genome. Taking advantage of properties of P22 and the transposable element Tn10, we recovered deletions which start at the Tn10 element and extend into the bla gene (Chan et al., 1972). The in vitro deletion (designated 2) which removes the DNA between the Pst and Pvu restriction sites was constructed in our laboratory (Shortle et al., 1980). All other in vitro deletions (218, 280, 287 and 241) were given to us by K. Talmadge (Talmadge et al.,1980).

deletions 165 and 13 and by deletion 68 harbor 20 of the 27 suppressible frameshift mutations. The reasons for the clustering of these mutations will be discussed later.

2. Two factor crosses

Two factor crosses were performed between two point mutations in bla which mapped to the same deletion interval or between a point mutation and a deletion endpoint which were suspected of being very tightly linked. Two phages carrying different bla alleles were crossed and the bla$^+$ recombinant phage were scored by the quantitative lysogen test (see Methods). As few as one recombinant per 10^7 phage was detected by this test.

Two factor crosses were used to analyze in more detail putative point mutations which mapped in the amino terminal region of the protein (see correlation of physical and genetic map). These mutations were crossed against each other and against phage which contained the deletions of known nucleotide sequence (Talmadge et al., 1980). Subsequently the changes in the nucleotide sequence in many of these putative point mutations were determined (Shortle and Botstein, 1981; this work). Knowledge of the nucleotide sequence of each mutant not only confirmed this fine structure mapping but it also allowed us to examine the resolution of this fine structure mapping method. These data are presented in Table 4 and Figure 5.

Table 4. Two-factor crosses for mapping *bla* alleles.

Cross	*bla* alleles	[a]Recombination frequency ($\times 10^{4}$)	[b]Reversion frequency ($\times 10^{4}$)	[c]Base pairs between alleles
1	*oc*B507 x ∇218	.024	<.01	2
2a	*oc*H18 x *oc*H87	.04	.005	2
b	*oc*H57 x *oc*H87	.09	<.003	2
c	*oc*H105 x *oc*H87	.04	.005	2
3a	*oc*U18 x ∇287	.7	<.02	5
b	*oc*U18 x ∇280	<.01	<.005	
4a	*oc*U122 x *oc*H12	.8	<.003	5
b	*oc*U122 x *oc*H58	.7	<.006	5
c	*oc*U122 x *oc*H94	.9	<.007	5
5a	*oc*H18 x *oc*H57	.005	.005	-
b	*oc*H57 x *oc*H105	.004	.004	-
c	*oc*H18 x *oc*H105	<.02	.005	-
6a	*oc*H12 x *oc*H58	<.01	<.01	-
b	*oc*H12 x *oc*H94	<.005	<.005	-
c	*oc*H12 x *oc*H127	<.01	<.01	-
d	*oc*H58 x *oc*H94	<.006	<.006	-
e	*oc*H58 x *oc*H127	<.004	<.004	-
f	*oc*H94 x *oc*H127	<.005	<.005	-

7a	*oc*U8 x *oc*U74	<.001	.001	-
b	*oc*U8 x *oc*U20	<.005	<.008	-
c	*oc*U8 x *oc*U62	<.006	<.006	-
d	*oc*U20 x *oc*U62	<.001	<.007	-
e	*oc*U87 x *oc*U79	<.007	<.007	-
f	*oc*B507 x *oc*U8	<.006	<.01	-
g	*oc*U8 x *am*U8r5	<.001	<.008	-
h	*oc*U8 x U8r10	<.003	<.003	-
8a	*am*H46 x *am*H61	.08	.08	-
b	*am*H46 x *am*H66	1.0	1.0	-
c	*am*H61 x *am*H66	.6	1.0	-
9	*fs*I36 x *fs*I20	<.006	<.006	-
10	V241 x B510	<.4	<.005	8
11	*fs*(14,21) x V280	2.0	<.004	13
12	*fs*(18,21) x V280	5.7	<.005	13
13	B501 x V280	4.0	<.01	13
14	B517 x V280	10	<.004	13
15	pro_{20}→leu x V280	5.0	<.003	15
16	" "	4.0	<.003	15
17	*oc*U8 x V241	2.0	<.03	25
18	B501 x B510	.7	<.05	29
19	B510 x B517	.7	<.03	31
20	B510 x V280	5.0	<.01	47
21	U8r10 x B517	6.0	<.01	48,49,50
22	B501 x *oc*B507	2.0	<.02	51
23	*oc*B808 x V280	10	<.01	64
24	*oc*B507 x V280	20	<.01	64
25	*fs*(14,21) x *am*H97	12	<.004	500
26	H8 x *oc*H87	6.0	<.01	<400

[a] Recombination frequency is expressed as the frequency of P22 bla$^+$ progeny phage over the total progeny P22 bla phage.

[b] The reversion frequency of each parental allele was measured and is expressed as the frequency of P22 bla$^+$ progeny phage over the total progeny P22 bla phage. The reversion frequency of the parental allele with the higher reversion frequencies is listed.

[c] Base pairs between alleles as determined by DNA sequencing and physical correlation between physical and genetic map (see below).

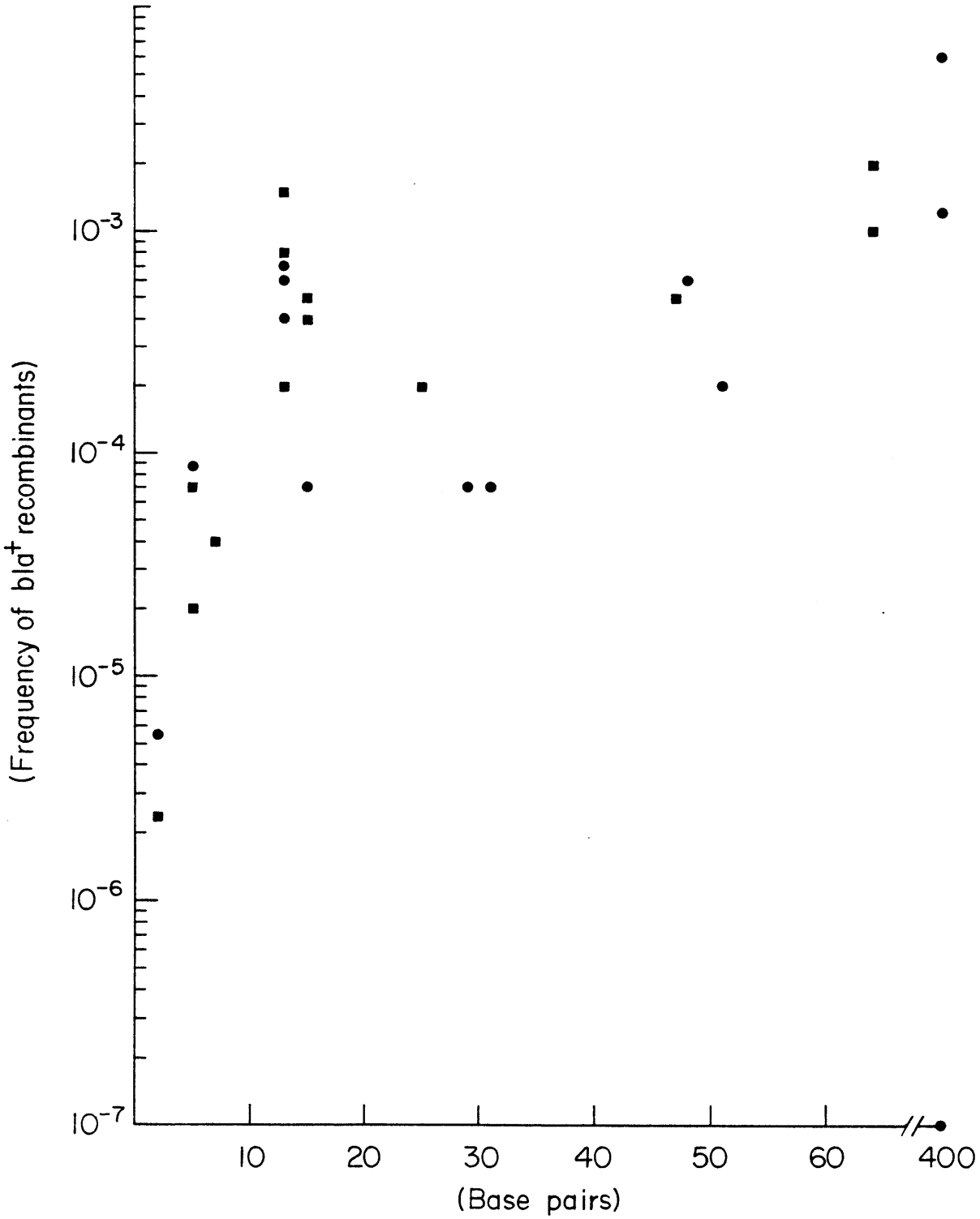

(Frequency of bla+ recombinants)
10-3
10-4
10-5
10-6
10-7
10
20
30
40
50
60
400
(Base pairs)

Figure 5: The correlation between recombination frequency and physical distance in bla. The frequency of bla+ recombinants between alleles at known positions is plotted as a function of the nucleotide base pairs that separate them. The position of each mutation was determined by nucleotide sequencing or by methods described in this work (see text and figure 6). The recombination frequencies between the various alleles are presented in Table 4. For most pairs of alleles, only one cross was performed to determine the recombination frequency between them. In those cases where the same cross was performed twice (Table 4; crosses 15 and 16, and crosses 23 and 24) the recombination frequency erred by no more than two fold. , point x point; point x deletion.

The data in Table 4 and figure 5 show that we can resolve two mutations which lie very close together. When alleles are separated by as few as two bases bla+ recombinants were detected at a frequency of .0002% (Table 4, crosses 1 and 2a-c).

Table 4 and figure 5 also show that the recombination frequency between two bla alleles increases as the number of nucleotides between them increases. Mutations separated by 400 nucleotides gave bla+ recombinants at a frequency of .2% to .6% of the total progeny. When alleles were separated by distances intermediate between 2 and 400 bases, in general intermediate recombination frequencies were obtained. However two mutations separated by only 13 bases gave a high frequency of bla+ progeny (Table 4, cross 14). Therefore, one must be careful in using two factor crosses to order mutations in bla or to judge physical distances between bla alleles because the correlation between physical distances and recombination frequencies is not absolute.

Further examination of figure 5 reveals that we can compare the recombination frequency between two alleles as a function of the type of allele. For a given distance the recombination frequency between two point mutations and between a point mutation and deletion are approximately the same, even though these deletions are at least 400 bases long. The recombination frequency between two point mutations separated by two bases (Table 4, cross 2a-c) is only two fold greater than the recombination frequency between a point mutation and deletion separated by two bases (Table 4, cross 1). We conclude that the recombination frequency between two alleles of bla is not affected drastically if one of

the alleles is a point mutation or a deletion.

Having established that two factor crosses could resolve bla alleles separated by as few as two bases, we performed several crosses to investigate if each of the clusters of ochre mutations represented mutations at more than one site, or if these clusters represented multiple isolates at the same site. Many of the ochre mutations in each cluster were crossed against each other. Six of the seven UV induced ochre mutations in the most amimo terminal interval fail to recombine with each other (Table 4, crosses 7a-g). These data suggest that these six UV induced ochre mutations represent multiple isolations of a single mutation at the same site. However clusters of hydroxylamine induced ochre mutations gave different results. ocH87 recombines with ocH18, ocH57 and ocH105 (Table 4, crosses 2a-c). ocH18, ocH57 and ocH105 fail to recombine with each other (Table 4, crosses 5a-c). ocH122 recombines with ocH12, ocH58 and ocH94 (Table 4, crosses 4a-c). ocH12, ocH58, and ocH94 fail to recombine with each other and with ocH127 (Table 4, crosses 6a-f). These data suggest that the group of mutations, ocH18, ocH57 and ocH105 represent multiple isolates of the same ochre mutation. The second group (ocH12, ocH58, ocH97 and ocH127) represent multiple isolates of another ochre mutation. ocH87 and ocH122 are very tightly linked to but at distinct sites from the sites defined by the first and second groups of ochres respectively.

E. Correlation of genetic and physical map

1. Orientation of the bla gene on P22 and orientations of bla deletions

The physical orientation of the deletions of the bla gene generated in DB7606 was deduced from our knowledge of: the DNA sequence of Tn1 (Heffron et al., 1979), the orientation of the Tn1 element in the prophage DB7606 (Weinstock and Botstein, 1980) and the nature of deletions generated by a Tn10 element in the a1 region of P22 under these selective conditions. The orientation of the Tn1 element in the prophage DB7606 was assumed to be identical to the Tn1 element in its parent, P22Ap30. The orientation of the Tn1 in P22Ap30 was deduced from the following facts. BamH I cuts asymmetrically and only once in the Tn1 element. The nucleotide sequence of Tn1 showed that the bla gene is contained in the smaller of these two asymmetric fragments and is oriented with its amino terminus proximal to the BamH I site (Heffron et al., 1979). The Tn1 element in P22Ap30 is genetically in the non-polar orientation. In this orientation the smaller of the two asymmetric BamH1 fragments is proximal to gene 9 and distal to P_{late} (Weinstock et al., 1979). From these two pieces of information we determined the physical orientation of the bla gene in the lysogenic map of DB7606 as $immC_L$ - gene 8 - $_{NH3}bla_{CO2}$ - gene 1 - gene 9 - Tn10. The deletions from this strain with endpoints in the bla gene rescue markers in gene8 but not gene1 or genes to the right of gene1 (unpublished data). Therefore the deletions of bla generated from DB7609 enter the gene from

the carboxy terminus and extend toward the amino terminus.

The physical orientation of these deletions relative to the bla structural gene was confirmed by several independent experiments. Recombination studies between deletions of known nucleotide sequence (deletions 218, 280, and 287) and the bla point mutations show that most of the mutations which are assigned to the amino portion of the structural gene by mapping data with deletions from DB7606 fail to recombine with these sequenced deletions which remove the amino terminal 2/3 of the structural gene. However many of the mutations which are assigned to the carboxy end of the bla gene do recombine with these sequenced deletions (figure 3). In addition the orientation of structural gene in relation to these in vivo deletions was confirmed by examining the polypeptide products of chain terminating mutations. As these mutations map closer to the amino end of the gene (as determined by their ability to recombine with DB7606 deletions) the polypeptide products of the bla gene decrease in size (see Chapter 3)

2. Physical location of bla mutations in bla gene

One method to determine the physical location of bla point mutations and deletions in the bla gene is to determine the nucleotide sequence of various bla lesions. Sequencing data showed that the mutations B507, B510, B517, pro_{20}->leu, fs(18,21) and fs(14,21) alter bases in the following codons: B507, codon 4: B510, codon 9; B517, codons 20 and 21; pro_{20}->leu, codon 20; fs(18,21), codons 18, 20 and 21; and fs(14,21)

codons 14, 20 and 21 (Shortle and Botstein, 1981; this work). The physical locations of the endpoints of deletions 218, 280, 287, 241 and 2 are also known (Talmadge et al., 1980; Shortle et al., 1980). The left (amino terminal) endpoints of deletions 4391, 241, 4393, 4394 and 2 are at codons 5, 12, 26, 28 and near codon 138 respectively. The right (carboxy terminal) endpoints of these deletions are all within a few bases of codon 183.

However, studies on the lacI gene (Miller et al., 1977; Coulondre and Miller, 1977a; and Schmeissner et al., 1977) showed that a mutation need not be sequenced in order to determine its physical location in the gene. They showed that lesions responsible for certain chain terminating mutations could be localized to specific codons in the DNA sequence by: the knowledge of the specificity of the mutagen used to induce the mutations, the knowledge of protein sequence, and the nature of the chain terminating mutations (ocre, amber, or frameshift). In bla we had the knowledge of these essential elements, and we had additional information including the DNA sequence of the entire gene and the physical location of the mutations described in the preceding paragraph. Using the strategy outlined for the lacI gene (Miller et al., 1977) we were able to localize mutations to specific codons in the nucleotide sequence of bla.

Ochres:

The primary nucleotide alterations due to hydroxylamine mutagenesis is a C → T transition (Drake, 1970). From the knowledge of the nucleotide sequence of the bla gene hydroxylamine should cause conversions from a sense codon to the nonsense codon, UAA (ochre), at codons for glutamine

(CAA) which occur at amino acid positions 4, 86, 88, 203 and 204. Hydroxylamine mutagenesis gave two groups of ochre mutations. One group recombines with deletion 2 but not with deletion 165 while the other group recombines with both deletions 165 and 2 but fails to recombine with deletion 287. This places the first group at codon 183 or greater and the second group between codons 25 between 138. Data from two factor crosses between ochre mutations within the same group indicate that both these groups can be divided into two extremeley tightly linked subgroups. The recombination frequency distinguishing the subgroups in the first set of ochres (Table 4, crosses 2a-c) is smaller than the recombination frequency distinguishing the subgroups in the second set (Table 4, crosses 4a-c). All these data are consistent with the assignment of the first group of ochres to codons 203 and 204 and the second group of ochres to codons 86 and 88.

Ochre mutations at two other codon were also identified. A representative, ocU8, from the group of ochre mutations that fail to recombine with each other recombines at very low frequency with deletion 218 (data not shown) and not at all with ocB507, a sequenced ochre mutation at amino acid position 4 (Table 4, cross f). Thus, this group of ochre mutations is assigned to the codon 4. A second ochre mutation recombines with deletion 287 but does not recombine with deletion 280 (Table 4; crosses 3a-b). Only one (GAA) of two codons in this deletion interval can be converted to an ochre mutation by a single base change. We assign this mutation to the condon of amino acid 26.

Ambers:

Mutations which alter sense codons to the nonsense codon UAG were also isolated using hydroxyalanine mutagenesis. Codons which have the potential to change to an amber mutation by a single C-T transition are located at positions 37, 97, 163, 208, 227, 265, 275 and 286. All the bla amber mutations isolated by hydroxylamine mutagenesis recombine with deletion 2 but fail to recombine with deletion 165, placing them between codons 183 and 287). amH46, amH61 and amH66 all mapped to the penultimate deletion internal and apparently fail to recombine with each other (Table 4, crosses 8a-c). amH97 maps between the right endpoint of deletion 218 (codon 183) and the deletion endpoint defined by deletion 48. The apparent molecular weight of the mature protein products of amH97 and amH46 are 19.5 K daltons and 27.5 K daltons (Chapter 3). Therefore, amH97 can tentatively be assigned to either codon 208 or 227, while amH46 is located at either codon 265, 275, or 286.

An amber mutation at a third site was isolated by reverting an ochre mutation. Ochre mutations are not suppressed by amber suppressors. By selecting revertants of an ochre mutation in an amber suppressor background, one can detect mutations from UAA (ochre) to UAG (amber) as well as from UAA to sense codons. With this strategy in mind, revertants of the ochre mutation, ocU8, were selected in an amber suppressor background. An isolate, amU8r5, was obtained which has very little bla^+ activity in an Su^- background but has nearly wild type activity in backgrounds containing the different amber suppressors. This mutation fails to recombine with its parent ocU8 which is located at codon 4;

therefore we assign amU8r5 to codon 4.

Frameshifts:

The mutagen ICR-191 causes the addition or loss of a base pair(s) usually in G/C runs (Newton, 1970; Oeschger and Hartman, 1970). Frameshift suppressors were isolated (Riddle and Roth, 1970 and 1972) which suppress only +1 frameshifts of the type, CCCU/C (suf A: Yourno and Kohno, 1972; Yourno, 1971; Yourno and Heath, 1968) or GGGG (suf D; Yourno, 1972). For suppressible frameshift mutations, we tentatively assume that the site of suppression is at or near the site of mutation because both the mutagen and the suppressors act as runs of G/C and because proteins usually cannot tolerate stretches of jumbled amino acids and maintain activity. However, it should be kept in mind that frameshift suppressors need not act at the site of mutation (Roth, 1974).

sufA suppressible frameshift mutations:

The sufA suppressible mutations, fsI36 and fsI20, map between the deletion endpoints of 218 and 280 and are indissolubly linked. Two proline codons, CCU and CCC, exist in this interval at codons 12 and 20 respectively. We assign this mutation near to or at codons 12 or 20.

SupD suppressible mutations:

The sufD frameshift suppressor has an extra C in the CCC anticodon of a glycine tRNA (Riddle and Carbon, 1973). Because a C residue in the anticodon does not apparently wobble this suppressor should only read the four base codon GGGG. This four base codon preexists in the nucleotide sequence of the bla gene or can arise due to the insertion of G at codons 85, 154, 239, 248 and 263.

The allele, fsI7, maps in the interval defined by deletion 2 and therefore lies between codons 138 and 183. Though several runs of G/C base pairs exist in this region, only one run is a potential site for suppression by the sufD suppressor. It occurs around codon 154. These data suggest that the mutation fsI7 is at or near codon 154. Consistent with this hypothesis is the fact that the polypeptide products of fsI7 in an Su^- background has an apparent molecular weight predicted for a +1 frameshift in this region (see Chapter 3).

The alleles, fsI10 and fsI40, map to the right of ocH18 and therefore must lie between codon 204 and 287. fsI40 maps to the right of fsI10 and therefore must lie closer to the carboxy terminal end of the bla gene than fsI10. The three potential sites for suppression by sufD in this region of the bla gene are at codons 239, 248 and 263. The apparent molecular weight of the product of fsI40 (see chapter 3) is most consistent with the addition of a base around codon 263. Therefore we suggest that the lesion fsI40 affects a codon near codon position 263 and that the lesion fsI10 is located near or at codon 239 or 248.

Knowing the physical location of these point mutations we can place limits on the physical location of the endpoints of the in vivo deletions. Deletion 68 recombines with amH46 and therefore must remove at most the coding region of the carboxy terminal 22 amino acids. Deletion 48 fails to recombine with amH46 and fsI40, but does rescue amH97 and fsI10. Its endpoint must lie between codons 238 and 265. Deletion 165 fails to recombine with deletion 2 but does rescue fsI7. Its endpoint must lie between codons 154 and 188. Deletions 13 and 60 fail to recombine with

fsI7 but do rescue ocH12. These endpoints must lie between codons 88 and 154. Finally deletion 74 fails to recombine with ocH12 but does rescue ocU18. Its endpoint must lie between codons 88 and 26. These results are summarized in figure 6.

II. Discussion

In this chapter we presented a fine structure genetic analysis of the structural gene for TEM β-lactamase. We used in vitro and in vivo methods of localized mutagenesis to isolate over 250 point mutations and 6 new deletions in the bla gene. Five other deletions were made available to us and allowed us to divide the gene into 12 distinct deletion intervals. A subset of these deletions and point mutations were oriented on the physical map of the bla gene by nucleotide sequencing and by a strategy similar to the one outlined by Miller et al. (1977).

A. A general strategy for isolating mutations in a particular region of a gene.

The strategy that we used to isolate signal sequence mutations in bla is generally applicable to any problem in which one is interested in isolating mutations in a small predetermined region of a gene. This strategy involves three steps, the mutagenesis of the desired region of the gene, the demonstration that the mutants produced by this mutagenesis are in that region and the assignment of these mutations to the same or

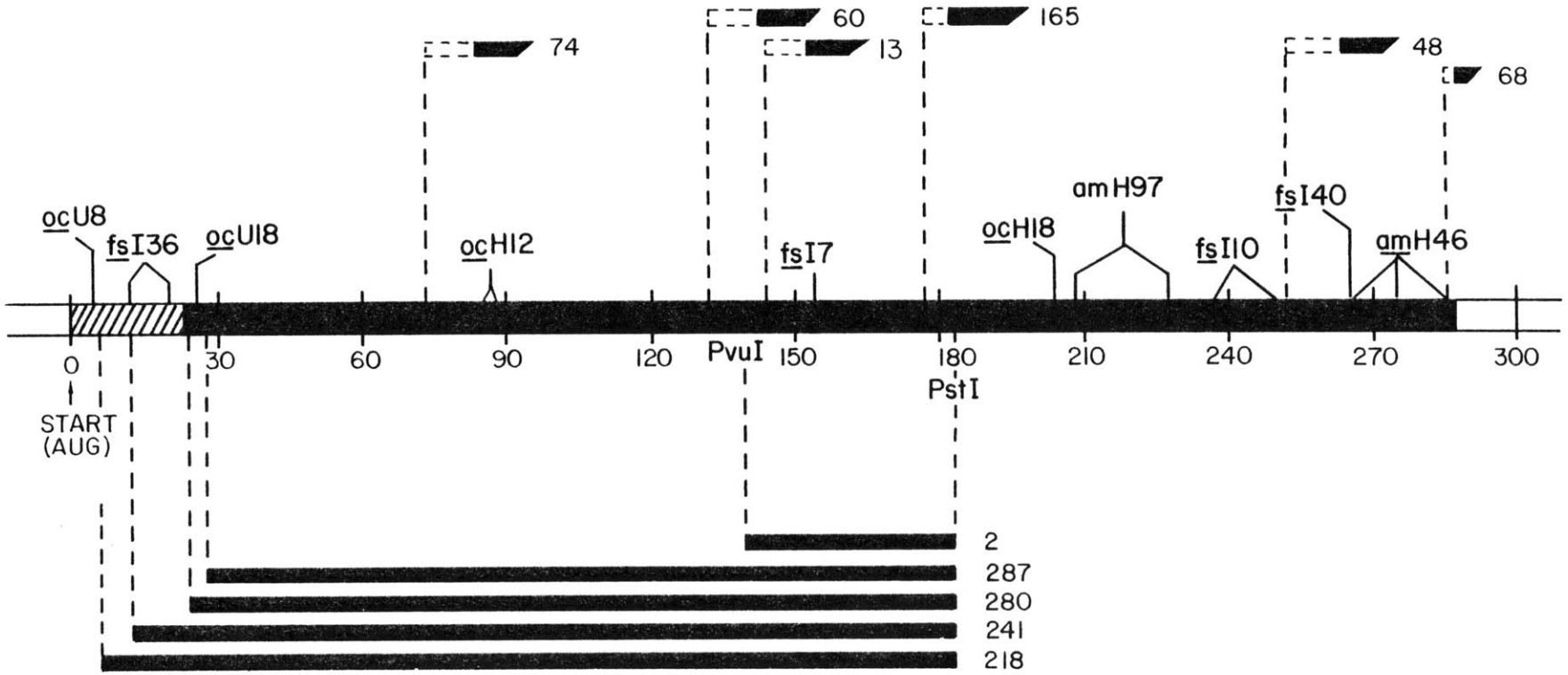

74
60
13
165
48
68
ocU8
fsI36
ocUI8
ocHI2
fsI7
ocHI8
amH97
fsI10
fsI40
amH46
0
30
60
90
120
PvuI
150
180
PstI
210
240
270
300
START
(AUG)
2
287
280
241
218

Figure 6: The physical map of bla. The position of each point mutation and deletion endpoint was determined by nucleotide sequencing (Talmadge et al., 1980; also this work) or by methods descirbed in this thesis (see the text). The position of a point mutations is indicated by a line connecting the point mutation and the gene. Ambiguities in the position of a point mutation are indicated by the presence of more than one line per mutant. Similarly, ambiguities in the position of deletion endpoints are indicated by an open bar. The right endpoint of all deletions drawn above the gene extend well beyond the carboxy terminus of the bla gene. The hatched area represents the signal peptide and the solid area represents the mature portion of β-lactamase.

different sites within that region.

The strategy outlined above might be carried out strictly by classical genetic methods or by more recent methods involving the use of recombinant DNA, *in vitro* mutagenesis and DNA sequencing. However, the methods used to isolate signal sequence mutations in *bla* illustrate how a combination of the classical methods and these more recent methods can be a much more powerful means of accomplishing the steps of this strategy than either method alone.

Signal sequence mutations were isolated very inefficiently by classical genetic methods. The inefficiencey of this method is most likely due to fact that signal sequence mutations have Bla phenotypes which are indistinguishable from either the *bla*$^+$ allele (pro_{20}->ser, *fs*(7,9) and val_9->ala) or any number of mutations that alter the mature portion of the protein and reduce its enzymatic activity (*fs*(14,21), *fs*(18.21) and pro_{20}->leu). The inability to focus classical methods of mutagenesis to the signal sequence means that the only way to find such mutations by this method is to screen for them among the total collections of *bla*$^-$ mutations. Given the target size of the signal sequence relative to the mature portion of β-lactamase, it is not surprising that the majority of the *bla*$^-$ are not in the desired region.

In contrast an *in vitro* method (Shortle *et al.*, 1980) efficiently produced mutations in the signal sequence of β-lactamase because this method allows one to direct mutagenesis to the signal sequence. The signal sequence mutations now represent a very large fraction of the *bla*- mutations. By the same reasoning it is clearly more efficient to isolate

deletions which define the signal sequence by *in vitro* methods (Talmadge *et al.*, 1980) than by *in vivo* methods. This idea is supported by the fact that none of the deletions isolated by classical genetic methods have endpoints that neatly define the signal sequence.

On-the-other-hand, classical genetic methods are much more efficient than these other methods for rapidly demonstrating that a mutation lies in the signal sequence and that two mutations are at the same or different sites. To document that 5 or 30 of the signal sequence mutations isolated by these *in vitro* methods are indeed in the signal sequence and that they are or are not at distinct sites requires only several days work by classical genetic methods but by *in vitro* methods (i.e. DNA sequencing) would require at least several weeks. The example in this paragraph and the preceding paragraph illustrate how the combination of the more recent *in vitro* methods and the classical genentic methods seems clearly to be the most efficient method to isolate different mutations in a desired region of a gene.

B. Resolution of mapping methods

In this study we document the resolution of the crosses used to construct this map. The quick mapping method which was used to construct the majority of the map has a resolution of one recombinant per 10^5 progeny. Mutations, which are near a deletion endpoint may recombine with that deletion at frequencies below 1 in 10^5. Therefore it should be emphasized that a subset of the mutations which failed to give detectable

recombination with any given deletion actually may be near the endpoint of that deletion and below the resolution of the quick mapping method. A case in point is fsI40 which failed to give recombinants with deletion 68 by the quick mapping method but did give recombinants with this deletion when higher resolution mapping methods were used.

The potential of several recombination systems to resolve mutations which are separated by as few as two bases has been documented in the lacI gene (Coulondre and Miller, 1977a), in the his operon (Johnston and Roth, 1981a,b), in the lamB gene (Emr and Silhavy, 1980) and now in bla. A striking feature of figure 5 is that the resolution of this system is not dramatically affected by the nature of the lesion in the cross. Crosses of point mutations by point mutations gave similar recombination frequencies to crosses of point mutations by deletions even when the lesions are separated by as few as 2 base pairs. A similar observation was made by Sodergren and Fox (1979) when crosses with phage lambda were performed though they did not examine recombination between such tightly linked markers. They propose that a non-homology (deletion or insertion) is excluded from heteroduplex structure. Therefore one might expect the non-homology to decrease the frequency of recombination between these mutations because branch migration of the heteroduplex structure can only enter the region between the point mutations and deletion from one side. However they also suggest that the non-homology not only prevents the extension of the heteroduplex structure but it also provokes the resolution of the heteroduplex structure. Perhaps in a cross of a point mutation by a deletion these two factors counterbalance each other to give

an overall recombination frequency similar to a cross of a point mutation by point mutation separated by the same distance. If this is the case, then these data suggest that the resolution of the heteroduplex structure provoked by the non-homology occurs essentially at the base where the non-homology begins.

The weak correlation between distance and recombination frequency documented in these two factor crosses is not surprising since such effects have been observed previously in Escherichia coli (Yanofsky et al., 1964; Drapeau et al., 1968; Norkin, 1970; Stadler and Kariya, 1973) and for bacteriophage T4 (Fessman, 1965; Katz and Brenner,1968). More recently the relationship between recombination frequency and distance was investigated more extensively on the lacI gene (Coulondre and Miller, 1977a). Their findings are virtually identical to the findings presented in this work.

C. Hot spots

The physical distribution in bla of ochre mutations isolated by hydroxylamine, an in vitro mutagen, contrasts sharply with the physical distribution of ochre mutations as isolated by UV, an in vivo mutagen. Five codons in the bla gene can be changed by hydroxylamine (assuming the G → A specificity) to ochre. We have isolated a group of ochre mutations at two independent sites and each group can be divided into two subgroups. These data indicate that we have isolated ochre mutations at 4 out of the 5 possible codons. Given that only nine independent ochre mutations have

been isolated then by Poisson distribution we predict that we should fail to isolate an ochre mutation at 1 out of every 6 codons with the potential to go to ochre. Clearly the difference between the predicted 1 out of 6 and the observed 1 out of 5 is not statistically significant. The apparent clustering of ochre mutations induced by hydroxylamine is due to the physical clustering of codons with the potential to go to ochre by a G→A transition and is not due to a mutagenic "hot spot" for hydroxylamine.

The ochre mutations isolated by UV light show a substantially different distribution. UV mutagenesis apparently can cause all four transversions and both transitions. As a result the number of codons which by a single mutation can go from sense to ochre in bla is much greater for UV (33) than hydroxylamine (5). Yet 7 of the 10 independently isolated ochre mtuations map to codon 4. Mutagenic "hot spots" in a gene were first documented by Benzer. Subsequenty Coulondre and Miller (1977b) showed that UV mutagenesis, in particular, produed several "hot spots" in the lacI gene. The UV induced ochre mutations at codon 4 in bla provide another example of a mutagenic hot spot.

The contrast in the physical distribution of ochre mutations isolated by hydroxylamine versus UV exemplifies the advantage and disadvantages of in vitro and in vivo mutagenesis. Hydroxylamine, an in vitro mutagen, gives a statistically even distribution of ochre mtuations among the potential ochre sites. However, the numbers of codons which can be changed to ochre by this mutagen are limited because it is extremely specific. In contrast UV, an in vivo mutagen, has a broader specificity (producing transversions as well as transitions) than hydroxylamine and

therefore has a greater potential target size for the production of ochre mutations in bla. However, the physical distribution of ochre mutations isolated by UV also is limited apparently because this mutagen is subject to a "hot spot" at codon 4.

Studies of lacI and in hisC reveal "hot spots" for ICR-191 (Roth, 1974). An examination of ICR-191 induced mutations in bla shows that many of these mutations though independently isolated, map to the same deletion interval and are suppressed by sufD. These mutations may represent preferred sites for the action of ICR-191.

D. Distribution of point mutations among conditional and non-conditional mutations

The frequency of nonsense mutations among the bla^- mutations induced by hydroxylamine and UV is 7% and 12% respectively. Similarly lacI nonsense mutations occur among lacI mutations at a frequency of 10-20% for a variety of different mutagens (Miller et al., 1977). The fact that a large proportion of these bla^- nonsense mutations are ochres is probably insignificant. In the case of hydroxylamine induced nonsense mutations, the number of ochre muations (9) relative to amber mutations (4) is not statistically different. On the other hand, the fact that 11 out of the 12 nonsense mutations induced by UV are ochre seems clearly significant. Since 7 out of these 10 ochre mutations map to a single site, this large proportion of ochre mutations may result from a "hot spot" for mutagenesis at codon 4 (see above) rather than reflect an actual bias for

ochre mutations over amber mutations.

The fraction of bla$^-$ mutations isolated by ICR-191 that are suppressible is substantially larger than the fraction of mutations isolated by ultraviolet light or hydroxylamine that are suppressible. This result is not surprising because ICR-191 prefers to add G/C base pairs to runs of G/C (Roth, 1974). These runs are also potential sites for suppression by sufA and sufD. Unlike ICR-191, UV or hydroxylamine mutagenesis does not have a specificity for nucleotide sequences which are more likely to be suppressed by amber, ochre or frameshift suppressors (Coulondre and Miller, 1977).

E. Correlation of Genetic and Physical Map

The genetic and physical maps of bla were correlated by directly determining the nucleotide sequence of a subset of lesions and by deducing the physical location of some bla lesions using a strategy similar to the one outlined by Miller et al. (1977). The latter method under the best of circumstances can predict the location and change in the nucleotide sequence due to a particular lesion. In bla some mutations have been localized to one or two codons while others are localized to a region in the protein. A comparison of the lacI system and the bla system indicates that our inability to localize more precisely many of the bla mutations stems from the availability of only a limited number deletion intervals in bla. However enough information was obtained from this study so that in general any new mutation can be assigned to approximately a 30-40 amino

acid region of the protein or 90-120 base pair region of the gene.

The availability of this relatively crude mapping information will prove invaluable in future studies of β-lactamase. For example suppose some mutation causes a particularly interesting phenotype (such as a signal sequence mutation). By using this genetic method to determine its approximate location in the bla gene one immediately limits the amount of DNA (or protein) which must be sequenced to determine the nature of lesion. In addition and perhaps more importantly, one is assured that the change in sequence which one uncovers in this region is responsible for the altered phenotype. Without such a map one would be faced not only with sequencing the entire gene to determine the nucleotide changes in any particular lesion but also with sequencing the entire DNA of the vector or host organism to show that the detected nucleotide change in bla is the only possible variable to account for the altered phenotype.

In conclusion we emphasize that bla is a model for any gene which one has cloned on a phage or plasmid with the intent to study its expression or the function of its product. The relative ease of constructing a fine structure genetic map and correlating it with a physical map, coupled with the clear advantages of having such a system at ones's fingertips makes this approach attractive for any gene.

Chapter 4: Secretion of β-lactamase

In the previous chapter we described the isolation and genetic characterization of a large number of mutants in the bla gene. In this chapter we identify the products of the wild-type and mutant alleles of bla and characterize their cellular location by cell fractionation experiments and trypsin accessibility experiments. By these criteria the products of the wild-type and mutant alleles of bla exhibit novel and unexpected phenotypes.

I. Results

A. Analysis of the protein products of wild-type and mutant β-lactamase genes by SDS-polyacrylamide gel electrophoresis

Phage-specified proteins are easily identified using SDS-polyacrylamide gel electrophoresis because phage-specified proteins can be labeled specifically after infection of a host which has been irradiated with ultraviolet light (Studier, 1973). We have used this system to investigate the synthesis and maturation of the β-lactamase protein encoded by P22bla and its derivatives in Salmonella tymphimurium. β-lactamase could be readily visualized under conditions essentially identical to those used to identify most of the P22 phage proteins (Botstein, Waddell and King, 1973; Poteete and King, 1977).

1. Identification of protein products of the wild-type bla gene

To identify the products of the bla+ gene carried on P22bla+ we examined autoradiograms of SDS-polyacrylamide gels on which were displayed the labeled proteins synthesized in irradiated cells after infection with bla^+ phages (see Methods). It was possible to identify three bands as products of the bla gene because all were absent or shifted to positions corresponding to lower molecular weights when the infection was with phage carrying chain-terminating bla^- mutations. All these bands were missing when a phage carrying a deletion of the entire bla gene was analyzed. The existence of three bla^+ bands (a single band and a faster-migrating doublet: see Figure 8, lane a) is interpreted as follows: the doublet co-migrates with purified mature β-lactamase, which migrates as a doublet due to incomplete denaturation under the standard conditions for preparation of samples for SDS-polyacrylamide gel electrophoresis (J. Knowles, personal communication and our unpublished results); often only a single band is visible. The slower-migrating band corresponds in molecular weight to a protein about 2500 daltons larger than the mature β-lactamase. Its size is what might be expected of a precursor in which the 23-residue signal peptide predicted by comparison of the β-lactamase protein sequence and the β-lactamase structural gene DNA sequence (Ambler et al., 1978; Sutcliffe, 1978) has not been removed.

2. Identification of protein products of mutant β-lactamase harboring mutations in the mature protein

Analysis of the bands produced after infection with phage carrying *bla*$^-$ mutations allowed us to distinguish three different phenotypic classes, all of which had in common a pair of bands differing in apparent molecular weight by about 2500 daltons. As summarized in Table 5, class I mutants showed a set of bands indistinguishable from those found with wild-type; class II were missing the bands at the wild-type positions, but had two new bands which migrate more rapidly although they are still separated by an apparent molecular weight difference of about 2500; finally, class III mutants were like those of class II but also contained a third, much more rapidly migrating band.

We interpret class I mutations as being missense mutations. This interpretation is supported by their revertibility, their failure to be suppressed by nonsense or frameshift suppressors, and their production of normal amounts of (presumably inactive) protein. Class II mutations are interpreted as chain-terminating mutations, which is proven directly by the observation that every one of them is suppressible by host nonsense or frameshift suppressors. In one case, *am*H97, the intensities of both the faster-migrating bands characteristic of the mutant were diminished, and bands which co-migrated with the products of the wild-type gene reappeared when a host carrying an amber suppressor was used (data not shown). The correct identifications of bands corresponding to all the amber, ochre and frameshift peptides found are supported by the correlation between

Table 5. Molecular weights of beta-lactamase-associated proteins in chain-terminating *bla*$^-$ mutants

Allele	Predicted molecular weight	Observed molecular weight	Differences in molecular weight; precursor-mature[a]
bla$^+$	32	30.5	
	29.5	27.5	3.0
Class I mutants: missense			
H3	32.	30.5	
	29.5	27.5	3.0
Class II mutants: chain-terminating (two beta-lactamase bands)			
*am*H46	29.1 or 30.3	29.5	
	26.6 or 28.8	27.5	2.0
*fs*I40	26.0 or 29.0	28.0	
	23.5 or 26.5	26.0	2.0
*fs*I10	26.0 or 29.0	27.0	
	23.5 or 26.5	24.5	2.5
*fs*17	17.6	16.5	
	15.1	14.0	2.5

Class III mutants: chain-terminating (three beta-lactamase bands)			
ocH18	22.0	21.5	
	19.5	29.0 (m1)	2.5 (p-m1)
		15.0 (m2)	
amH97	23.0 or 25.0	22.5	
	20.5 or 22.5	19.5 (m1)	3.0 (p-m1)
		16.0 (m2)	

[a]Figures in these columns are given to the nearest 0.5 kilodalton.

The predicted molecular weights (in kilodaltons) are derived from genetic mapping of the mutations as summarized in Figure 1, using the assumption that an average amino acid has a molecular weight of 110. In the case of frameshift mutations we know the number of amino acids because the suppressibility of the mutations requires that the frame be shifted into the +1 frame (Roth, 1974). The next termination codon was found in this frame on the nucleotide sequence (Sutcliffe, 1978) and the length so determined was used to calculate the molecular weight. The observed molecular weights were calculated from autoradiograms of SDS-polyacrylamide gels using as molecular weight standards: BSA, 64,000; tomato bushy stunt virus protein, 42,000; and lambda repressor, 26,500. (p), (m1) and (m2) indicate the putative precursor, the highest molecular weight mature species and the lower molecular weight mature species, respectively.

observed apparent molecular weights and the molecular weights predicted from the positions of the mutations in the bla gene (Table 5). Class III mutations are also suppressible and thus appear to be chain-terminating mutations. Both members of this class are close together in map position, and we suppose that the fragment produced by these mutants is extraordinarily sensitive to proteolytic degradation, producing the third and fastest-migrating band.

The observation that all the class II and class III (chain-terminating) mutants produce bands differing in apparent molecular weight by about 2500 daltons suggests that the removal of the 23 amino acid signal sequence from the amino terminus occurs in all of them. If β-lactamase is indeed synthesized as a precursor which is subsequently processed from the amino end, then the presence of the carboxy end of the protein is apparently not essential for the processing.

3. Identification of the protein products of the signal sequence alleles

In the previous chapter, we reported the isolation of mutations which alter the signal sequence of β-lactamase. Segment directed mutagenesis produced pro_{20}->leu and two frameshift mutations, B501 and B510, which were subsequently reverted to a Bla^+ phenotype (pro_{20}->ser, fs(14,21), fs(7,9) and val_9->ala). To identify the products of the bla gene harboring these signal sequence mutations, we examined autoradiograms of SDS-polyacrylamide gels on which were displayed the labeled proteins

synthesized in irradiated cells after infection with the appropriate P22bla phage (Methods). Three criteria were used to identify a band as the product of a particular allele of the bla gene: the absence of the band(s) when the irradiated cells were infected with chain terminating mutations early in bla; the co-migration of the protein with either precursor or mature β-lactamase; and extraction from the gel of the putative protein in the band followed by partial amino acid sequence of its amino terminus (Methods). The two phages which harbored both the point mutation and frameshift mutation (B510 and B501) produce no bands in the 27-32 Kdalton region of the gel (figure 7). However, when the protein products of the phage which carried bla^+ revertants of these two mutations were examined, bands reappear in this region of the gel (figure 8; lanes d, f, h, l and n). The putative precursor forms of fs(14,21) (figure 8; lane f) and ala_9->val (data not shown) co-migrate with the precursor form of the wild-type gene (lane d), and the mature forms of fs(7,9) (figure 8; lane l), pro_{20}->ser (figure 8; lane n) and ala_9->val (data not shown) co-migrate with the wild-type mature species. The precursor forms of pro_{20}->leu, fs(18,21), pro_{20}->ser and fs(7,9) migrate slightly faster than the precursor product of the wild-type gene (figure 8; lanes h, j, m, l and d respectively).

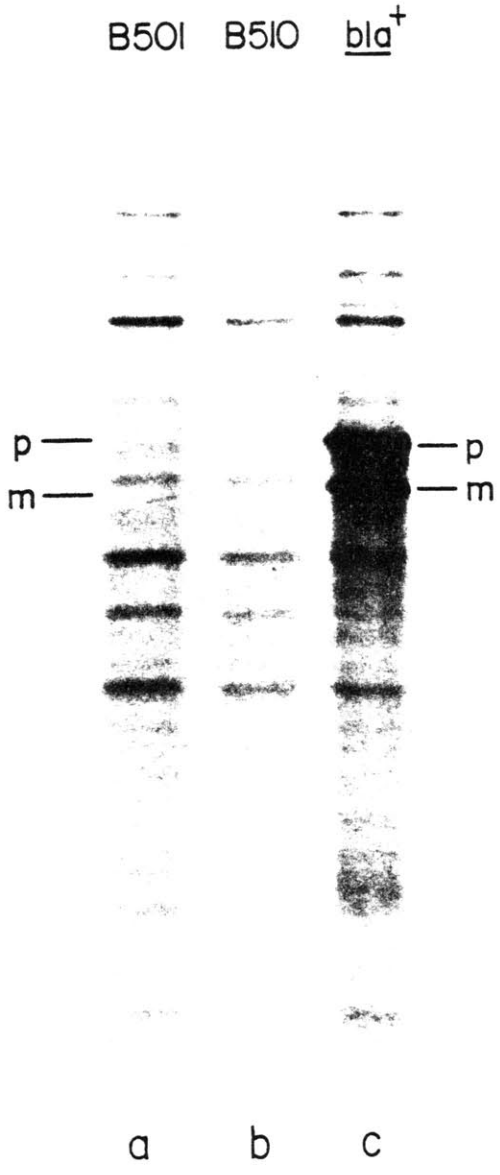

B501
B510
bla+
p
m
p
m
a
b
c

Figure 7: ^{35}S-methionine labeled cells infected with bla-B501, bla-B510 and bla+ phage. A culture of DB4381 was irradiated and infected with bla-B501, bla-B510 or bla+ phages at a multiplicity of 10. The cells` were labled with ^{35}S-methionine for 1 minute (see Methods). 20 µl aliquots were loaded onto each slot. Electrophoresis was on a 12.5% gel, and the autoradiogram was exposed for 10 hours. p and m indicate the position of the precursor and mature forms of β-lactamase respectively.

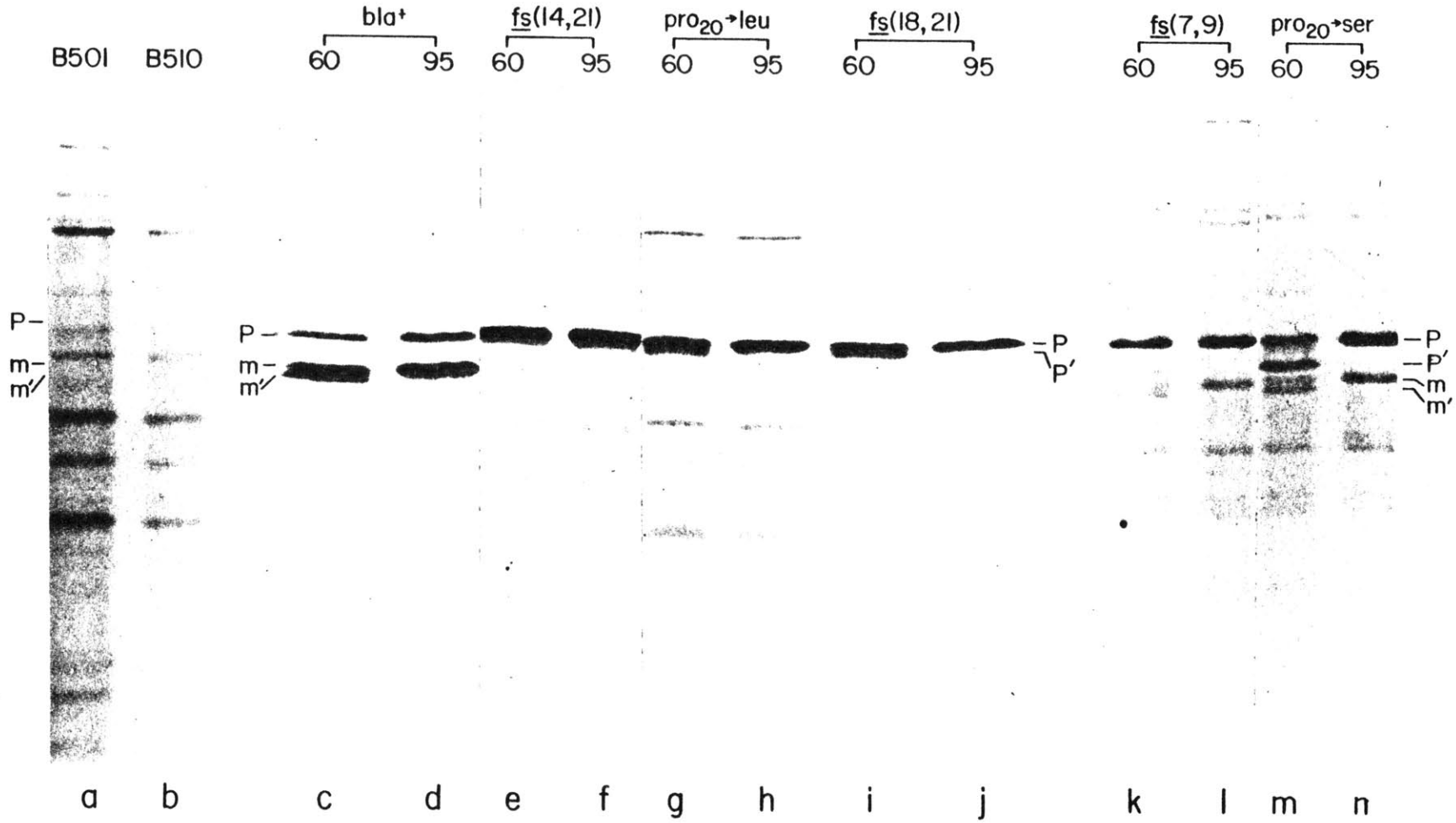

B501
B510
bla+
60
95
fs(14,21)
60
95
pro20→leu
60
95
fs(18,21)
60
95
fs(7,9)
60
95
pro20→ser
60
95
P
m
m′
P
m
m′
P
P′
P
P′
m
m′
a
b
c
d
e
f
g
h
i
j
k
l
m
n

Figure 8: ^{35}S-methionine labeled cells infected with P22bla phages containing signal sequence alleles of bla. A culture of DB4381 was irradiatied and infected with P22bla phages containing signal sequence alleles at a moi of 10. The infected cells were labeled with ^{35}S-methionine for 3 minutes and then sample buffer was added. The sample was split in two. One half was heated to 60°C for 2 minutes (doublet forming conditions) and the other half was heated to 95°C for 5 minutes (non-doublet forming conditions). 20µl aliquots were loaded on each slot. Electrophoresis was on two 12.5% gels and the autoradiograms were exposed for 20 hours. Lanes c-f and g-j are a composite of lanes from the same gel. Lanes k-n are for a separate gel. The allele and the temprture to which the sample was heated is listed above each lane. p, precursor form; p', precursor form that appears only under doublet forming conditions; m, mature form; m', mature form that appears only under doublet forming conditions.

B. Partial amino acid sequence of the amino terminus of bla encoded proteins

To establish unambiguously that these putative precursor and mature molecules are the product of the bla gene and that the precursors migrate more slowly than the wild-type mature species due to the presence of a signal sequence at their amino termini, the partial amino acid sequences of the amino termini of these proteins were determined (see Methods). Cells infected with the appropriate bla phage were labeled with either ^{3}H-isoleucine and ^{3}H-phenylalanine (to sequence the precursor forms of bla) or ^{3}H-alanine and ^{3}H-valine (to seqence the mature forms of bla), and the labeled proteins subjected to SDS-polyacrlamide electroporesis. The labeled bla encoded proteins were purified from the gel and subjected to automated Edman degradations. In some cases, the steps of the degradation procedure that contained peak amounts of label were checked for their amino acid composition.

Table 6 summarizes the protein sequencing data. The peaks of radioactivity for the mature forms, amH46, fsI7 and wild-type, occur in steps that correlate exactly with the peaks one would expect given the published amino acid sequence of the bla^{+} mature form (Ambler et al., 1978; Sutcliffe, 1978) and the labeled amino acids used in these experiments. The peaks of radioactivity for the different precursor species also occurs exactly in the expected steps given the amino acid sequence of the precursor form deduced from the DNA sequence of the gene (Sutcliffe, 1978), the labeled amino acids used in these experiments and

Table 6. Partial amino acid sequence of precursor and mature forms of different bla alleles.

Mature

Allele	Sequence step	his 1	pro 2	gly 3	thr 4	leu 5	val* 6	lys 7	val* 8	lys 9	asp 10	ala* 11	glu 12
bla$^+$		688	456	515	577	626	1389	911	1613	1015	821	1418	789
fsI7		1172	1115	1068	1350	1355	3608	2056	3700	2051	1509	2419	780
≠amH46		1522	1524	1131	1053	1754	3404	3016	3505	1657	1244	ND	ND

Precursor

Allele	Sequence step	met	ser 1	ile** 2	gln 3	his 4	phe** 5	arg 6	val 7	ala 8	leu 9	ile** 10	pro 11	phe** 12
bla$^+$			103	657	195	173	1157	ND	232	216	215	620	317	1184
fs(7,9)			136	1984	254	421	2304	425	323	140	432	1455	655	1093
pro$_{20}$→leu			264	1310	321	524	2281	1255	394	439	524	1223	984	ND

*^{3}H-labeled amino acids used to label mature species.
**^{3}H-labeled amino acids used to label precursor species.
≠For amH46, ^{3}H-val alone was used to label mature species.

Numbers are counts/20 minutes of ^{3}H-isotype. Peak fractions are underlined. For preci
assume met at position 1 is removed. ND, not done.

the assumption that the amino terminal methionine is removed. The amino terminal methionine is removed from many proteins in gram negative bacteria.

These data show that the apparent larger molecular weight of the precursor species of the alleles pro_{20}->leu, fs(14,21) and wild-type are due to the presence of the signal sequence at their amino termini. These data also show that the processing of the wild-type precursor form that occurs in phage infected and irradiated cells is apparently identical to the processing of normal cells. Finally they show that the fidelity of processing does not require the carboxy terminus of β-lactamase.

C. Precursor-product relationships

1. Products of bla genes with wild-type signal sequences

The preceding results prompted a direct test of the possibility that the two bands differing by about 2500 daltons in wild-type exhibit a precursor-product relationship. If infected cells are exposed to a short pulse of radioactive amino acids, then the precursor form (by hypothesis, the slower-migrating species) should be labeled preferentially. After increasing time of incubation with unlabeled amino acids (chase, stoichiometrically related amounts of radioactivity should disappear from the putative precursor and appear in the product [by hypothesis, the faster-migrating band(s)].

We performed an experiment in which irradiated host cells were infected with P22*bla*$^+$ and subjected to a 30 sec exposure to ^{35}S-methionine followed immediately by the addition of excess unlabeled methionine. Samples were added directly to sample buffer at 90^{o}C immediately after addition of the unlabeled methionine and every 30 sec thereafter. Figure 9 shows an autoradiogram of an SDS-polyacrylamide gel analysis of these samples, and figure 10 shows a plot of the fraction of radioactivity found in the putative precursor and product bands on that gel as measured with a scanning densitometer (see Methods). From these figures it is clear that at least 80% of the radioactivity is present in the putative precursor band at the beginning of the chase, while more than 90% of the radioactivity is in the putative product after 270 sec. After 30 seconds of chase, the radioactivity in the sum of all the β-lactamase-related bands remained constant, supporting the conclusion that by this time the incorporation of ^{35}S-methionine into *bla* encoded proteins no longer occurred. This conclusion was also supported by analysis of radioactivity in the band corresponding to the P22 coat protein, which also remained constant during the chase.

The results of this experiment satisfy the conditions for a meaningful pulse-chase analysis, and we conclude that most of the β-lactamase synthesized in the cell after infection with P22 *bla*$^+$ is made first as a precursor about 2500 daltons larger than the mature enzyme. The conclusion does not depend upon the irradiation of the host with ultraviolet light, since a similar experiment using unirradiated cells also gave results consistent with a precursor-product relationship

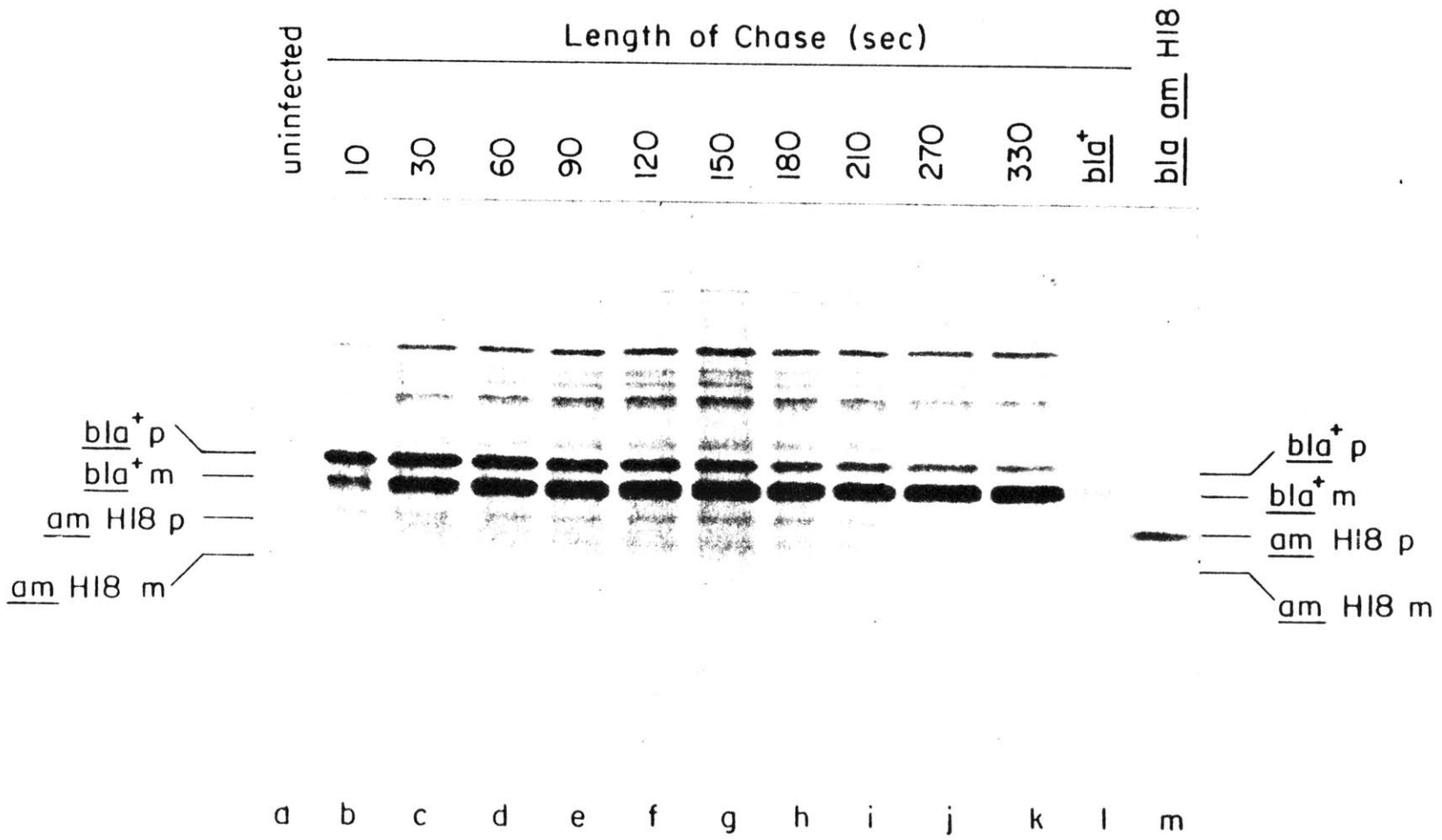

uninfected
Length of Chase (sec)
10
30
60
90
120
150
180
210
270
330
bla+
bla am H18
bla+ p
bla+ m
am H18 p
am H18 m
bla+ p
bla+ m
am H18 p
am H18 m
a b c d e f g h i j k l m

Figure 9: Autoradiogram of ^{35}S-methionine-labeled extracts from pulse-chase analysis of bla^{+} gene products. The details of the pulse-chase experiment are described in Experimental Procedures. Lanes (b-k) are samples taken from cells infected with bla^{+} phage, pulse-labeled for 30 sec with ^{35}S-methionine and chased with excess cold methionine. The number above each slot indicates the time (in seconds) after the addition of the chase. The multiplicity of infection was 10. A constant volume of 20 µl was loaded in each slot. Electrophoresis was on a 12.5% gel and the autoradiogram was exposed for 22 hr. Lane (a) contains an extract of uninfected cells pulsed with ^{35}S-methionine for 30 sec. Lane (l) contains an extract of irradiated cells infected with bla^{+} phage and pulsed with ^{14}C amino acids followed by a short chase. Lane (m) contains an extract of bla-amH18-infected cells pulsed with ^{35}S-methionine for 30 sec.

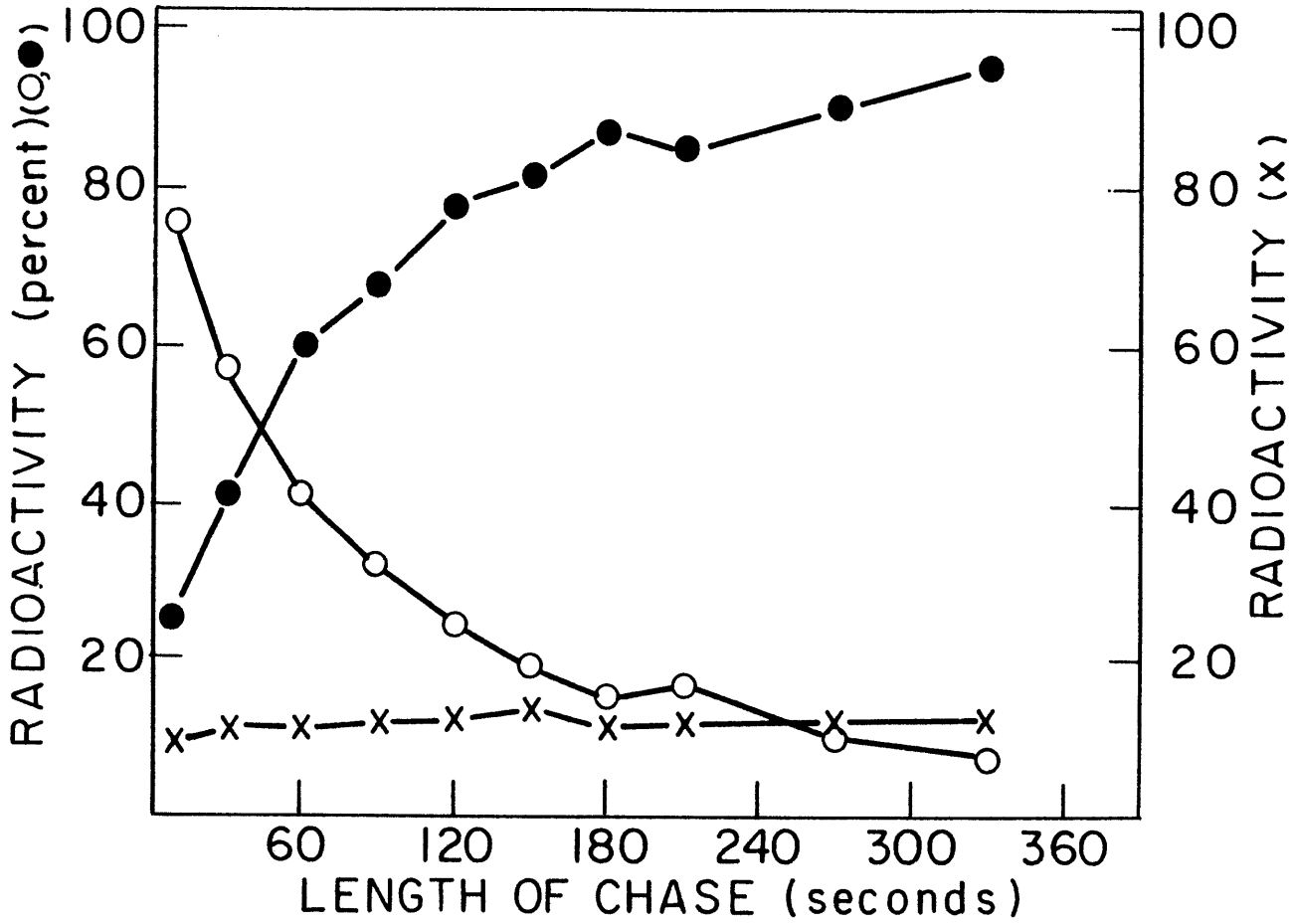

RADIOACTIVITY (percent)(○,●)
100
80
60
40
20
RADIOACTIVITY (x)
100
80
60
40
20
60
120
180
240
300
360
LENGTH OF CHASE (seconds)

Figure 10: Quantitation of pulse-chase analysis of bla$^+$ protein products. The radioactivity in specific bands of the autoradiogram of the gel presented in Figure 2 was measured by quantitating the intensity of those bands with a Seineh Soft Laser Scanning Densitometer. To ensure that the intensities of the bands in this autoradiogram were linear with time of exposure, three separate exposures of 5.5, 11 and 22 hr were made on preflashed film. On the right ordinate is plotted the percentage of the total radioactivity in bla$^+$ bands present in either precursor or mature forms. On the left ordinate is the total radioactivity present in a specific band corresponding to the P22 phage head protein p5. Plots of radioactivity in the precursor and mature bands versus time are virtually superimposible with the plot of the percentage of total radioactivity in bla$^+$ bands in the precursor and mature bands versus time shown above. (O O) percentage of total radioactivity in bla$^+$ bands present in the precursor form; (O O), percentage of total radioactivity in bla$^+$ bands present in the mature form; (X X) total radioactivity in p5.

(Josefsson and Randall, 1981). Another experiment (also not shown) using uniformly labeled ^{14}C amino acids instead of ^{35}S-methionine gave similar results, eliminating the possibility that the result represents an artifact of the use of ^{35}S-methionine.

When similar experiments were carried out using P22 phages carrying various chain terminating alleles of the bla gene, the results again showed a precursor-product relationship. In every case the slowest-migrating β-lactamase-related protein contains most of the radioactivity after the pulse, and the faster-migrating bands contain most of the radioactivity after 5 min of chase (data not shown).

2. Products of bla genes with altered signal sequences

The different bla alleles that alter the signal peptide can be divided into two groups: those that produce a single band which co-migrate with or near the wild-type precursor (fs(14,21), pro_{20}->leu and fs(18,21): figure 8; lanes f, h and j respectively) and those that produce in addition to this band a second band that co-migrate with mature β-lactamase (pro_{20}->ser, fs(7,9) and ala_9->val). However the percentage of the total label in the mature product for pro_{20}->ser and fs(7,9) after three minutes of labeling is reduced relative to wild-type (figure 8; lanes n, l and b respectively). The reduction of label at the position of the mature species relative to wild-type suggests that the rate of processing of precursor to the mature species is altered for these alleles as well as for the alleles, fs(14,21), fs(18,21) and pro_{20}->leu, for which no

processed form of β-lactamase is detected.

To investigate this phenomenon further, we performed pulse chase experiments with these alleles similar to the experiment described above for the wild-type allele. When the infection was with phage carrying the bla alleles, fs(14,21), fs(18,21) or pro_{20}->leu, the intensity of the band at the precursor position remains constant over the length of the chase (as long as 10 minutes) and no band appears at the position of the mature species (figure 11, panel D).

When the infection was with phage carrying the bla alleles, fs(7,9) or pro_{20}->ser, a result was obtained that differed from the results of fs(14,21), fs(18,21), pro_{20}->leu or wild-type. In the insets to figure 11, the autoradiograms from a pulse chase experiment for fs(7,9) and pro_{20}->ser are shown. The radioactivity in the precursor and product bands was quantitated for each lane of these gels and plotted as the percent of total label present in bla encoded species (Methods). The rate of transfer from the precursor to the mature species for these two bla mutants is much slower than the rate observed for wild-type (figure 11; compare panels A,B and C). In addition the percentage of total label in bla encoded proteins that is in precursor even after an extensive chase for pro_{20}->ser is greater than that seen for wild-type. For ala_9->val the rate of transfer of label from precursor to the mature species and extent of processing are indistinguishable from the wild-type (data not shown).

Since these experiments fulfill the requirements for a meaningful pulse chase experiment, we can make the following conclusions. The alleles fs(14,21), fs(18,21) and pro_{20}->leu alter the amino acid sequence

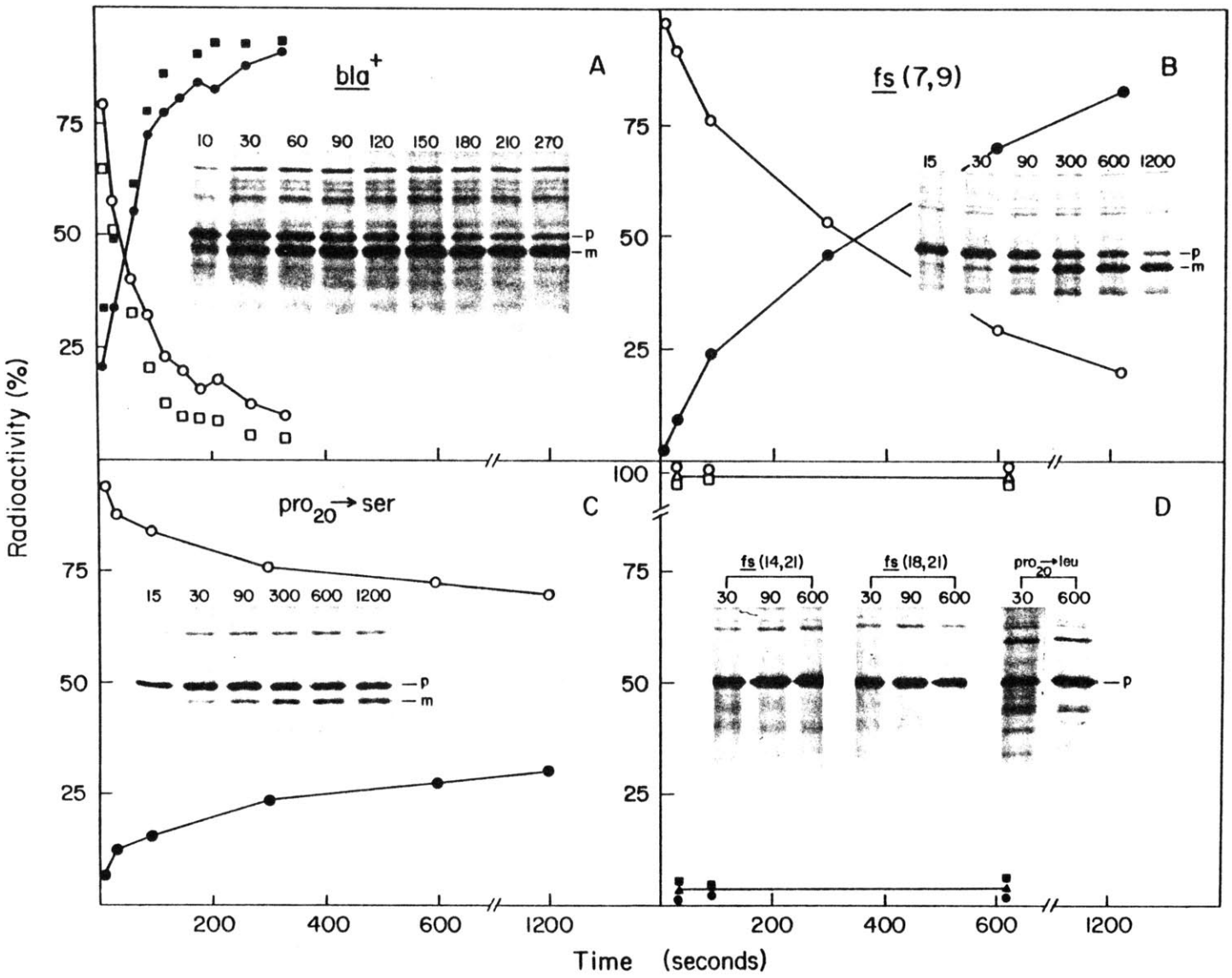

bla+
A
10 30 60 90 120 150 180 210 270
–p
–m
fs (7,9)
B
15 30 90 300 600 1200
pro20→ ser
C
15 30 90 300 600 1200
fs (14,21)
fs (18,21)
pro20→leu
D
30 90 600
30 90 600
30 600
Radioactivity (%)
Time (seconds)
200 400 600 1200

Figure 11: Pulse-chase analysis of the gene products of signal sequence allels. Pulse-chase experiments for the bla alleles, tsH41, fs(7,9), pro_{20}->ser, fs(14,21), fs(18,21) and pro_{20}->leu were performed exactly as described in the legends to figures 9 and 10 with the following modications. The time of sampling during the chase varied for different experiments. Choloramphenical instead of cold methionine was used to stop the incorporation of label in the pulse-chase experiments with the alleles, fs(7,9) and tsH41. The autoradiograms for these pulse-chase experiments are presented as insets in each panel (p, precursor form and m, mature form) except for the autoradiogram of tsH41 which is not shown. For each panel the percentage of the total radioactivity in bla encoded proteins in either precursor or mature form is plotted on the abscissa, and the length of the chase in seconds is plotted on the ordinate. Panel A: ○ , bla+ precursor form; ● , bla+ mature form; □ , tsH41 precursor form; ■ , tsH41 mature form. Panel B: ◇ , fs(7,9) precursor form; ◆ , fs(7,9) mature form. Panel C: ◇ , pro_{20}->ser precursor form; ◆ , pro_{20}->ser mature form. Panel D: ○ , fs(14,21) precursor form; ● , fs(14,21) mature form; □ , fs(18,21) precursor form; ■ , fs(18,21) mature form; △ , pro_{20}->leu precursor form; ▲ , pro_{20}->leu mature form. The data from figures 9 and 10 for the bla+ allele are repeated in panel A for ease of comparison.

of the signal sequence of β-lactamase such that less than 5% (resolution of the autoradiograms) of these proteins are processed to give the mature species. The alleles fs(7,9) and pro_{20}->ser alter the signal sequence such that precursor is processed to the mature species but at a much slower rate than precursor containing the wild-type signal sequence.

D. Cellular location of bla encoded proteins by cell fractionation

The ability to identify the protein products of the several alleles of the bla gene allowed us to examine the effect of the changes in the structure of β-lactamase on its cellular location. Since β-lactamase is normally secreted from the cell into the periplasm, we adapted the osmotic shock procedure of Neu and Heppel (1965) to distinguish proteins located in the periplasm from proteins present in the cytoplasm or attached firmly to the cyotplasmic membrane. Irradiated cells were infected with P22 bla^+ or the various P22 bla^- phages and labeled after infection with either ^{35}S-methionine or uniformly labeled ^{14}C amino acids. The cells were transferred from hypertonic to hypotonic medium by centrifugation and resuspension as described in Methods. In the end, three samples were produced: the periplasmic fraction (supernatant), the cytoplasmic/membrane-bound fraction (pellet) and an unfractionated control.

1. *bla*$^+$ (*mature species*)

The results of such an experiment with P22 *bla*$^+$ (wild-type β-lactamase) are shown in Figure 12 (lanes g, h, l). Among the labeled proteins (which include many phage proteins as well as the β-lactamase related ones) only the mature form of β-lactamase is found in the periplasmic fraction. This results verified the periplasmic location of β-lactamase in the case of the P22-infected *S. typhimurium*. The remaining proteins, notably the β-lactamase precursor form, remain in the cytoplasmic/membrane-bound fraction. Quantitation of these results by measurement of the intensity of the bands in the autoradiograms (Table 7) showed that about 90% of the mature β-lactamase is in the periplasmic fraction, whereas about 90% of the precursor is in the cytoplasmic/membrane-bound fraction.

2. *Chain terminating alleles of bla*

Using the various *bla*$^-$ mutants, we examined the cellular locations of the precursor and product forms of the β-lactamase in experiments similar to the one described above for the wild-type. For mutants which cause premature termination of the polypeptide chain (that is, mutants *am*H46, *fs*I40, *oc*H18, *am*H97; see Figure 4), the two bands which differ by 2500 daltons were both found in the cytoplasmic/membrane-bound fraction after fractionation. This unexpected result suggests that deletion of even a small part of the carboxy terminus of β-lactamase results in failure of

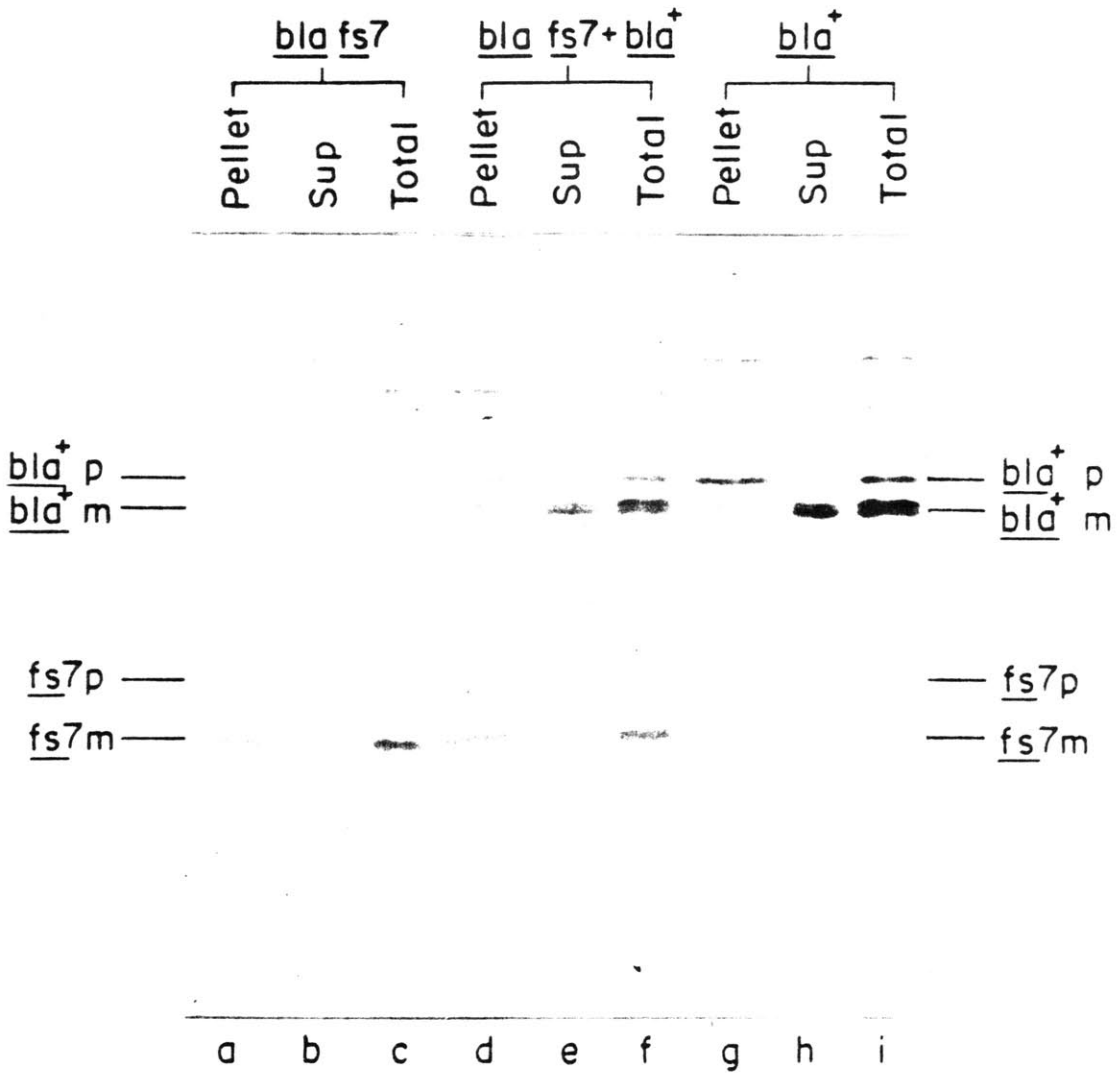

bla fs7
bla fs7+ bla+
bla+
Pellet
Sup
Total
Pellet
Sup
Total
Pellet
Sup
Total
bla+ p
bla+ m
fs7p
fs7m
bla+ p
bla+ m
fs7p
fs7m
a b c d e f g h i

Figure 12: Osmotic shock analysis of ^{14}C-labeled cells infected with bla-fsI7 and bla^{+} phage. A culture of DB4381 was irradiated and infected with bla-fsI7, bla^{+} or a mixture of the two phages. The multiplicity of infection was held constant at 20; for the mixed infection each phage was used at a moi of 10. The infected cells were labeled with ^{14}C amino acids and osmotically shocked (see Experimental Procedures). 20 µl aliquots were loaded on a slot. Electrophoresis was on a 15% gel and the autoradiogram was exposed for 24 hr. (Pellet) represents the membrane-bound and cytoplasmic fraction. The supernatant (Sup) represents periplasmic proteins. (Total) is an equivalent aliquot of the same labeled cells which were not shocked.

Table 7. Fractionation of beta-lactamase-related peptides by the cold osmotic shock procedure

Infection	Band[a]	Recovery (%)[b]	Periplasmic (supernatant) (%)	Nonperiplasmic (pellet) (%)
Mutant: bla-fsI7 (terminates after 167 residues)				
Wild-type	p(bla$^+$)	100	10	90
	m(bla$^+$)	113	90	10
Mutant	p(bla$^-$)	111	10	90
	m(bla$^-$)	100	18	82
Mixed	p(bla$^+$)	100	12	88
	m(bla$^+$)	108	73	27
	p(bla$^-$)	72	<10	>90
	m(bla$^-$)	99	15	85

Mutant: bla-amH97 (terminates after 206 or 226 residues)				
Wild-type	p(bla$^+$)	86	4	96
	m(bla$^+$)	106	87	13
Mutant	p(bla$^-$)	140	<10	>90
	m1(bla$^-$)	118	23	77
	m2(bla$^-$)	84	50	50
Mixed	p(bla$^+$)	110	<10	>90
	m(bla$^+$)	100	78	22
	p(bla$^-$)	75	<10	>90
	m1(bla$^-$)	110	9	91
	m2(bla$^-$)	69	44	56
Mutant: bla-fsI40 (terminates after 251 residues)				
Wild-type	p(bla$^+$)	86	<10	>90
	m(bla$^+$)	91	83	17
Mutant	p(bla$^-$)	175	<10	>90
	m(bla$^-$)	116	12	88

Mixed	p(both)	ND	ND	ND
	m(both)	100	60	40
Mutant: bla-amH46 (terminates after 264 or 274 residues)				
Wild-type	p(bla$^+$)	67	<10	>90
	m(bla$^+$)	94	83	17
Mutant	p(bla$^-$)	100	<10	>90
	m(bla$^-$)	98	13	87
Mixed	p(both)	112	<10	>90
	m(both	96	35	65
Mutant: bla-ts41				
Mutant	p(bla-)	100	<10	>90
	m(bla-)	95	65	35
Mixed	P(both)	95	<10	>90
	m(both)	98	85	15

[a](p) means precursor band, (m) means mature band; where there are two mature forms (class III mutants), (m1) is the larger and (m2) is the smaller as in Table 1, which gives the actual molecular weights.

[b]Recovery was determined by divising the sum of the radioactivity in particular precursor or mature bands in the pellet and supernatant fractions by the radioactivity in the same band(s) in the unfractionated sample. Equivalent amounts of each fraction (see Figure 4) were applied to each lane of the SDS-polacrylamide gel.

All the experiments were performed as described in the legend to Figure 4 (from which the relevant data here are derived). The autoradiograms were exposed for 20-40 hr on preflashed film and scanned as described in Experimental Procedures. The results for another class III mutant (not shown) are very similar to the ones given here for amH97. (ND) Not determined.

secretion of the polypeptide into the periplasm despite the apparently normal processing of the precursor into a "mature" form.

A typical experiment using P22 bla-fsI7 is shown in Figure 12. As in the case of wild-type, 90% of the precursor-sized protein was found in the cytoplasmic/membrane-bound fraction (pellet); however, in contrast to wild-type, 90% of the mature fsI7 protein is also found in this fraction (Figure 12, lanes a and b). To eliminate possible artifacts attributable to the necessity of carrying out the osmotic shock procedure on different samples, we carried out a control infection in which the same cells were infected with both P22 bla-fsI7 and P22 bla^+ phages simultaneously. Since the prematurely terminated β-lactamase proteins synthesized by the mutant migrate to a position in the SDS-polyacrylamide gel electrophoresis distinct from the position of the wild-type β-lactamase proteins, the fractionation of both mutant and wild-type proteins can be observed in the same samples. The results (Figure 12, lanes d and e) clearly show that in the mixed infection the mature wild-type β-lactamase appears as expected in the periplasmic fraction while most of the mutant protein stays in the cytoplasmic/membrane-bound fraction. Quantitation of these results is given in Table 7.

These results were unchanged when the cellular location experiments were performed as above, with the exception that the periplasmic proteins were released by conversion of infected labeled cells to spheroplasts (Witholt et al., 1976) instead of by osmotic shock (not shown).

Figure 12 also provides evidence that the synthesis of wild-type β-lactamase is proportional to the multiplicity of infection (lanes d-f

represent moi = 10; lanes g-i represent moi = 20). However, the same relative amount of the total β-lactamase protein is found as the mature species in the periplasm, indicating that neither the secretion nor protein processing mechanisms is saturated.

Table 7 also summarizes the results of osmotic shock experiments similar to the one in Figure 12 with other chain-terminating mutations in the bla gene. In every case (including one mutation, amH46, which terminates either 11 or 21 amino acid residues from the normal carboxy terminus of the protein) the precursor and the mature protein (that is, the two forms that differ by about 2500 daltons) are not recovered in the periplasmic fraction (less than 20% of the total), but instead remain in the cytoplasmic/membrane-bound fraction. It should be noted that some of the mutants tested (fsI40 and amH46) produce protein fragments whose molecular weight is close enough to that of the wild type β-lactamase to preclude resolution of mutant and wild-type bands on the SDS-polyacrylamide gels of the mixed infections. In these cases it was observed that the radioactivity migrating at the position of mature wild-type protein was divided about equally between the periplasmic and cytoplasmic/membrane fractions, as expected if the mutant form is not free in the periplasm while the wild-type form is.

Further examination of the data in Table 7 reveals that the chain-terminating mutants which produce a third band in addition to the precursor and mature forms (class III) show a slightly different behavior: about half of the extra band appears in the periplasmic fraction, although no precursor or mature protein does. This further processing (or

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[page 146]

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degradation) of the protein appears to facilitate secretion, since the third band is recovered in the periplasmic fraction much more efficiently than either of the larger β-lactamase-related peptides made by these mutants.

The preceding experiments suggest that the prematurely truncated forms of β-lactamase are either soluble cytoplasmic or weakly bound to the surface of a membrane. These results suggests that the carboxy terminus of the β-lactamase protein is essential for the proper execution of either an initial step (translocation across the inner membrane) or perhaps a terminal step in secretion (release from the inner membrane). Since we examined a mutant in which the chain is terminated at residue 265 (or 275) of the protein sequence, the last 21 (or 11) amino acid residues are essential.

To examine the nature of the association of the mature forms of the prematurely terminated peptides with osmotically shocked or spheroplasted cells, we fractionated proteins bound to membranes from soluble proteins using the method of Silhavy *et al.* (1976). Irradiated cells were infected with P22 *bla*$^+$ or P22 *bla*$^-$ phages and then labeled with ^{14}C amino acids. The labeled cells were converted to spheroplasts and lysed by sonication or osmotically. Intact cells (comprising less than 10% of the radioactivity) were removed and the membranes were collected by centrifugation at 100,000 x g for 2 hr. The membrane pellet and supernatant fluid were separated and assayed for β-lactamase proteins by SDS-polyacrylamide gel electrophoresis as before. To monitor the success of the fractionation procedure, the SDS-polyacrylamide gel was stained

with Coomassie blue. The membrane fractions contained only a few staining protein bands, with molecular weights corresponding to the major membrane proteins of S. typhimurium (Osborn et al., 1972), while the cytoplasmic fractions contained a large number of staining protein bands, as expected. Examination of the intensities of Coomassie blue stain in several isolated membrane bands showed that the percentage of total membrane protein which remained in the soluble fraction was less than 20%.

The results of a membrane isolation experiment in which the cells were lysed by lysozyme-osmotic shock are shown in Figure 13 and Table 8. In every case we found that most of the processed β-lactamase-associated proteins are soluble. The majority of the precursor forms of these proteins are also found in the soluble fraction, although in the case of infections with amH46 and wild-type a substantial fraction of the total precursor protein is membrane-bound (see Table 8). The same results were obtained if the cells were lysed by sonication (data not shown). These observations suggest that the nonsecreted mature mutant forms are either soluble cytoplasmic or weakly bound to the surface of a membrane.

3. Localization of the precursor products containing wild-type and mutant signal sequence alleles of bla

We investigated the cellular location of the precursor products of the wild-type and signal sequence alleles of bla by cell fractionation experiments similar to the ones described above. However, the procedures were modified in order to detect the possible change in cellular location

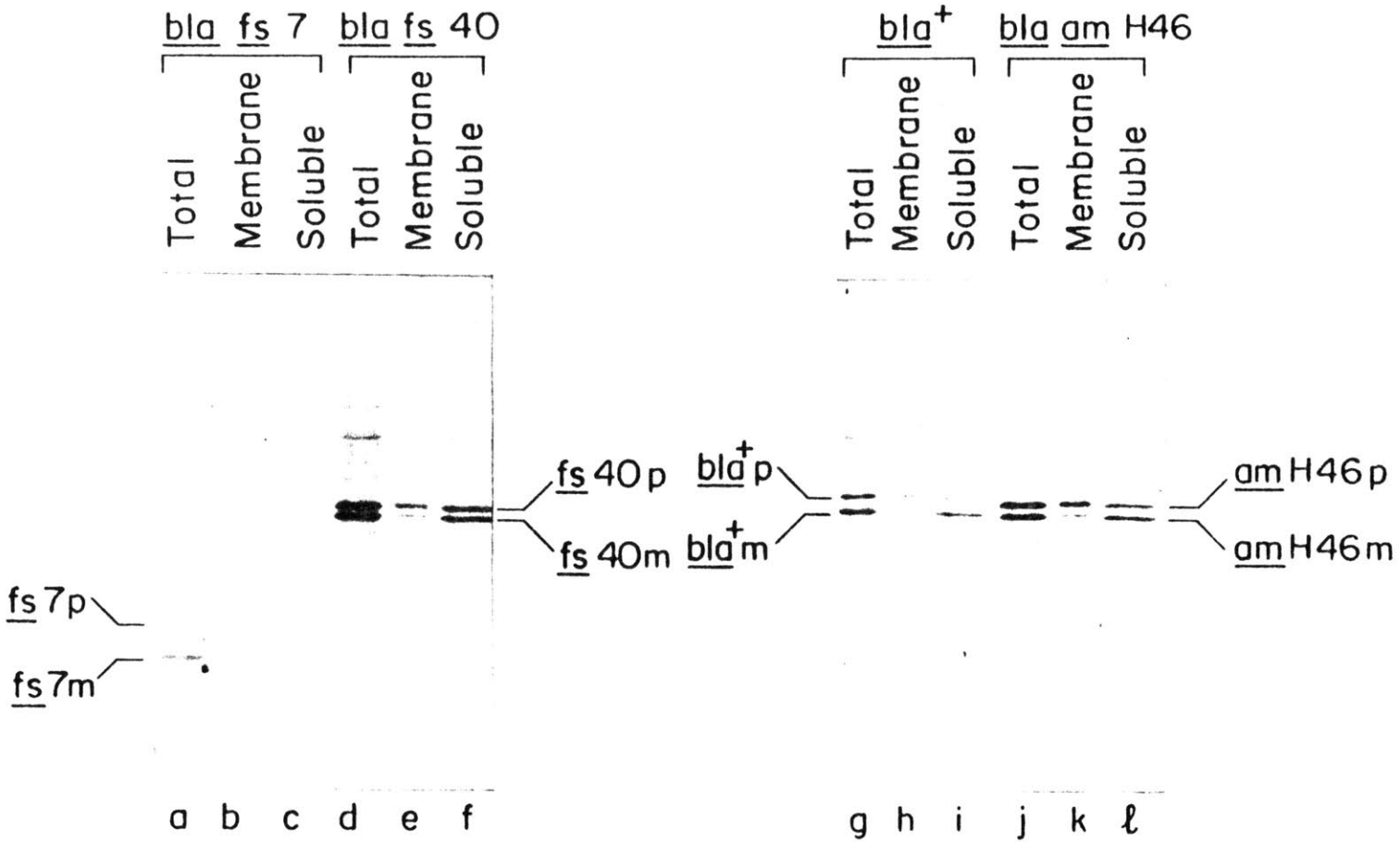

bla fs 7
bla fs 40
bla+
bla am H46
Total
Membrane
Soluble
Total
Membrane
Soluble
Total
Membrane
Soluble
Total
Membrane
Soluble
fs 40p
fs 40m
bla+p
bla+m
am H46p
am H46m
fs 7p
fs 7m
a b c d e f
g h i j k ℓ

Figure 13: Membrane isolation of cells infected with bla$^+$ and bla$^-$ phage. A culture of DB4381 was irradiated and infected with bla-fsI7, bla-fsI40, bla-amH46 or bla$^+$ phages at a moi of 20. The infected cells were labeled with ^{14}C amino acids as described in methods. These cells were lysed by lysozyme osmotic shock and their membranes were isolated by centrifugation as described in Experimental Procedures. (Total) represents the labeled cellular proteins after removal of the periplasmic fraction; the mature wild-type β-lactamase present represents the small fraction of this protein which was not released into the periplasmic fraction. 20 µl of each fraction were loaded on a slot; care was taken so that each sample represents an equivalent amount of culture. Electrophoresis was on a 15% gel and the autoradiogram was exposed for 8 hours.

Table 8. Fraction of beta-lactamase-related peptides by membrane isolation.

Infection	Band	Recovery (%)	Soluble (%)	Membrane (%)
*fs*17	p	ND	ND	ND
	m	66	78	22
*fs*40	p	100	78	22
	m	88	89	11
*am*H46	p	91	54	46
	m	95	77	23
bla^{+}	p	117	62	38
	m	106	91	9

The experiment was performed as described in the legend to Figure 5. The autoradiograms were exposed for 8-16 days on preflshed film and scanned as described in Experimental Procedures. (p), (m) and (Recovery) are defined as in the legend to Table 2. Apparently due to the differences in total protein loaded, the width of lanes containing unfractionated proteins or soluble proteins was substantially greater than that of those lanes containing only membrane proteins. Since the scanning densitometer measures the radioactivity in a band over a fixed width, these differences in lane width between fractions were taken into account in calculations of the amount of radioactivity present in a band of a given fraction. (ND) Not determined.

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[page 152]

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of these precursors as a function of time. Irradiated cells that were infected with the appropriate P22bla phage were labeled with ^{35}S-methionine for 30 seconds, and then cold methionine was added. Samples of the labeled cells were removed at various times after the additon of the chase and immediately chilled to 0°C. The cells in each sample were converted to spheroplasts, and then the periplasmic proteins were separated from the spheroplasts by centrifugation. The spheroplast were then lysed osmotically, and the membrane and other macromolecular structure were separated from the soluble proteins of the cytoplasm by ultra-centrifugation. The proteins in each fraction were separated by SDS-polyacrylamide gel electrophoresis. The total protein in each fraction was visualised by Coomassie blue stain while labeled proteins were visualised by autoradiography. The intensities of the labeled bands on the autoradiograms were quantitated with a scanning densitometer (Methods).

To obtain meaningful results from these pulse-chase cell fractionation experiments several conditions needed to be satisfied. We assumed that by placing the samples at 0°C we effectively ended the chase, that is if intermediates in the secretion process existed they could be trapped by chilling the samples to 0°C. For alleles of bla that produced mature β-lactamase this assumption could be checked. We reported above a series of pulse chase experiments in which the chase was ended by mixing the aliquot of labeled culture with sample buffer heated to 95°C. It is safe to assume that under these conditions all cellular processes including secretion stop almost instaneously. Therefore if chilling also stops

secretion, then the ratios of label in precursor to mature species at specific times during the chase in which the chase was ended by chilling should be equal to the ratio of label in precursor to mature species at the same times during the chase in which the chase was ended by transferring an aliquot to sample buffer at 95°C. These conditions are met for experiments with the fs(7,9) and bla$^+$ alleles but not for pro_{20}->ser (data not shown). The ratio of precursor to mature species for pro_{20}->ser at any time point is similar to wild-type when the chase is ended by chilling, but much greater when the chase is ended with heated sample buffer. This result indicates that a portion of the precursor in pro_{20}->ser continues to be processed and/or secreted at 0°C.

To be able to compare the behavior of the products of bla encoded proteins in separate fractionations, we examined the cell fractionation of unlabeled and labeled proteins not encoded by bla. These proteins partition to the same cellular fraction independent of whether they are from two separate fractionations within the same experiment (figure 14) or two fractionations from different experiments (data not shown). In addition the pattern of proteins of each cellular fraction give the expected result. The cytoplasmic fraction has the bulk of the Coomassie blue stained and labeled proteins (figure 14). The membrane fraction has a smaller number of protein bands than the cytoplasmic fraction and among these is included the majority of the outer membrane proteins. The periplasmic fraction has very few proteins as detected by Coomassie blue, but for those alleles in which mature β-lactamase is detected greater than

90% of the total mature protein present for that particular time point is recovered in the periplasm.

A typical autoradiogram is presented in figure 14 of a pulse-chase cell fractionation experiment for irradiated cells infected with P22bla phage. The quantitation of the intensities of bla$^+$ encoded proteins is presented in figure 14. The precursor species and mature species give very different patterns of fractionation. The precursor appears in all three fractions with the majority of label in newly synthesized precursor (soon after the addition of the chase) fractionating as soluble in the cytoplasm (figure 14; lanes b, e and h). In contrast the mature species is found almost exclusively in the periplasmic fraction (figure 4; lanes a, d, g and j). Because the majority of the label which apparently transfers from the precursor to the mature species comes from precursor which fractionates as soluble in the cytoplasm, we can conclude that precursor which is an intermediate to the formation of mature species is either soluble in the cytoplasm or weakly bound to the surface of a membrane. The percentage of newly synthesized precursor that fractionates as periplasmic is so small that the presence of precursor in this fraction can be explained by the cross contamination of one fraction with another (see legend to table 9).

The products of five of the six signal sequence alleles of bla were investigated by pulse-chase cell fractionation experiments identical to the one described for wild-type above, and these results are presented in Table 9. They clearly show that the precursor products of these five alleles are recovered predominantly in the membrane and cytoplasmic

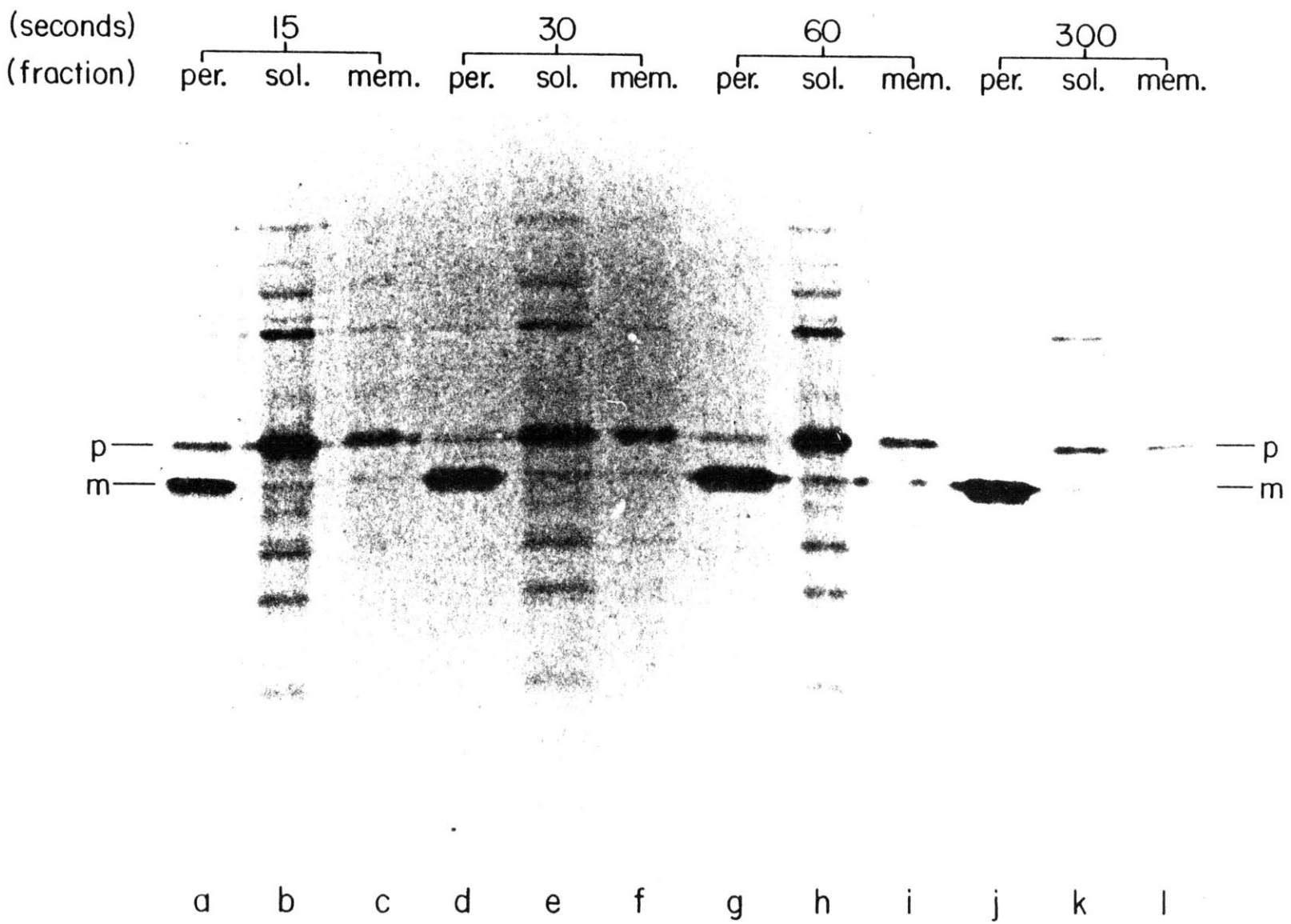

(seconds)
(fraction)
15
30
60
300
per.
sol.
mem.
per.
sol.
mem.
per.
sol.
mem.
per.
sol.
mem.
p
m
p
m
a
b
c
d
e
f
g
h
i
j
k
l

Figure 14: Pulse-chase cell-fractionation of cells infected with bla+ phage. A culture of DB4381 was irradiated and infected with P22bla+ phage at a moi of 20. The infected cells were pulse labeled with ^{35}S-methionine and chased with cold methionine. Samples were removed at various times during the chase and chilled to 0°C. The cellular proteins in each sample were partitioned by cell fractionation (see Methods) into three fractions, periplasmic (per.), soluble cytoplasmic (sol.) and membrane bound (mem.). 20µl of each fraction were loaded on a slot. Electrophoresis was on a 12.5% gel and the autoradiogram was exposed for 2 days. The length of the chase (in seconds) and the cellular fractions are listed above each lane. p, precursor form. m, mature form.

Table 9. Pulse-chase membrane-fractionation of wild-type and signal sequence alleles of *bla*.

			% total label in bla encoded proteins		
Allele	Chase (seconds)	Band	Periplasm	Soluble cytoplasm	Membrane
wt	15	Mature	31	0	0
		Precursor	5	53	10
	30	Mature	49	0	0
		Precursor	1	41	9
	60	Mature	55	0	0
		Precursor	0	38	7
	300	Mature	81	0	0
		Precursor	0	11	8

fs(7,9)	15	Mature	10	0	0
		Precursor	7	60	24
	90	Mature	22	0	0
		Precursor	3	44	31
	600	Mature	40	0	0
		Precursor	1	18	41
	1200	Mature	42	0	0
		Precursor	0	11	45
pro_{20}→ser	15	Mature	30	0	0
		Precursor	6	35	29
	90	Mature	41	0	0
		Precursor	3	21	35
	600	Mature	41	0	0
		Precursor	2	8	49
	1200	Mature	51	0	0
		Precursor	1	7	41

fs(14,21)	15	Precursor	3	67	30
	90	Precursor	5	59	36
	180	Precursor	3	37	60
	390	Precursor	6	34	60
fs(18,21)	15	Precursor	3	43	57
	90	Precursor	2	42	55
	180	Precursor	0	27	73
	390	Precursor	0	26	74
pro_{20}→leu	15	Precursor	14	36	50
	90	Precursor	5	28	67
	180	Precursor	9	23	68
	390	Precursor	4	23	73

All experiments were performed as described in the legend to Figure 14. The autoradiograms were exposed 20-40 hr on preflashed film and scanned as described in Methods. In all fractionations the apparent contamination of the periplasmic fraction with membrane and cytoplasmic fractions is less than or equal to 14%.

fractions. Like the wild-type precursor, the small amount of these precursors in the periplasmic fraction can be accounted for by contamination of this fraction with the cytoplasmic fraction. In addition, essentially no precursor of any of these alleles is recovered in the periplasmic fraction when this fraction is seperated from the rest of the cell by osmotic shock (data not shown). From these experiments we conclude that all of the precursors of bla (independent of the allele) are in a different cellular location than the wild-type mature species.

The distribution of the mutant precursors among the cytoplasmic and membrane fractions is similar to wild-type for all alleles except the precursors of fs(18,21) and pro_{20}->leu. These precursors behave noticeably different from the other precursors. They show a significantly greater association with the membrane at earlier time points in the chase.

E. Trypsin accessibility experiments

Since proteins can be localized to either surface of a membrane, proteins that appear membrane bound by cell fractionation experiments have ambiguous cellular locations. For example, the membrane bound precursors could be either on the cytoplasmic side or the periplasmic side of the inner membrane. If the precursor and mature forms of bla that fractionate as soluble in the cytoplasm are really weakly membrane bound prior to the lysis of the cell, then these proteins suffer the same ambiguity as the truly membrane bound proteins. To resolve whether the precursor or mature species of particular alleles are on the cytoplasmic or perplasmic side

of the inner membrane, we probed the localization of these proteins by trypsin accessibility experiments (Halegoua and Inouye, 1979).

The rationale for these experiments is simple. If a protein is localized in the periplasm or on the periplasmic side of the inner membrane, then it should be digested by proteases when cells are converted to spheroplast as well as when the spheroplast are lysed. However, if this protein is localized in the cytoplasm, then it should be protected from the degradative action of proteases when the cells are converted to spheroplasts but not when the spheroplasts are lysed.

The actual design of these protease experiments is very similar to the pulse chase cell fractionation experiments. Irradiated and infected cells were pulse labeled with ^{35}S-methionine for 30 seconds followed by the addition of unlabeled mehtionine (and in some cases chloramphenicol). Samples were removed approximately 20 seconds and several minutes after the addition of the chase and chilled immediatiely to 0^{o}C. Each sample was converted to spheroplasts and then split into two aliquots. The spheroplasts in one aliquot were lysed osmotically. Then the aliquot containing the intact spheroplasts and the aliquot containing the lysed spheroplasts were each subdivided and treated with increasing amounts of trypsin. The trypsin digests were ended after 10 minutes by transferring the digests to sample buffer preheated to 95^{o}C. Then these samples were electrophoresed on SDS-polyacrylamide gels. Autoradiograms of the gels were made and the intensties of the bla encoded proteins were quantitated as described in the Methods. In these experiments the trypsin accessibility of the non-bla encoded proteins is consistant with the

cellular fractionation of these proteins and with the state of the spheroplasts, lysed or intact (Methods).

1. The bla^+ allele

The results of the trypsin accessibility experiments with cells infected with bla^+ phage are shown in figure 17 (panels 3a and b). After 20 seconds of chase, 65% of the label in bla encoded proteins is in the precursor form. This protein is digested by trypsin when the spheroplasts are lysed but is not digested when the spheroplasts are intact. Five minutes after the addition of the chase, the small amount of precursor that remained (20% of the total label in bla encoded proteins) remains protected from trypsin in intact spheroplasts. The mature β-lactamase is not digestible by trypsin in either lysed cells or spheroplasts (see section F below).

2. The alleles tsH41, tsH1, fsI7 and amH46

The trypsin insensitivity of wild-type precursor in spheroplasts but not in lysed cells seemed due to its location on the cytoplasmic side of the inner membrane. However, an alternative interpretation of these data is possible. Since the wild-type mature species apparently exists in a trypsin resistant conformation, it is possible that the wild-type precursor also exists in a trypsin resistant conformation on the periplasmic side of the inner membrane. The precursor form, unlike the

mature form, is converted to a trypsin sensitive conformation upon lysis of the spheroplasts.

To distinguish among these two interpretations, we performed trypsin accessibiility experiments with alleles of bla whose precursor and mature forms are trypsin sensitive in crude lysates. If the precursor form of one of these alleles was trypsin resistant in intact spheroplasts, then we could be reasonably confident that the apparent trypsin resistance of the precursor form was due to its protection by the inner membrane and not due to the precursor form assuming a trypsin resistant conformation.

Four alleles whose precursor and mature forms are trypsin sensitive in crude lysates are: two temperature sensitive alleles of bla (tsH1 and tsH41) and two chain terminating alleles (fsI7 and amH46). Except for trypsin sensitivity, the products of the tsH41 allele are identical to the products of the wild-type allele by the criteria of cell fractionation (Table 7) and kinetics of processing (figure 11, panel A).

In figure 15 we show a typical autoradiogram from a trypsin accessibiltiy experiment for the tsH41 allele. An examination of lanes a and f (samples in which no trypsin was added) show that the majority of precursor labeled at 20 seconds is eventually processed to the mature species. Lanes b-c show that this chaseable precursor is not digested by trypsin in intact spheroplasts while the mature species is. When the spherolasts are lysed, then the precursor is digested (lane e). After 5 minutes of chase the small amount of precursor that remains is not digested by trypsin when spheroplasts are intact. In contrast, the majority of the newly processed mature protein is digested by trypsin

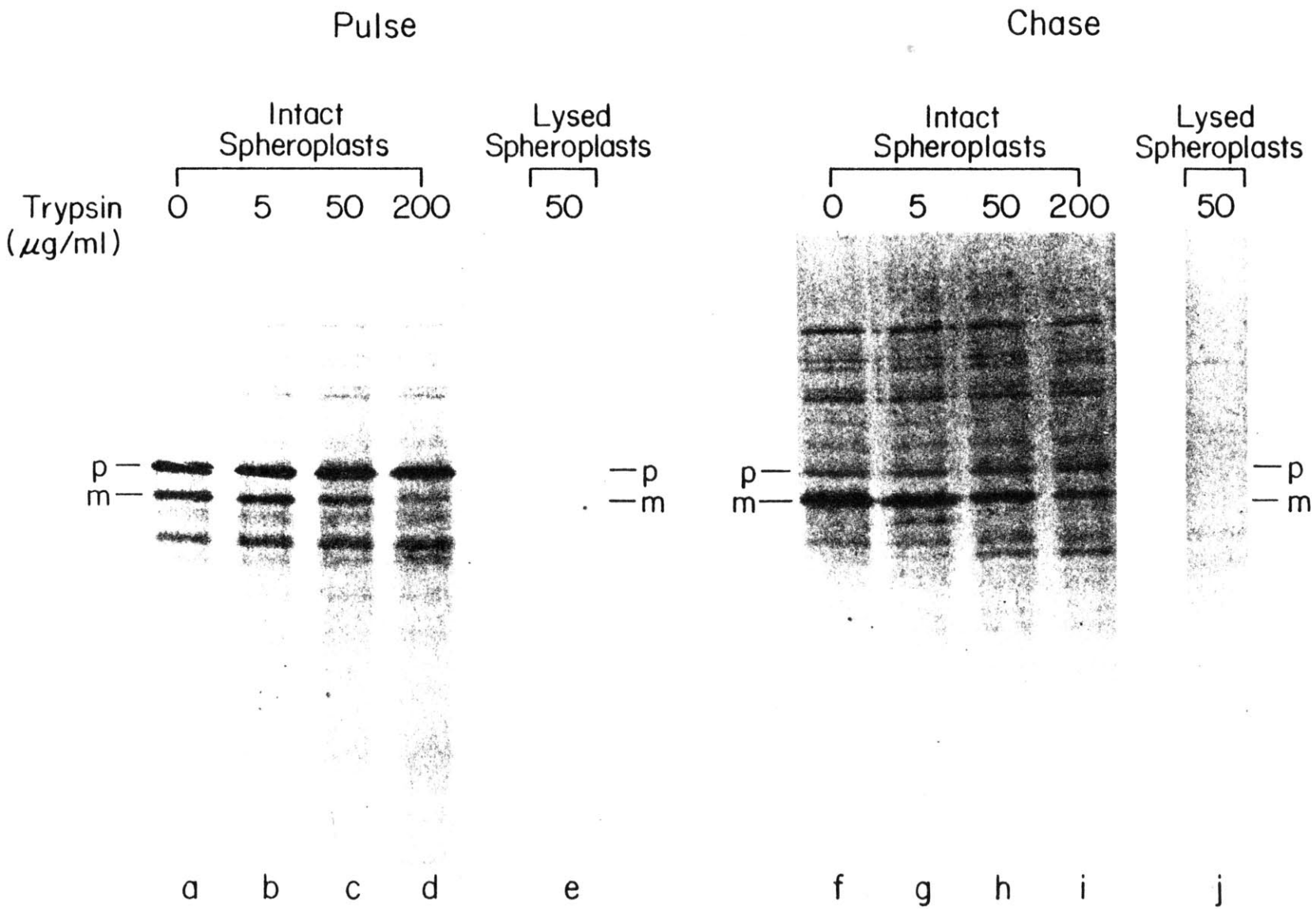

Pulse
Chase
Intact Spheroplasts
Lysed Spheroplasts
Intact Spheroplasts
Lysed Spheroplasts
Trypsin (μg/ml)
0
5
50
200
50
0
5
50
200
50
p
m
p
m
p
m
p
m
a
b
c
d
e
f
g
h
i
j

Figure 15: Pulse-chase trypsin accessibility experiment with cells infected with P22bla-tsH41. A culture of DB4381 was irradiated and infected with P22bla-tsH41 phage at a moi of 10. The infected cells were labeled with ^{35}S-methionine and chased in the presence of cold methionine and chloramphenical. Samples were removed immediately after the addition of the chase and 5-10 minutes later and chilled to 0^{o}C . The samples were converted to spheroplasts and then split in two. One half of the spheroplasts were pelleted and lysed osmotically (after the supernatant containing the periplasmic fraction was removed). Aliquots of the intact and lysed spheroplasts were exposed to increasing amounts of trypsin for ten minutes. 20µl of each digestion was loaded on a slot. Electrophoresis was on a 12.5% gel and the autoradiogram was exposed for 2 days. p, precursor form. m, mature form.

under these conditions (lanes f-i). The precursors and mature species of amH46 (figure 16; figure 17, panels 2a-d), fsI7 and tsH1 (data not shown) all behave similarilty to tsH41 in trypsin accessibility experiments.

These results strongly suggest that the precursors of these four alleles of bla are located on the cytoplasmic side of the inner membrane while the mature species to which they give rise are located on the periplasmic side of the inner membrane. They also carry implication that the apparent trypsin insensitivity of the wild-type precursor in spheroplasts is also due to its sequestration on the cytoplasmic side of the inner membrane and not due to some intrinsic trypsin resistance of the precursor form. Finally they suggest that the carboxy terminus of β-lactamase is not required for this protein to traverse the inner membrane.

3. The signal sequence alleles

The localization of the mutant precursors containing alterations in the signal sequence was also investigated by the trypsin accessibility experiments (figure 17). Examination of these results indicate that the signal sequence alleles of bla are divided into two classes. The precursors of one class (fs(14,21) and fs(7,9)) behave identically to the precursors of wild-type, amH46, fsI7, tsH41 and tsH1. They are digested by trypsin only after lysis of the spheroplasts (figure 17; panels 4a-b, 5a-b). For fs(14,21) the total amount of label in the precursor remains constant over the length of the chase as expected since this mutation

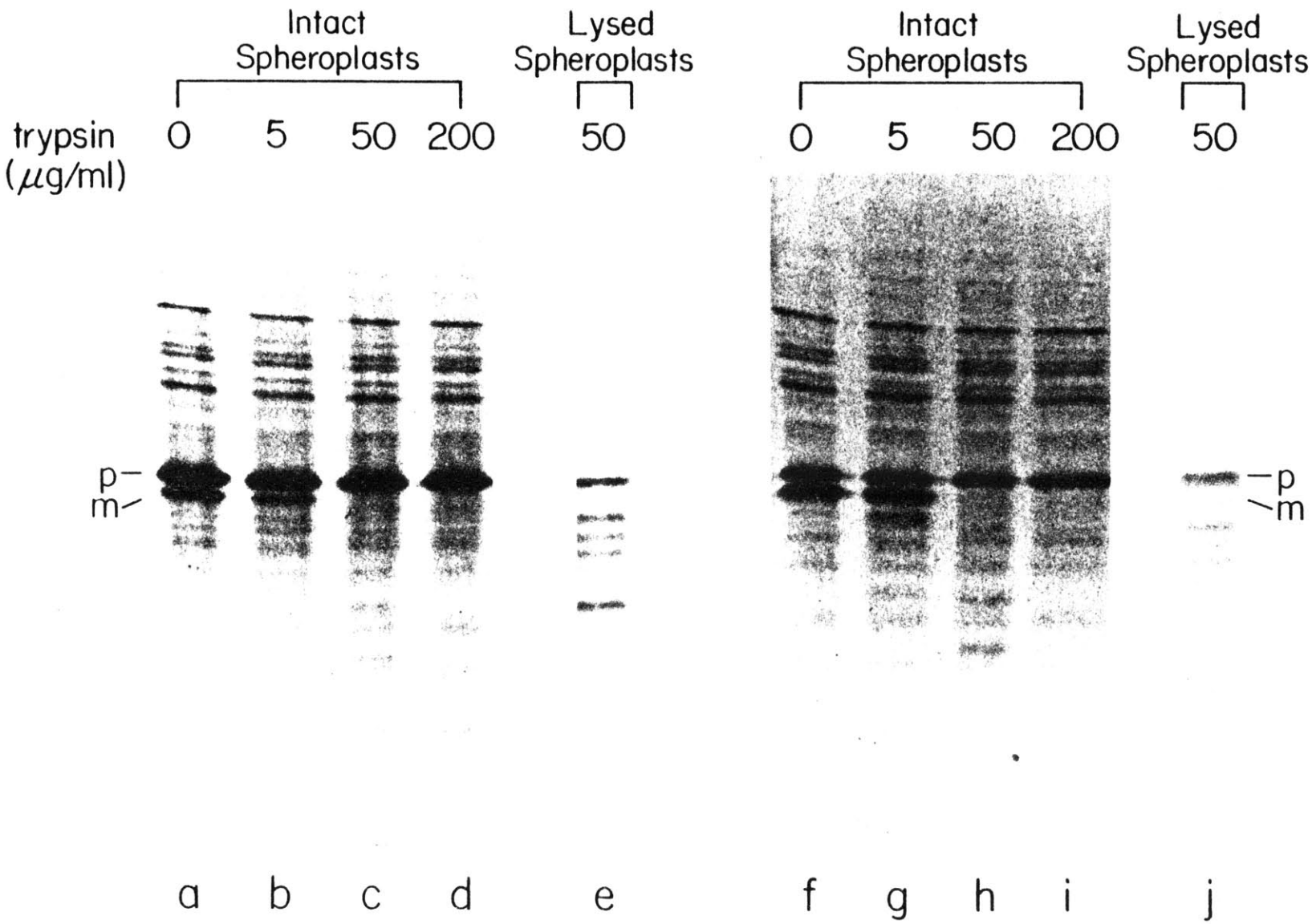
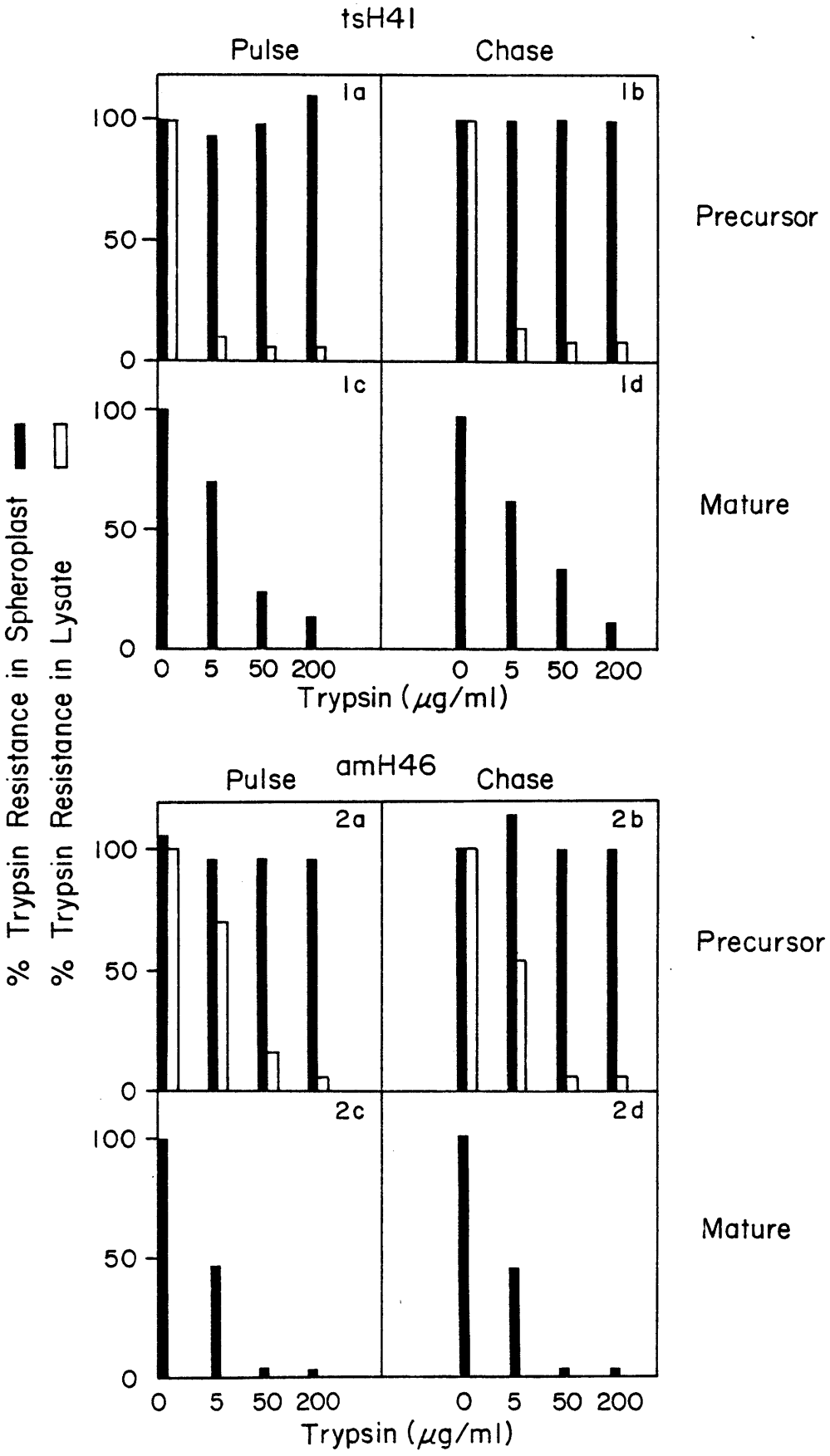
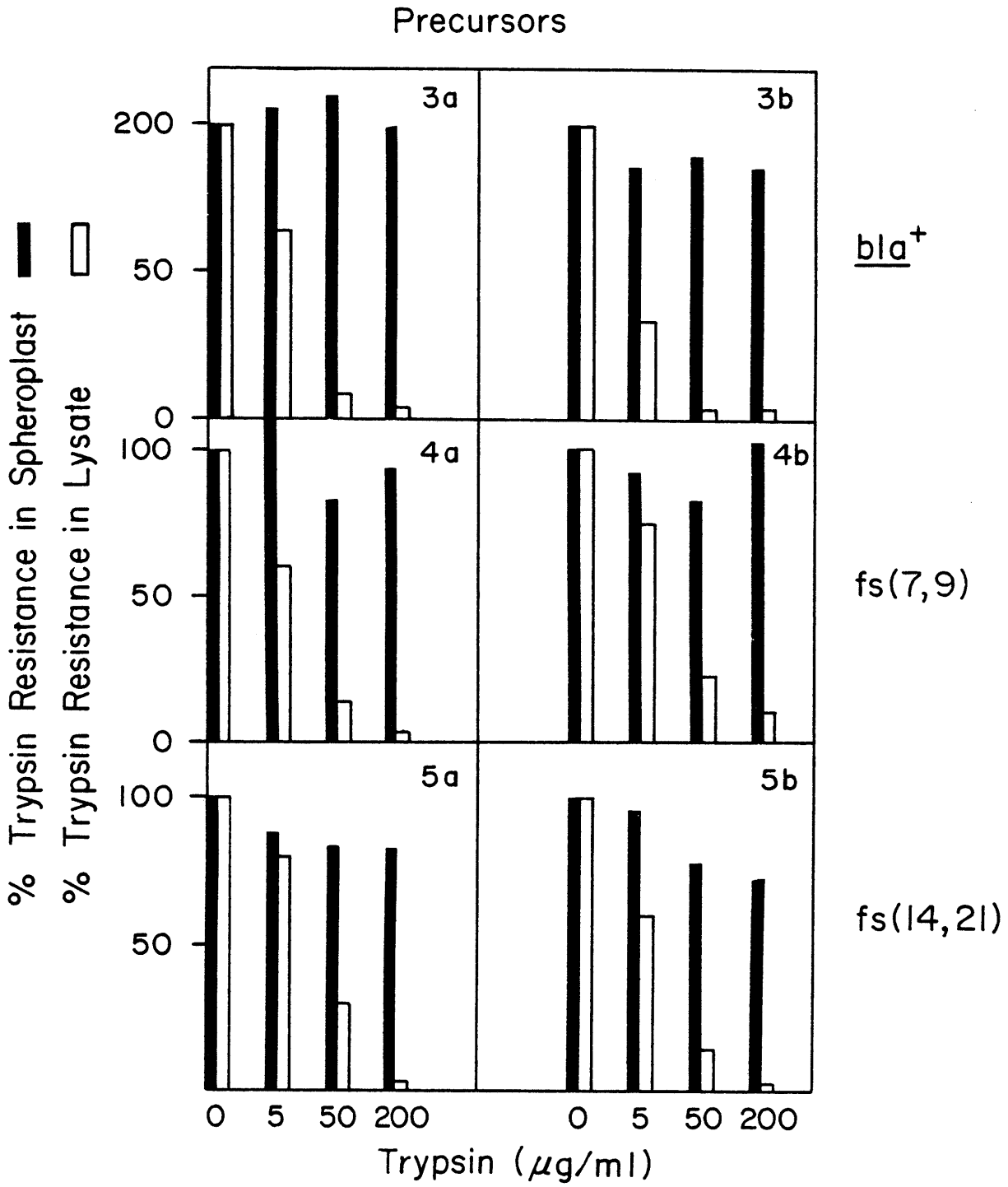

Intact Spheroplasts
Lysed Spheroplasts
Intact Spheroplasts
Lysed Spheroplasts
trypsin (μg/ml)
0
5
50
200
50
0
5
50
200
50
p
m
p
m
a
b
c
d
e
f
g
h
i
j

Figure 16: Pulse-chase trypsin accessibility expriment with cells infected with P22-amH46. A culture of DB4381 was irradiated and infected with P22-amH46 phage at a moi of 10. The rest of the experiment was performed exactly as described in the legend to figure 15 except chloramphenical was omitted.


tsH41
Pulse
Chase
1a
1b
1c
1d
100
50
0
Precursor
Mature
% Trypsin Resistance in Spheroplast
% Trypsin Resistance in Lysate
0 5 50 200
0 5 50 200
Trypsin (μg/ml)
amH46
Pulse
Chase
2a
2b
2c
2d
100
50
0
Precursor
Mature
0 5 50 200
0 5 50 200
Trypsin (μg/ml)


Precursors
3a
3b
4a
4b
5a
5b
200
50
0
100
50
0
100
50
% Trypsin Resistance in Spheroplast
% Trypsin Resistance in Lysate
0 5 50 200
0 5 50 200
Trypsin (μg/ml)
bla+
fs(7,9)
fs(14,21)

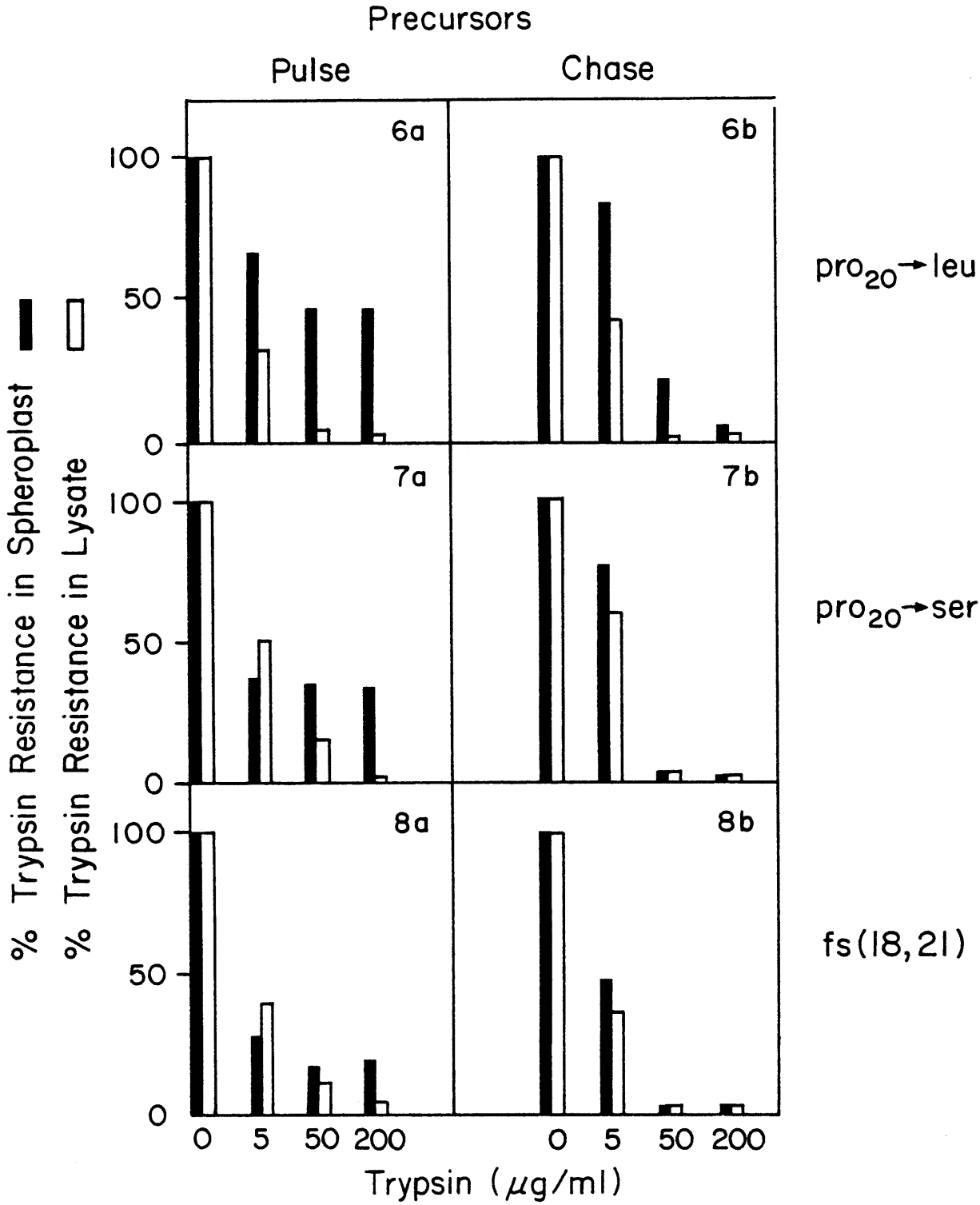

Precursors
Pulse
Chase
6a
6b
7a
7b
8a
8b
100
50
0
% Trypsin Resistance in Spheroplast
% Trypsin Resistance in Lysate
pro20→leu
pro20→ser
fs(18,21)
0
5
50
200
Trypsin (μg/ml)

Figure 17: Pulse-chase trypsin accessibility anlaysis of bla encoded proteins. Trypsin accessibility experiments were performed with cells labeled with ^{35}S-methionine and infected with P22bla phage carrying the allele, fs(7,9), fs(14,21), fs(18,21), pro_{20}->ser, pro_{20}->leu or wild-type as described in the legend to figure 15. The autoradiograms were exposed 20-40 hours. Then for each of the alleles above as well as the alleles, tsH41 and amH46, the intensities of the bands due to bla encoded proteins were quantitated as described in Methods. The solid bars indicate the percent of total label in precursor or mature form which remains after intact spheroplasts are treated with trypsin. The open bars indicate the percent of total label in precursor or mature form which remains after lysed spheroplasts are treated with trypsin.

abolishes processing of the signal sequence (see above). In the case of fs(7,9), 70% of the precursor that is trypsin inaccessible in spheroplasts is converted to the secreted mature species during the chase (data not shown). These experiments suggest that essentially all of the precursor molecules of fs(7,9) and fs(14,21) are sequestered in the cytoplasm.

The precursors of the second class (pro_{20}->leu, pro_{20}->ser, and fs(18,21)) behave differently from the precursors described above. 60 to 70 percent of the label in the precurors of fs(18,21) and pro_{20}->leu after 20 seconds of chase disappears when intact spheroplasts are exposed to trypsin (figure 17; panels 6a, 8a). The remaining 30 to 40 percent is digested if the spheroplasts are lysed (panels 6a, 8a). At the end of the chase more than 90% of the total label in these precursors disappears when intact spheroplasts are treated with trypsin (panels 6b, 8b). The precursor of pro_{20}->ser behaves similarly to the precursor of fs(18,21) and pro_{20}->leu in these experiments (panels 7a, 7b). However, like the cell fractionation experiments (see above) the chilliing of the samples apparently did not inhibit completely the processing and or secretion of this precursor. As a result we are unable to determine whether the processed molecules which accumulate during the chase come from precursor which is inaccessibile or accessible in spheroplasts.

These data suggest that after a short chase the precursor molecules of fs(18,21), pro_{20}->leu and pro_{20}->ser are located in two cellular compartments. Some precursor molecules are sequestered in the cytoplasm and some are accessible to the periplasmic side of the inner membrane. After a long chase, apparently all of the precursors of these alleles are

accessible to the periplasmic side of the inner membrane.

The fraction of molecules that have translocated acrosss the inner membrane can be measured by the trypsin accessibility of the precursor form and or the presence of mature β-lactamase. For wild-type, since apparently none of the precursor molecules are trypsin sensitive in intact spheroplasts, the fraction of molecules that have translocated the inner membrane after 20 seconds of chase is equal to the fraction of label in the mature form (35%, data not shown). For the alleles, pro_{20}->leu and fs(18,21), which are defective for processing, the fraction of molecules that have translocated the inner membrane after 20 seconds is equal to the fraction of precursor that is trypsin accessible in intact spheroplasts, that is 55% and 75% respectively (figure 17, panels 6a and 8a). These data suggests that the translocation across the inner membrane of the products of the alleles, fs(18,21) and pro_{20}->leu is either more rapid than the product of the wild-type allele or less inhibited by chilling to $0^{o}C$.

F. Intrinsic protease sensitivities of the bla^{+} precursor and mature forms

To investigate further the apparent protease insensitivity of the mature β-lactamase, we modified a standard trypsin accesibility experiment. After 20 seconds of chase, labeled cells infected with bla^{+} phage were chilled and subsequently lysed. Aliquots of this crude lysate were treated with increasing amounts of different pronases for 30 minutes.

The results from this experiment are shown in figure 18. A comparison of lanes a with the other lanes shows that while all other proteins including the wild-type precursor are digested completely by trypsin (figure 18; lanes h and i) and pronase XIV (figure 18: lanes e-g) the wild-type mature form appears to be virtually undigested by these enzymes. We conclude that the wild-type precursor and mature species must differ significantly in their conformation to exhibit such different sensitivities to these proteases in lysates.

G. The doublet phenonmenon

When samples containing labeled proteins from irradiated cells infected with bla$^+$ phage were prepared for SDS-polyacrylamide electrophoresis by mixing them with sample buffer containing β-mercaptoethanol and heating them to 60-90^{o}C for 2 minutes, the mature species of β-lactamase migrates as a doublet on the gel (figure 8, lane c). Subsequently we determined that if the same samples are heated for and additional 3 minutes at 100^{o}C, the mature species migrates as a single band which co-migrates with the slower migrating band of the doublet (figure 8, lanes c and d). When we investigated this phenomenon further, we found that if no β-mercaptoethanol is present in the sample buffer, the mature species migrates as a single band which co-migrates with faster migrating band of the doublet (data not shown). By the addition of β-mercaptoethanol and heat this faster migrating species can be converted to the slower migrating species and at intermediate time points mature

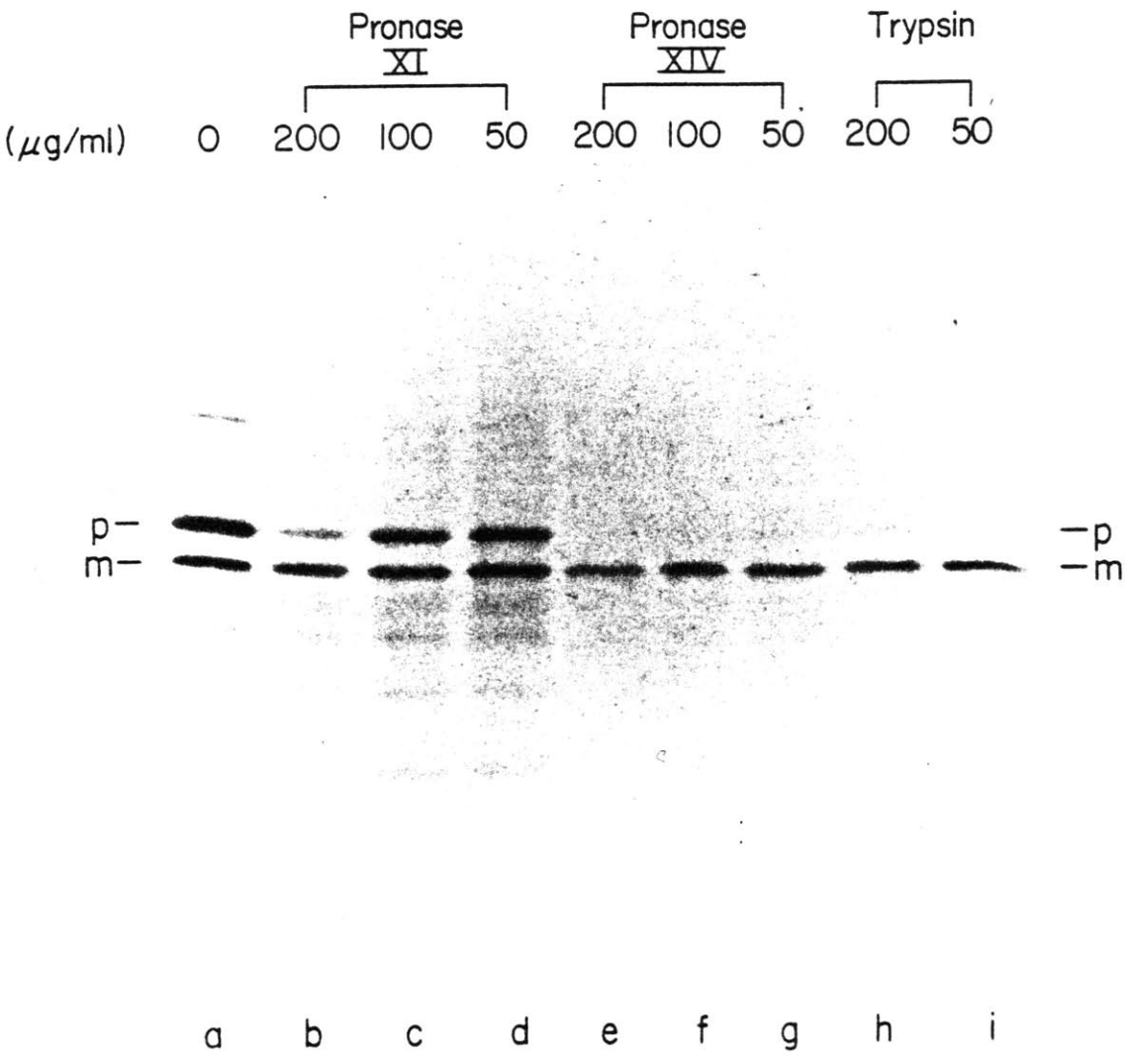

Pronase XI
Pronase XIV
Trypsin
(μg/ml)
0
200
100
50
200
100
50
200
50
p—
m—
—p
—m
a
b
c
d
e
f
g
h
i

Figure 18: Sensitivity of bla+ encoded proteins to proteases in crude lysates. A culture of DB4381 was irradiated and infected with P22bla+ phage at a moi of 10. The infected cells were labeled with ^{35}S-methionine for 30 seconds and then chilled to 0^{o}C. The cells were lysed as described in Methods and samples of this crude lysate were exposed to varying concentrations of different proteases for 30 minutes. 20 µl aliquots were loaded on to a slot. Electrophoresis was on a 12.5% gel and the autoradiogram was exposed for 15 hours. p, precursor form; m, mature form.

β-lactamase ran as a mixture of these two species, the doublet (figure 19). However, under conditions where the mature species produces a doublet the wild-type precursor did not (figure 19; lanes b, c, d).

Though we do not understand the cause of the doublet, it provides another phenotype besides molecular weight and cellular location which differs between the wild-type precursor and mature species. Therefore we investigated the ability of the proteins encoded by the signal sequence alleles bla to form doublets to see if this phenotype correlates strictly with the processed state of the protein or with its cellular location. bla encoded proteins for each signal sequence allele were labeled (Methods) and sample buffer containing β-mercaptoethanol was added. The samples were split in two and either heated to 60°C for 2 minutes (doublet forming conditions) or 95°C for 5 minutes (non-doublet forming conditions). The criteria that we used to decide if a precursor formed a doublet were the same as we used for the wild-type mature species. If a new band appeared under doublet forming conditions which we suspected of being precursor but altered in its mobility, then coincident with its disappearance under non-doublet forming condition should be an increase in the intensity of the other precursor band.

The results of this experiment are presented in figure 8. They show that the ability to form a doublet correlates with the cellular location of the bla protein and not the processed state of this protein. Precursors which accumulate on the cytoplasmic side of the inner membrane fail to form doublets under doublet forming conditions. The precursor of fs(14,21) behaves like wild-type precursor and fails to form doublets

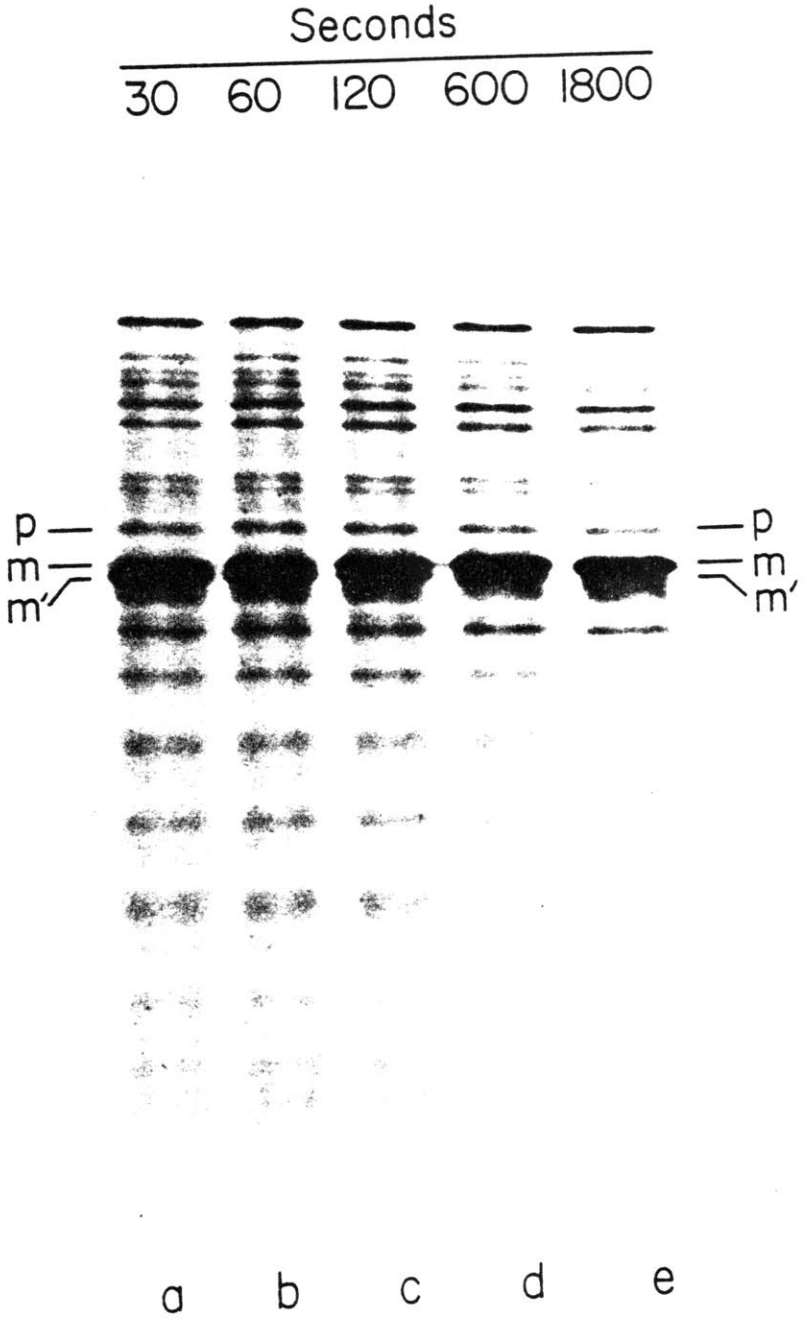

Seconds
30
60
120
600
1800
p
m
m′
p
m
m′
a
b
c
d
e

Figure 19: Analysis of the doublet formation of bla+ encoded proteins. A culture of DG4381 was irradiated and infected with P22bla+ phage at a moi of 10. The infected cells were labeled with ^{35}S-methionine for 3 minutes. The labeled cells were transferred to a tube containing an equal volume of 2 fold concentrated sample buffer preheated to 37°C. Samples were removed from the tube at the time intervals indicated above each lane and placed on ice. 20 µl aliquots were loaded on a slot. Electrophoresis was on a 12.5% gel and the autoradiogram was exposed for 20 hours. p, precursor form; m, mature form; m', mature form present only under double forming conditions.

under doublet forming conditions (figure 8, lane e). The precursor of fs(7,9) behaves mostly like wild-type though a small amount of the faster migrating species is apparent (figure 8, lane l). However in all cases in which we provided evidence that the product (precursor or mature) of the bla allele is located on the periplasmic side of the inner membrane, that product forms a doublet. When the mature species is present, it forms a doublet under doublet forming conditions regardless of the signal sequence allele (figure 8: pro_{20}->ser, lanes m; fs(7,9), lanes k; bla^+, lanes c). In addition the precursors encoded by the alleles of fs(18,21), pro_{20}->leu and pro_{20}->ser which apparently accumulate precursor on the periplasmic side of the inner membrane behave similarly to the wild-type mature species in two ways. They form easily visible doublets under the appropriate condidtions, and all the label in precursor ran at the slower of the two precursor positions under non-doublet forming conditions (figure 8; lanes g, i and m). We conclude from these experiments that the abiltiy of precursor or mature species to form a doublet is an intrinsic property of that protein which correlates strongly with its apparent cellular location.

H. Phenethyl alcohol and β-lactamase secretion

Phenethyl alcohol (PEA), a molecule that affects membrane fluidity, inhibits the processing of several secreted proteins. However the inhibition of processing by PEA can be attributed either to its ability to inhibit directly the processing enzyme or its ability to the inhibit the

secretion process such that the precursor fails to reach the processing enzyme.

We used PEA to inhibit the processing and or secretion of β-lactamase (Methods). The amount of PEA required to inhibit processing of β-lactamase was identical to the amount previously documented in Escherichia coli (Oxender, personal communication). The precursor of pro_{20}->leu and wild-type which accumulated after exposure to PEA were tested for their ability to form a doublet. For both alleles, the precursor that accumulates in the presence of PEA fails to form a doublet. This result is particlarly striking for pro_{20}->leu whose precursor forms a doublet in the absense of PEA (figure 21, compare lanes n and r with lanes a and f). Since a strong correlation exists between the ability of the bla encoded protein to form a doublet and its cellular location, these results suggest that the precursors which accumulate in the presence of PEA are on the cytoplasmic side of the inner membrane.

To test this hypothesis we performed the trypsin accessibility experiments in which half of the irradiated and infected cells were labeled and chased in the presence of PEA (Methods). The other half was labeled and chased in the absense of PEA, and then PEA was added. Standard trypsin accessibility experiment were performed with these samples (see above).

The results of these experiments for cells infected with the phage carrying the wild-type and pro_{20}->leu alleles are shown in figures 20 and 21. For the wild-type infection the precursor which accumulates as a result of PEA is digested with trypsin only in lysed but not intact

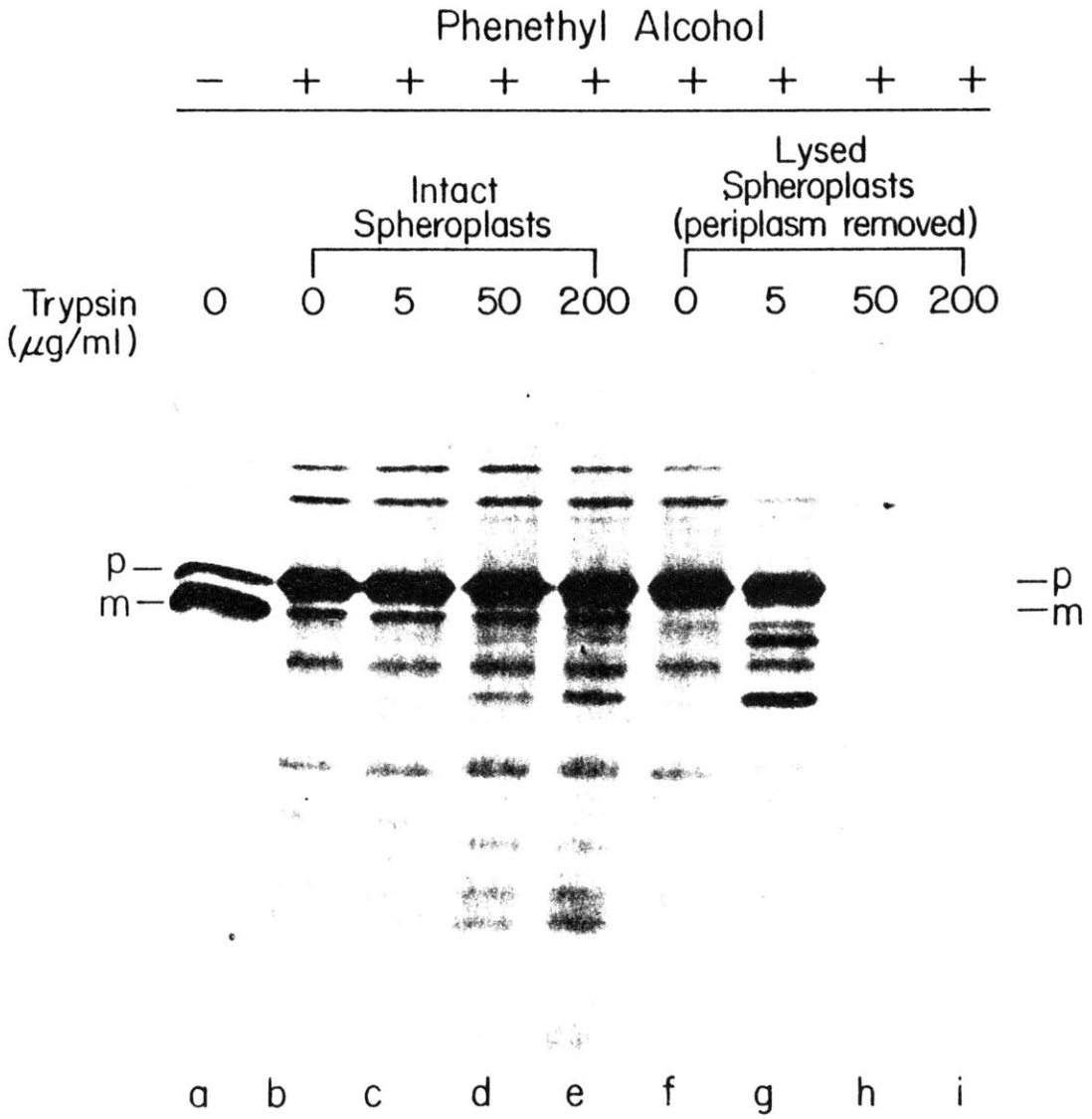

Phenethyl Alcohol
− + + + + + + + +
Intact Spheroplasts
Lysed Spheroplasts (periplasm removed)
Trypsin (μg/ml)
0 0 5 50 200 0 5 50 200
p
m
p
m
a b c d e f g h i

Figure 20: Trypsin accessibility analysis of proteins produced by cells treated with phenylethyl alcohol after infection with P22bla+ phage. A culture of DB4381 was irradiated and infected with P22bla+ phage at a moi of 10. The infected cells were labeled with ^{35}S-methionine for 30 seconds in the presence or absence of phenethyl alcohol and then chased with excess cold methionine (Methods). The cellular locations of bla+ encoded proteins in cells treated with PEA were analysized by trypsin accesibility experiments (see Methods and the legend to figure 15). 20 µl aliquots were loaded on a slot. Electrophoresis was on a 12.5% gel and the autoradiogram was exposed for 24 hours. p, precursor form; m, mature form; +, presence of PEA; -, absence of PEA.

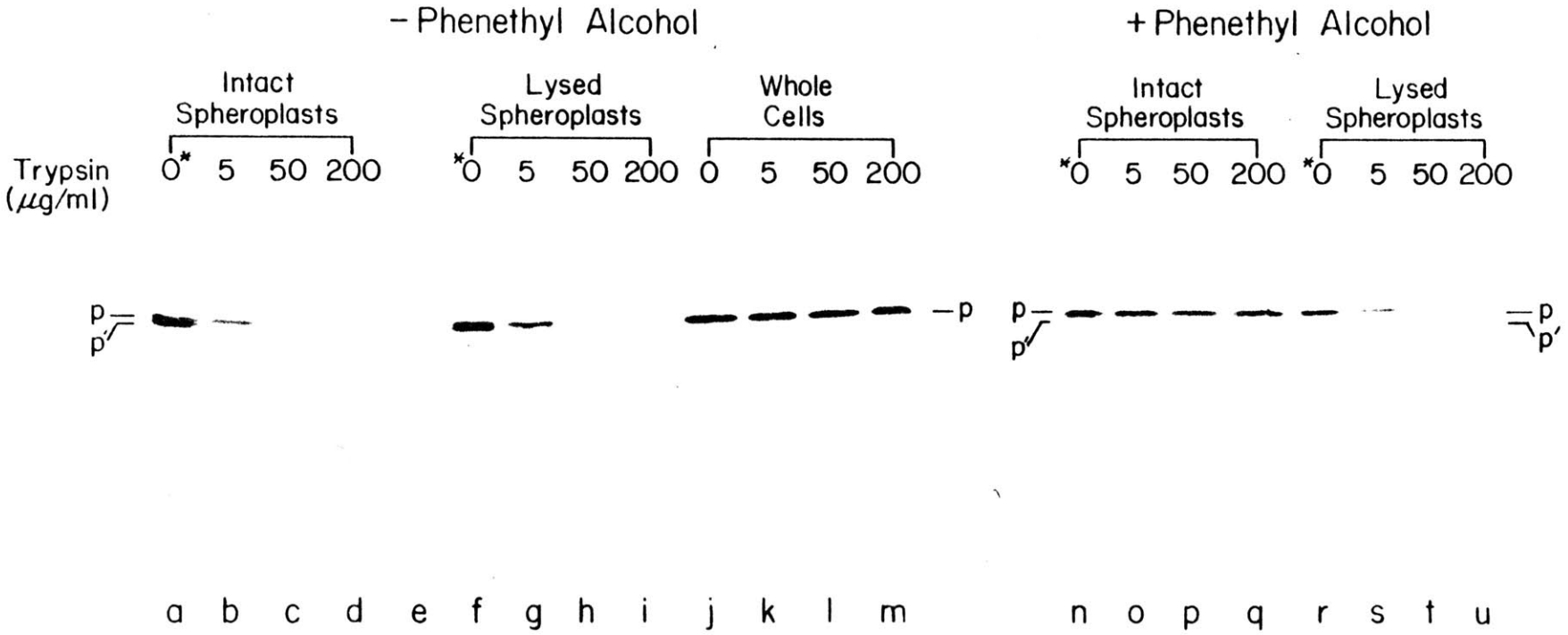

– Phenethyl Alcohol
+ Phenethyl Alcohol
Intact Spheroplasts
Lysed Spheroplasts
Whole Cells
Intact Spheroplasts
Lysed Spheroplasts
Trypsin (μg/ml)
0* 5 50 200
*0 5 50 200
0 5 50 200
*0 5 50 200
*0 5 50 200
p
p′
–p
a b c d e f g h i j k l m
n o p q r s t u

Figure 21: Trypsin accessibility analysis of proteins produced by cells treated with phenethyl alcohol after infection with P22bla-pro_{20}->leu phage. This experiment was performed exactly as described in the legend to figure 20 with the following exceptions. Irradiated cells were infected with P22bla-pro_{20}->ser instead of P22bla+. The cellular locations of bla encoded proteins labeled in the presnece and absence of PEA were analyzed by trypsin accessibity experiments. p, precursor form; p', precursor form present only under doublet forming conditions; * , samples treated under doublet formining conditions.

spheroplasts (figure 20). For pro_{20}->leu the precursor which is present after the cells are treated with PEA differs from the precursor when no PEA is present. In the absence of PEA, the precursor which accumulates after a 5 minutes of chase is digested by trypsin in intact spheroplasts (figure 21; lanes b, c and d). In the presence of PEA the precursor of pro_{20}->leu is trypsin insensitive in intact (figure 21; lanes n, o and p) but not lysed spheroplasts (lanes r, s and t). In all cases the same concentration of PEA was present in each digestion reaction indicating that this drug does not inhibit directly the protease activity of trypsin. We conclude that the effect of PEA is to cause the accumulation of precursor for both the wild-type and pro_{20}->leu alleles which is apparently on the cytoplasmic side of the inner membrane.

II. Discussion

In this study the products of the different bla alleles were characterized with respect to four criteria: their ability to be processed, their ability to form doublets under partial denaturating conditions, their behavior in cell fractionation experiments and their accesibility to trypsin in intact spheroplasts. By these criteria the products of the wild-type and some of the mutant alleles have novel properties not observed in studies of other secreted proteins.

Several precursors of bla which harbor mutations affecting their signal sequence have novel phenotypes apparently for two reasons. First,

the behavior of the wild-type precursor is different from the precursor of other secreted proteins (see below). Second, in the past signal sequence mutations were selected by methods that favored the isolation of mutations which prevented specifically the initiation of secretion (Bassford et al., 1979). In contrast, the mutations that alter the signal sequence of β-lactamase were isolated by selecting for the presence of some bla function or by screening for the absence of bla function. Neither of these methods contains an obvious bias that would favor a particular defect in secretion. Therefore we might expect to isolate mutations in bla that affect initial, intermediate or terminal steps in the secretion of β-lactamase. With this perspective the properties of these bla encoded proteins are summarized below.

When the phenotypes of the precursors of the different bla alleles are examined, the alleles can be divided into two classes. One class that includes the alleles wild-type, amH46, tsH1, tsH41, and fsI7 (precursors containing wild-type signal sequences) and the alleles fs(14,21) and fs(7,9) (signal sequence alleles) produces only one detectable form of precursor which has the following properties: accessibility to trypsin in lysed but not intact spheroplasts and inability to form a doublet on SDS polyacrylamide gels. In addition this class of precursors fractionate as if they are mostly cytoplasmic at early times during the chase. We call precursors with these properties P1.

The properties of the P1 precursors containing wild-type signal sequences are novel in two ways. First, they have particularly long half-lives as evidenced by the ease with which they are detected. As

mentioned in the introduction precursors of other secrected proteins have been difficult to detect apparently due to their very short half-lives. Second, P1 precursors containing the wild-type signal sequence are sequestered apparently on the cytoplasmic side of the inner membrane, yet they chase into a mature form which has traversed the inner membrane.

The signal sequence alleles in the P1 class, fs(7,9) and fs(14,21), encode for precursors that exaggerate these novel properties. The precursor encoded by the allele fs(7,9) has all the properties of precursors containing the wild-type signal sequence except its half-life is even longer. The precursor encoded by fs(14,21) is sequestered on the cytoplasmic side of the inner membrane but unlike the other P1 precursors does not chase to a secreted mature form.

A second class of mutants, comprised of alleles whose lesions affect the signal sequence (fs(18,21), pro_{20}->leu and pro_{20}->ser), accumulate precursors which differ from the P1 class of precursors because they are trypsin accesible in intact as well as lysed spheroplasts and in addition form a doublet. We call this second class of precursor, P2. The P2 precursor forms of bla are the first documented case of precursor forms of a secreted protein that have traversed the inner membrane but have not been processed.

We can also define two classes of mature protein. One class of alleles, the chain terminating alleles, produce a mature species that is associated with the spheroplasts and not the periplasm after substantial chase, trypsin accessible in intact and lysed spheroplasts and forms a doublet. The mature product of fsI7 share with these alleles the first

two of the three propeties. These properties define a class of mature species we call M1.

Finally the wild-type and tsH41 alleles produce mature proteins that are soluble periplasmic. They define another class, M.

A. Translocation of β-lactamase across the inner membrane

The observation that the P1 class of precursors which are apparently located on the cytoplasmic side of the inner membrane can give rise to mature products with either M or M1 properties strongly suggests that the translocation of β-lactamase across the inner membrane can occur posttranslationally. Furthermore the finding that the allele, fs(7,9) results in the substantial delay of the conversion of a precursor with P1 properties to the mature species but does not alter dramatically the efficiency of this conversion suggest that the translocation step can be slowed down dramatically without eliminating the successful secretion of this protein.

The models of protein secretion all address two basic questions. What is the energy souce for transporting the nascent chain or completed peptide across the membrane? Do proteins which are destined to be secreted contain all the structural and informational elements required for their translocation across the membrane in their amino acid sequence or are they assisted in this process by a membrane imbedded machinery?

The conclusion that β-lactamase traverses the membrane post-translationally only addresses the first of these two questons. This

constraint implies that the hydrolysis of GTP during protein elongation is not the energy source which drives β-lactamase across the inner membrane. The properties of the P1 class of precursors in cell fractionation experiments and the inherent ambiguities of all such experiments (see below) prevent us from discerning whether some initial interaction between P1 precursors and the cytoplasmic side of the inner membrane must occur as the proteins are synthesized or if P1 precursors can exist totally as a soluble protein in the cytoplasm prior to their interaction with the membrane.

The idea that β-lactamase can post-translationally traverse the inner membrane is not inconsistant with any of the facts known about secretion in eukaryotes or prokaryotes. Some proteins can traverse co-translationally the membrane of the endoplasm reticulum while other protiens can traverse post-translationally the membranes of the mitochondria and chloroplast. Similarly in prokaryotes apparently some proteins such as alkaline phosphatase can traverse the inner membrane co-translationally while another protein, the coat protein of the filamentous phage M13, can apparently insert into the inner membrane post-translationally.

Mutations in the signal sequence of the maltose binding protein and the lambda receptor protein that apparently abolish secretion can be suppressed by mutations which that map to the ribosomal cluster. This result suggests that a ribosomal protein may play a role in secretion. Our results do not eliminate the possibility that the ribosomes play a role in the secretion of β-lactamase. For example, the ribosomes may

catalyze the interaction of the signal sequence with the membrane. They do suggest that the traversal of the membrane by β-lactamase is not obligatorily co-translational, that is necessarily coupled to translation.

The conclusion that β-lactamase traverses the inner membrane post-translationally can be challenged in two ways. Either one could challenge the use of pulse chase experiments to establish the precursor as an intermediate in secretion or the use of trypsin and cell fractionation as a means to localise the precursor.

The physical association of a protein with a microsomal vesicle and its digestion by trypsin only if these vesicles are disrupted have been the two major criteria used by Blobel and Dobberstein to establish the sequestration of proteins into the microsomes of higher eukaryotes. However, the localization of the P1 class of precursors to the cytoplasmic side of the inner membrane by these criteria is complicated by two facts, the intrinsic resistance of the wild-type mature species and the potential existence of undiscovered cellular compartments.

Since the mature form of the wild-type protein is trypsin resistant the formal possibility exists that the wild-type and fs(7,9) precursors are localized to the outside of the inner membrane and assume a trypsin resistant confromation. Upon lysis these precursors change their confromation to a trypsin sensitive form. This conclusion is less tenable because the precursors of the chain terminating and ts alleles of β-lactamase are protected in intact spheroplasts even though neither the precursor nor the mature species of these alleles apparently assume a trypsin resistant conformation. Furthermore, all P2 precursors and mature

species of the chain terminating alleles are apparently membrane bound but trypsin digestable. Therefore, trypsin has access to membrane bound proteins, and membrane binding alone apparently is not sufficient to confer upon a bla encoded protein resistance to trypsin. These experiments strongly suggest that the apparent trypsin resistance of P1 precursors of wild-type and fs(7,9) in intact spheroplasts is not due to some intrinsic resistance of these precursors but is due to some physical barrier which prevents trypsin from gaining access to them.

The second possibility is that this barrier is not the inner membrane. Perhaps P1 is localized to cellular compartments external to the inner membrane which prevent access of trypsin to P1. To invent such compartments may seem excessively imaginative. However, the recent work of Novick et al. (1979a,b) and coworkers in yeast clearly indicates that organelles in eukaryotes can be sufficiently short lived that they are only discovered when the lifetime of the organelle is increased by mutation. However since there exists no evidence for compartments other than the cytoplasm and the periplasm which have the inherent property of sequestration in prokaryotes, our experiments are best interpreted to mean that precursors of the P1 class are localized to the cytoplasmic side of the inner membrane.

It is clearly important to establish that precursor with P1 properties is an actual intermediate in the secretion process. In this study we use a kinetic analysis to establish precursor as an intermediate to the mature species. In this type of analysis we assert that the disappearance of label from the precursor and the appearance of label in the mature species

represents the the conversion of precursor to mature species under the properly controlled conditions. One prediction from this assertion is that the conversion of precursor to mature species should be independent of protein synthesis. In agreement with this prediction precursor is converted to mature species in the presence of chloramphenical. A second prediction from such an assertion is that if the disappearance of label from precursor and the appearance of label in the mature species are related then a mutation which slows down the disappearance of label from precursor should slow down equally the rate of appearance of label in the mature species. Two alleles of bla, fs(7,9) and pro_{20}->ser give exactly these results. By combining such pulse chase experiments with trypsin accessibility experiments and cellular fractionation experiments we provide evidence that this chaseable precursor is in the P1 state.

B. Role of the removal of the signal sequence in secretion

The properties of the P2 class of precursors (trypsin accessibility in intact spheroplasts and association with membranes) localize these precursors of β-lactamase to the surface of a membrane in the periplasm. Because they differ from mature species only by the addition of 23 amino acids of the signal sequence, the signal peptide must cause the differrence in cellular location between the P2 class of precursor and the mature species. These data suggest that in order for β-lactamase to achieve its soluble state in the periplasm the signal sequence must be removed. An amino acid subsitution in the f1 coat protein causes a

reduction in the rate of processing of its signal peptide and the accumulation of f1 coat precursor. Failure to find this precursor as part of the soluble phage led the authors to suggest that processing of this protein is required in order that it free itself from the surface of the membrane (Russel and Model, 1981). A model such as the one proposed by Inouye (see Dirienzo et al., 1978), in which the signal sequence interacts with the cytoplasmic side of the inner membrane as a loop, accounts nicely for the requirement of the removal of the signal sequence to obtain release of the translocated protein from the periplasmic surface of the inner membrane.

The fraction of β-lactamase molecules that have translocated the inner membrane after a short chase may be significantly greater for the alleles fs(18,21) and pro_{20}->ser than for the wild-type allele. The altered signal peptides of these alleles might increase the rate of translocation across the inner membrane or improve the ability of β-lactamase molecules to translocate the membrane at low temperatures. Mutations that improve the function of a biological process have been observed, for example mutations in promoters which increase the rate of the initiation of transcription (Rosenberg and Court, 1979).

At the moment the nature of the defect in the P2 precursors encoded by fs(18,21) and pro_{20}->leu which results in the inhibition of processing is not understood. The amino acid alterations are near the cleavage site. Therefore these mutations may directly alter a site which is recognized by the protein responsible for removing the signal sequence from the precursor, or the change in amino acid sequence may alter the entire

structure of the precursor such that this structure somehow blocks access of the processing enzyme to the cleavage site. Alternatively the signal sequence may contain the information which directs the protein to the proper membrane, as well as information necessary for its passage through a membrane (Blobel, 1980). The change in the signal sequence of P2 might cause the localization of the precursor to some compartmemt in which the processing enzyme is absent. For example P2 precursors may be on the inner surface of the outer membrane while the processing protein is on the outer surface of the inner membrane.

The latter hypothesis suggests that P2 precursors are altered in such a way that they are no longer on the normal pathway for secretion. If a mutant existed in which P2 precursor was accumulated and then was processed to give the secreted mature form, then we could demonstrate P2 precursor as an intermediate, and the later model could be eliminated. Though pro_{20}->ser may be such a mutant, such experiments have been unsuccessful because this mutant produces precursor in which a portion of it continues to be processed even after chilling to $0^{o}C$. As a result, it is impossible to accumulate enough P2 precursors in the absense of any mature species such that we can establish unambiguously that P2 precursors are intermediates to the secreted mature form. However, it is interesting that the cleavage defective mutant of the fd coat protein has the same phenotype as pro_{20}->ser in this respect (Russel and Model, 1981).

Though it is impossible to determine whether the precursors which accumulate with P2 properties are intermediates to the formation of the mature species, the processes that localize these two forms of β-lactamase

apparently share at least one step. Both the precursors with P2 properties and the wild-type mature species traverse the inner membrane and in both cases the traversal step is blocked by phenethyl alcohol. Since other secreted and exported proteins are not effected by this reagent, its effect on the localization of these two forms of β-lactamase suggests that it is affecting a specific step in localization that these two proteins share.

C. Role of the carboxy terminus of β-lactamase in secretion

The processed forms of β-lactamase missing the carboxy end of the protein are cytoplasmic or weakly membrane bound as determined by cell fractionation experiments. Trypsin accessibility experiments indicate that these peptides are accessible to the periplasmic side of the inner membrane. The latter experiment apparently resolves the ambiguity of the localization of these proteins and suggests that they are weakly bound to the surface of a membrane in intact spheroplasts. This weak interaction is disrupted when the cell undergoes osmotic lysis therby making the protein appear cytoplasmic. The absence of any large protected peptide indicated that the bulk of the mature species is most likely on the periplasmic side of the membrane. These data suggest that the carboxy terminus of β-lactamase apparently is not required for β-lactamase to traverse the inner membrane in spite of the fact that these proteins apparently traverse the inner membrane post-translationally. Similar experiments with the maltose binding protein and the arginine binding

protein were reported which suggest that the carboxy terminus of these proteins are not required for their tranlocation across the inner membrane (Ito and Beckwith, 1981; Celis, 1981).

The weak interaction between the membrane and the peptides encoded by chain terminating mutants has several possible explainations. The protein, as a result of its missing carboxy terminus, may assume a denatured confromation which is naturally sticky to a membrane. On the other hand the wild-type protein must pass through and eventually be released from the inner membrane. The removal of the carboxy terminus may greatly reduce the efficiency of this terminal step of secretion. For example in eukaryotes, the molecule IgM is found in two forms , a truly secreted form and a membrane bound form in which almost the entire molecule has traversed the plasma membrane. These two molecules differ only by a peptide at their carboxy termini. At this time we cannot resolve the ambiguity between these two plausible models among others.

D. Intrinsic properties of bla encoded proteins

1. Resistance to proteases in crude lysates

The P1 precursors, the P2 precursors and the wild-type mature species differ from each other in at least two of three intrinsic properties; trypsin sensitivity in lysates (as opposed to trypsin accessibility in spheroplasts), ability to form a doublet, and ability to confer upon the cell resistance to varying concentrations of ampicillin. The substanial

difference in trypsin sensitivity between the two precursor forms of β-lactamase and the wild-type mature protein strongly suggests that the processed and unprocessed forms of β-lactamase are in different conformations. We do not know whether this trypsin sensitivity reflects a major or minor conformational difference. However similar results were obtained when the trypsin sensitivity of another secreted protein was examined (Oxender et al., 1980).

2. The doublet phenomenon

The P1 precursors fail to form a doublet when essentially all other form of β-lactamase do. This difference can not be accounted for by a difference in how the sample were treated since these experiments were often internally controlled (i.e. both doublet and non-doublet forming species were present in the same tube). The reason mature species or P2 precursors can form doublets while P1 precursors cannot is not well understood. However, this phenotype does correlate with the apparent cellular location of the bla encoded protein. Those bla encoded proteins on the cytoplasmic side of the inner membrane do not form doublets while those bla encoded proteins on the periplasmic side do. One could conjecture that this difference is due to some conformational change or to some unspecified protein modification which occurs as the protein traverses the membrane. Modifications of proteins which cross the inner membrane have been reported. The other obvious possibility is that the difference reflects some conformational difference such as the formation

of a disulfide bond. Once secreted the bla protein forms a disulfide bond which is difficult to reduce. Two cysteine amino acids exist in the mature polypeptide though it is unknown whether these two amino acids form a disulfide bond.

Several facts suggest that the ability of certain bla encoded proteins to form a doublet is not due to specific protein degradation. Since the faster migrating species of the doublet can be converted to the slower migrating species, these data suggests that the doublet is due to a lower molecular weight form of β-lactamase being converted to a higher molecular weight form. We have determined the partial amino acid sequence of these proteins. These proteins have the normal amino termini for precursor and mature species as predicted from the DNA sequence. This result eliminates proteolysis at the amino terminus at least as the cause of doublet formation. The fact that many of the mature products of the chain terminating alleles form doublets (Koshland and Botstein, 1980 and unpublished data) suggests that the doublet is not due to specific proteolysis of the carboxy terminus. The shortest peptide that we have identified, the mature product of fsI7, apparently fails to from a doublet suggesting that a site responsible for the formation of a doublet lies in the amino acid sequence defined by this lesion and the next most carboxy terminal lesion whose mature species did form a doublet.

3. Ability to confer ampicillin resistance

The resistance of the cell to increasing amounts of ampicillin also seem to correlate with the cellular location and processed state of the protein encoded by the particular bla allele. fs(14,21) which apparently accumulated precursor only on the cytoplasmic side of the inner membrane confers upon the cell resistance to 25μg/ml of ampicillin. fs(18,21) and pro_{20}->leu accumulate precursor on the periplasmic side of the inner membrane and confer upon cells resistance to 200μg/ml. All bla alleles that accumulate the wild-type mature species conferred resistance to the cell of 1000μg/ml. Thus the closer the bla protein apparently got to its destination as a soluble protein in the periplasm, the higher the resistance of the cell to ampicillin. One could postulate that all forms of β-lactamase which contain at least the amino acid sequence of the wild-type mature protein have the same enzymatic activity but the effectiveness of this activity toward protecting the organism from ampicillin depends upon the cellular location of that enzyme activity. Alternatively one could postulate that the precursor forms of these enzymes may have substantially less activity than the mature species but the processing step is inherently leakier than the translocation step.

E. Cell fractionation and cellular location

Many experiments which study in vivo the secretion process in prokaryotes have relied upon the use of cell fractionation experiments to

localize proteins to particular cellular compartments. Our experience with the prematurely truncated mature peptides of β-lactamase suggest that these methods may not have sufficient resolution to conclude that a protein (a precursor to a secreted protein for example) which is found in the cytoplasmic fraction is not weakly bound to either side of the inner membrane and released by the particular fractionation procedure. Recently other inadequacies in this method of localization were reported. Ito and Beckwith showed that the apparent membrane association of the hybrid protein between the lambda receptor protein and β-galactosidase (previously assumed to a membrane protein) depended upon the salt concentration. Our results coupled with these other reports clearly indicate that the localization of a protein by cell fractionation must be treated as only a first approximation of the true localization of a protein and by no means provides a definitive answer to this question.

Chapter 5: Conclusion

We present a working model for the secretion of β-lactamase. This model is summarized in figure 22. The model supposes that the secretion of β-lactamase can be described by a simple pathway in which the precursor is synthesized on the cytoplasmic side of the inner membrane. The resulting species, P1, is then at least partially translocated across the inner membranee to give the P2 form. The signal sequence of P2 is removed. Finally, if the cleaved P2 then assumes its proper configuration, it is released from the membrane to give M.

We can examine each allele of *bla* in relationship to this speculative model. We explain the accumulation of wild-type P1 by assuming that for the secretion of the wild-type protein the transport of P1 across the inner membrane is the rate determining step. This suggested slowness of this step could be due to the slow insertion of P1 into the membrane or to the failure of the signal sequence to be recognized rapidly by some component of a cellular secretion machinery. In this light it should be remembered that the *bla* gene only recently has been introduced into *Salmonella typhimurium* and *Echerichia coli* as the result of transposition and studies in recombinant DNA (Heffron *et al.*, 1975). The suggested slow function of the *bla* signal sequence in *Salmonella typhimurium* relative to other secreted proteins of *Salmonella typhimurium* and *Escherichia coli* may result from the fact that the organism in which the *bla* gene evolved and in which the *bla* signal sequence was selected to function is taxonomically quite distant from *Salmonella typhimurium*.

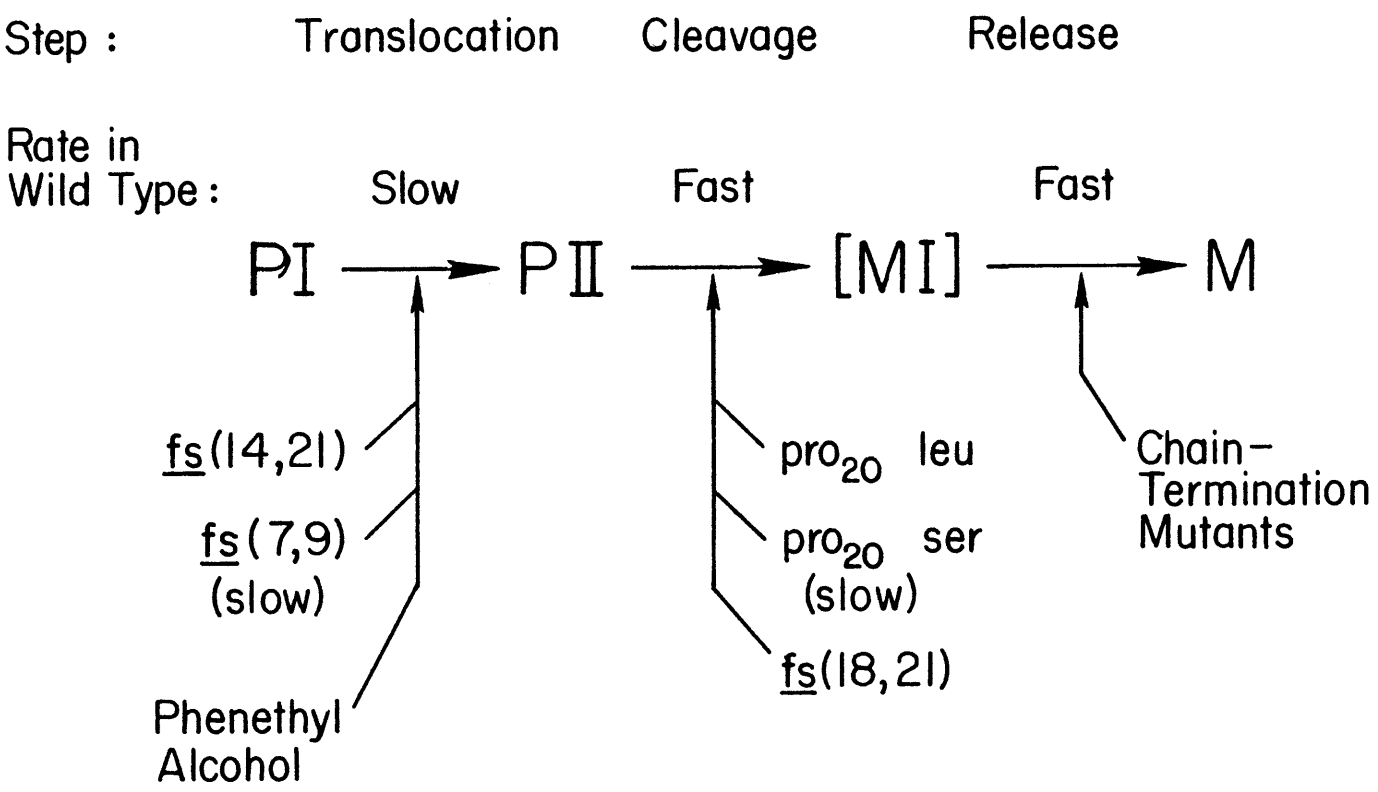

Step :
Translocation
Cleavage
Release
Rate in Wild Type :
Slow
Fast
Fast
PI
PII
[MI]
M
fs(14,21)
fs(7,9) (slow)
Phenethyl Alcohol
pro20 leu
pro20 ser (slow)
fs(18,21)
Chain-Termination Mutants

Figure 22: A working model for the secretion of β-lactamase. During the synthesis of wild-type β-lactamase the signal peptide may or may not interact with the inner membrane. Once the synthesis of the precursor is completed, it traverses the inner membrane (the translocation step) but fails to become a soluble periplasmic protein until the signal peptide is removed and the carboxy terminus performs some as of now undetermined function. For the secretion of the wild-type protein we postulate that the translocation step is the rate determining step in this pathway and therefore the only intermediate form of β-lactamase that accumulates is precursor with P1 properties. Mutations in this protein can change the rate of any one of these three steps and cause the accumulation of intermediates with the properties of P1, P2 or M1. The step at which a particular bla mutation or phenethyl alcohol exerts its effect are indicated in the diagram.

The translocation step is affected by two mutant alleles of bla, fs(7,9) and fs(14,21); both lesions lie within the region encoding the signal sequence. Therefore these mutations should affect the function of the signal sequence per se. One possible explanation of the phenotype of the allele, fs(7,9), is to notice that the arginine residue normally at codon 7 in wild-type is moved to codon 9 and as a result the hydrophobic core is shrunk by two amino acids. If the length of the hydrophobic core does affect the rate of the interaction of the protein with the membrane or some component of the membrane, then the phenotype of this mutant would be explained. The allele, fs(14,21), results in the substitution of a charged amino acid for a hydrophobic amino acid in the middle of the hydrophobic core. This allele is similar to the signal sequence mutations which affect the maltose binding protein (Bedouille et al., 1980) and the lambda receptor protein (Emr et al., 1980).

The lesions of the other three signal sequence alleles, fs(18,21), pro_{20}->leu and pro_{20}->ser, affect amino acid residues near the site where the signal peptidase cleaves the signal sequence from the precursor form. All three mutations apparently affect processing and not the translocation step. In the model, the amino acid substitutions that result from these mutations can be thought of as inhibiting the processing of β-lactamase by the signal peptidase. A comparison of these alleles suggests that apparently subtle changes in the amino acid sequence in this region can drastically alter the fate of the protein since the proline to serine change at codon 20 does produce mature protein, although at a reduced rate, while the proline to leucine change at the same codon does not.

To explain why some of the precursor molecules of pro_{20}->ser are processed in the cold while all precursors containing the wild-type or fs(7,9) amino acid sequence apparently do not, we assume that the translocation of precursor across the membrane is a cold sensitive step while the processing of precursor is not. The observation that the translocation step is cold sensitive is not particularly surprising because the secretion of another periplasmic protein, alkaline phosphatase is also apparently cold sensitive (Pages et al., 1979).

The inhibition of secretion at low temperature has been attributed to the effect of temperature on membrane fluidity. Experiments indicate that membrane fluidity is required for the proper localization of several proteins (Lazdunski et al., 1979; Pages et al., 1979; DiRienzo and Inouye, 1979). Our observation that phenethyl alcohol inhibits the transformation of P1 precursors to P2 precursors suggests a role for membrane fluidity in the translocation step for β-lactamase precursor.

The final step in the model is invoked because of the properties of the chain terminating alleles of bla. Since the mature forms of these alleles remain associated with a membrane instead of becoming free in the periplasm, we suggest that the efficent release of β-lactamase from the membrane hinges upon it assuming a proper configuration.

In conclusion we summarise the virtues and weaknesses of this model. The major virtue of this model is that it accounts simply for all the properties of the products of the different alleles of bla. Second, it introduces the idea that the transport of proteins across the inner membrane might be broken down, using mutants, into relatively defined

steps like the morphogenesis of a phage.

The weaknesses of this model include the fact that no direct evidence exists which shows that P2 precursor forms or M1 mature forms are normal intermediates to the formation of M. Further, the order of the steps defined by the P2 and M forms is uncertain; all we know is that the execution of both steps is required for the complete release of the protein from the membrane.

This model does not specify the actual mechanism by which β-lactamase is transferred across inner membrane other than to specify that it can occur (although need not necessarily occur) post-translationally. This is required by our data for β-lactamase. Many models have been proposed which will accomodate post-translational insertion or translocation of a protein across a membrane (Blobel, 1980; Engelman and Steitz, 1981; Ito et al., 1979; Daniels et al., 1981). Hopefully future studies will provide an answer to the actual mechanism of this step.

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