

MOLECULAR ANALYSIS OF THE MOSQUITO LARVICIDAL TOXINS OF  
*BACILLUS SPHAERICUS* 1593M

Thesis submitted for the degree of  
Doctor of Philosophy  
at the University of Leicester.

by

Vidya Rajan, BSc, MSc (India)  
Department of Genetics  
University of Leicester

July 1991

UMI Number: U037081

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U037081

Published by ProQuest LLC 2015. Copyright in the Dissertation held by the Author.  
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against  
unauthorized copying under Title 17, United States Code.



ProQuest LLC  
789 East Eisenhower Parkway  
P.O. Box 1346  
Ann Arbor, MI 48106-1346



X-75-174841-1

X-75-174841-1

## ABSTRACT

### MOLECULAR ANALYSIS OF THE MOSQUITO LARVICIDAL TOXINS OF *BACILLUS SPHAERICUS* 1593M. by Vidya Rajan

*Bacillus sphaericus* produces a sporulation associated toxin specific for the larvae of dipteran insects: mosquitoes that are vectors for the transmission of human diseases such as malaria and filariasis. Two dissimilar DNA sequences conferring larvicidal activity on recombinant *Escherichia coli* had previously been cloned from *B.sphaericus* 1593M (Souza et al., (1988), J. Biotechnol., 7, 71-82). Proteins encoded by one of these DNA sequences are studied in this thesis.

The DNA sequence cloned from *B.sphaericus* 1593M is here shown to encode two proteins of 41 and 59KDa that are together required for toxicity to mosquito larvae. These proteins were previously hypothesized to exist in the crystal of *B.sphaericus* as high molecular weight oligomers (Baumann et al., (1985), J. Bacteriol., 163, 738-747). I now show that these two proteins are distinct from the high molecular weight protein which appears to constitute the majority species of the crystal. I have identified this protein as the Surface layer protein of *B.sphaericus*. I also show that the 59KDa species is a distinct and separate constituent of the crystal.

As a prerequisite for raising antibodies to study regulation of synthesis of these proteins, purification following overproduction or secretion was investigated. Thus, attempts were made to purify the 41 and 59KDa proteins from *E.coli* cells using the C-terminal signal sequence of Haemolysin, a protein normally secreted from *E.coli*. Furthermore, attempts were made to express the gene encoding the 41KDa protein in *E.coli* to a high level from  $\lambda$  P<sub>R</sub> and P<sub>L</sub> promoters. I show that this protein could not be overexpressed in this way. This was attributed to a lack of transcript termination on the vector due to the presence of a faulty *fd* transcription terminator downstream of the gene.

The nucleotide sequence of the gene encoding the 59KDa protein was determined. This sequence was then used to design an approach which led to the overexpression of the gene from the T7 RNA polymerase-recognized  $\Phi$ 10 promoter on the plasmid pET3a. This in turn allowed the production of antibodies.

The expression of the mRNA transcripts of the genes encoding the 41 and 59KDa proteins were also studied in *B.sphaericus* and  $\beta$ -galactosidase fusions were constructed to serve as "reporters" for the expression of the 41 and 59KDa proteins in *E.coli* and *B.subtilis*. I propose that the regulation of expression of the genes is complicated, and depends upon the use of two, temporally regulated promoters.

### ACKNOWLEDGEMENTS

I am grateful to Prof. I. B. Holland for giving me the opportunity to work in his laboratory, for his supervision, scientific rigour, enthusiasm, and for his suggestions on the writing of this thesis. I also wish to thank Prof. K. Jayaraman for permitting me to use the genes cloned in her laboratory in this work, for advice, and for permitting me to carry out larvicidal assays in her laboratory.

I am grateful to all the members of the Department of Genetics, especially to those of Lab. 145, for providing a stimulating and enjoyable working atmosphere. I wish to thank Serge Casaregola, Brendan Kenny, Mark Blight, John Jenkins, Stephen Streatfield, Isobel Hopkins and Simone Seror for advice on technical matters. I would also like to thank Terry Lynn and Margaret Peake for cheerfulness and helpfulness beyond the call of duty.

The amino acid sequencing described in Chapter 4 was carried out by Dr. J. Keen at the University of Leeds. The oligonucleotides used in this thesis were synthesized by John Keyte in the Department of Biochemistry, University of Leicester.

There are many people who contributed to making my stay in Leicester such an enjoyable one. In particular, I should like to thank Serge, Steve, Rob, Bren, John, Parul, Isobel, Christina and Ila for many happy times, and for introducing me to the joys of Jokespace, Helsinki's, the Spectrum, and the Leicestershire countryside. And thank you to the Phoenix for those late night shows, and to Brian Redhead for making me look forward to Monday mornings.

Finally, I should like to thank my parents for their constant love, moral and corporeal sustenance, and for the use of the computer. To Steve, who battled through the initial drafts of this thesis, and who gave me encouragement and support during the final stages, thanks.

---

To my Mother and Father

### Abbreviations

bp	base pair
BSA	Bovine serum albumin
Ci	Curie
°C	degrees Celsius
DNA	Deoxyribonucleic acid
dNTP	2'-deoxy (N) 5'-triphosphate N=adenosine (A), cytidine (C), guanosine (G), thymidine (T)
EDTA	ethylenediamine tetra-acetic acid
IPTG	isopropyl-β-D-galactopyranoside
Kb	Kilobase
KDa	Kilodalton
KV	Kilovolt
L	Litre
μg	microgram
min	minutes
μL	microlitre
mg	milligram
mRNA	messenger RNA
nt	nucleotide
ONPG	o-nitrophenyl-β-D-galactopyranoside
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
rDNA	ribosomal deoxyribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
TEMED	N, N, N', N'-tetra-methylethylenediamine
tRNA	transfer ribonucleic acid
v/v	volume for volume
w/v	weight for volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
μF	micro Farad
Ω	Ohms

## Contents

	<u>Page</u>
<u>Chapter 1</u>	
<u>Introduction</u>	1
1.1 Insect pathogens.	2
1.1.1 Entomopathogenic viruses.	2
1.1.2 Fungal pathogens.	3
1.1.3 Protozoa and nematodes.	3
1.1.4 Bacterial pathogens.	4
1.2 The response of insects to pathogens.	5
1.3 The toxic factors of entomopathogenic Bacilli.	5
1.4 The toxin encoding genes.	8
1.4.1 Genes from <i>B.thuringiensis</i> encoding toxicity to insect larvae.	9
1.4.2 Genes from <i>B.sphaericus</i> reported to encode toxicity to mosquito larvae.	11
1.4.2.1 <i>B.sphaericus</i> 1593.	11
1.4.2.2 <i>B.sphaericus</i> 2362.	14
1.4.2.3 <i>B.sphaericus</i> SSII-1.	14
1.5 Regulation of toxin synthesis.	16
1.6 Mode of action of the crystal related toxins.	19
1.7. Perspectives.	23
1.8 Aims of this project.	24
 <u>Chapter 2.</u>	
<u>Materials and Methods.</u>	
2.1 Genetical and molecular methods associated with Bacillus strains.	26
2.1.1 Strains.	26
2.1.2 Media, growth and storage.	26
2.1.2.1 Antibiotics.	27
2.1.3 DNA isolation from Bacillus strains.	27
2.1.3.1 Chromosomal DNA extraction from Bacillus strains.	27
2.1.3.2 Plasmid DNA isolation from <i>B.subtilis</i> .	28
2.1.4 DNA mediated transformation of <i>B.subtilis</i> .	29
2.1.4.1 Preparation of competent <i>B.subtilis</i> cells for transformation.	29
2.1.4.2 Preparation of <i>B.subtilis</i> cells for transformation by electroporation.	29

2.1.5	Crystal preparation from <i>B.sphaericus</i> strains.	30
2.1.5.1	Crystal preparation using NaBr density gradient centrifugation.	30
2.1.5.2	Crystal purification by discontinuous sucrose density gradient centrifugation.	31
2.1.6	Preparation of spores from <i>B.sphaericus</i> .	31
2.2	Genetical and molecular methods associated with <i>Escherichia coli</i> .	32
2.2.1	Strains.	32
2.2.2	Media, growth and storage.	32
2.2.2.1	Antibiotics.	33
2.2.3	Transformation of <i>E.coli</i> .	33
2.2.3.1	Rapid calcium chloride transformation.	33
2.2.3.2	Rubidium chloride transformation.	34
2.2.3.3	Transformation by electroporation.	34
2.3	Methods associated with DNA.	35
2.3.1	Plasmids and phage.	35
2.3.2	Small scale plasmid isolation by the minipreparation method.	36
2.3.3	Large scale plasmid DNA extraction and CsCl purification.	37
2.3.4	Ethanol precipitation of DNA.	38
2.3.5	Phenol-chloroform extraction of DNA.	38
2.3.6	Digestion of DNA with restriction enzymes.	38
2.3.7	Agarose gel electrophoresis and fragment recovery.	39
2.3.7.1	DNA fragment recovery using "Glass milk".	39
2.3.7.2	Fragment recovery from Low Gelling Temperature agarose gels.	40
2.3.7.3	Fragment recovery by the "Death Wish" method.	40
2.3.8	Filling-in of recessed ends and digest back of protruding ends of DNA fragments.	41
2.3.8.1	Filling-in of 3' recessed ends.	41
2.3.8.2	Digestion of 5' recessed ends.	42
2.3.9	Alkaline phosphatase treatment of DNA.	42
2.3.10	Ligation of DNA fragments.	43
2.3.11	DNA fragment labelling with <sup>32</sup> P-dCTP.	43
2.3.12	Southern transfer of DNA fragments.	44
2.3.12.1	DNA fragment transfer.	44
2.3.12.2	Colony transfer.	46
2.3.13	Hybridization with radiolabelled probe DNA.	46

2.3.14	Stripping radiolabelled probe DNA from Hybond-N membranes.	47
2.3.15	Oligonucleotide directed <i>in vitro</i> mutagenesis of DNA.	47
2.4	Methods associated with proteins.	48
2.4.1	Preparation and labeling of minicells.	48
2.4.1.1	Fractionation of minicell proteins.	49
2.4.2	<i>In vitro</i> coupled transcription-translation assays.	50
2.4.3	Labelling of whole cells of <i>E.coli</i> .	51
2.4.4	Preparation of cell and supernatant samples of <i>E.coli</i> .	52
2.4.5	SDS polyacrylamide gel electrophoresis.	52
2.4.6	Visualizing proteins separated by SDS polyacrylamide gel electrophoresis.	53
2.4.7	Inclusion body preparation.	54
2.4.8	Electroelution of protein from SDS polyacrylamide gels.	54
2.4.9	N-terminal amino acid sequencing of the 125KDa protein.	55
2.4.10	Lowry estimation of protein concentration.	55
2.5	Nucleotide sequence analysis of DNA.	56
2.5.1	Preparation of single stranded (ss) DNA from M13 phage.	56
2.5.2	DNA nucleotide sequencing.	57
2.5.2.1	Sequencing of ssDNA.	57
2.5.2.2	Sequencing double stranded (ds) DNA.	59
2.5.3	Preparation of the sequencing gel.	59
2.5.3.1	Preparation of the gel mould.	59
2.5.3.2	Pouring the sequencing gel.	59
2.6	Immunological methods.	61
2.6.1	Preparation of antigen for immunization.	61
2.6.2	Serum preparation.	61
2.6.3	Western immunoblotting of proteins.	61
2.6.4	Reprobing Western immunoblots.	63
2.6.5	Immunoprecipitation.	63
2.7	Tissue culture methods.	64
2.7.1	Cell lines.	64
2.7.2	Growth, storage and handling.	64
2.7.3	<i>In vitro</i> toxicity assays.	65
2.8	<i>In vivo</i> toxicity assays.	65
2.9	RNA methods.	65

2.9.1	Preparation of total <i>B.sphaericus</i> and <i>E.coli</i> RNA.	66
2.9.2	Analysis of RNA following transfer onto nylon membranes using radioactive probes.	67
2.9.2.1	Northern blot filter hybridization.	67
2.9.2.2	"Slot-blot" analysis of RNA.	67
2.9.3	Hybridization of RNA with labelled DNA probes.	67
2.9.4	Densitometric analysis of autoradiographs.	68
2.10	Computing.	68
2.11	Source of materials.	68
2.12	Containment and safety.	69

### Chapter 3.

#### Identification of the toxin gene products from *B.sphaericus* 1593M.

3.1	Introduction.	70
3.2	Screening for the presence of the 3.6Kb insert in other strains of <i>Bacillus sphaericus</i> .	71
3.3	Identification of the toxin gene products.	73
3.3.1	Analysis of deletions of the plasmid pAS233HA in minicells.	75
3.4	The 41 and 59KDa proteins are localized to the cytoplasmic fraction of minicells.	76
3.5	Attempts to set up an <i>in vitro</i> assay system using cultured mosquito cells.	78
3.6	Analysis of larvicidal activity of pAS233HA and the deletion derivatives <i>in vivo</i> .	80
3.7	Summary.	81

### Chapter 4.

#### Analysis of the 125KDa crystal protein of *B.sphaericus* 1593.

4.1	Introduction.	83
4.2	Isolation and dissociation analysis of the 125KDa crystal protein produced by <i>B.sphaericus</i> 1593.	84
4.3	Antibodies to the 125KDa crystal protein.	86
4.4	Attempts to immunoprecipitate the 41 and 59KDa proteins from minicells.	88
4.4.1	Immunoprecipitation of minicell extracts containing the 41 and 59KDa proteins with the "anti-125KDa" antibody.	89

4.4.2	Immunoprecipitation of minicells containing the 41 and 59KDa proteins with "anti-crystal" antibody.	89
4.5	Comparison of the properties of the "anti-125KDa" antibody and the "anti-crystal" antibody.	90
4.6	N-terminal amino acid sequencing of the 125KDa crystal protein.	92
4.7	Presence of the 125KDa protein at various stages of growth of <i>B.sphaericus</i> 1593.	94
4.8	Summary.	94

## Chapter 5.

### Analysis of the gene encoding the 41KDa toxin protein of *B.sphaericus*, and the attempted high-level expression of this gene.

5.1	Introduction.	97
5.2	Secretion of the 41KDa protein from <i>E.coli</i> using the haemolysin secretion pathway.	98
5.2.1	Secretion from <i>E.coli</i> of the N-terminal 13KDa of the 41KDa protein fused to the 23KDa signal region of haemolysin.	99
5.2.2	Secretion from <i>E.coli</i> of the entire 41KDa protein fused to the 23KDa C-terminal signal region of haemolysin.	100
5.3	Attempts to achieve high level expression of the gene encoding the 41KDa protein.	102
5.3.1	Attempts to clone the gene encoding the 41KDa protein into the <i>E.coli</i> expression vector, pET3c.	103
5.3.2	Cloning of the gene encoding the 41KDa protein into the <i>E.coli</i> expression vector, pJLA504.	104
5.4	Detection of the 41KDa protein expressed from the plasmid pJLA504-41 in <i>in vitro</i> transcription-translation assays.	105
5.5	Analysis of the stability of the 41KDa protein.	106
5.5.1	Analysis of the stability of the 41KDa protein <i>in vitro</i> .	106
5.5.2	Attempts to determine the half-life of the 41KDa protein <i>in vivo</i> .	107

5.6	Identification of the mRNA transcript of the 41KDa protein encoding gene <i>in vivo</i> .	108
5.6.1	The transcription stop signal downstream of the gene encoding the 41KDa protein is not efficient.	109
5.7	Summary.	110

## Chapter 6.

### DNA sequencing and high-level expression of the gene encoding the 59KDa toxin protein of *B.sphaericus*.

6.1	Introduction.	112
6.2	Secretion from <i>E.coli</i> of a portion of the 59KDa protein using the haemolysin secretion system.	113
6.3	DNA sequence analysis of the gene encoding the 59KDa protein.	114
6.4	Mutagenesis and overexpression of the gene encoding the 59KDa protein.	118
6.5	Cloning of the gene encoding the 59KDa protein into the vector pET3a to achieve high-level expression.	118
6.5.1	Secretion of the S10-59SER fusion protein from <i>E.coli</i> BL21-DE3 <i>via</i> the haemolysin secretion system.	120
6.6	Toxicity analysis of cells producing high levels of the S10-59SER fusion protein by <i>in vivo</i> bioassays with mosquito larvae.	122
6.7	Raising antibodies to the S10-59SER protein.	124
6.8	Presence of the 59KDa protein in purified crystals of <i>B.sphaericus</i> .	124
6.9	Summary.	125

## Chapter 7.

### Preliminary studies on the regulation of expression of the genes encoding the 41 and 59KDa toxin proteins during the growth phase in *E.coli*, *B.subtilis* and *B.sphaericus*.

7.1	Introduction.	127
7.2	Construction of fusions of the <i>E.coli lacZ</i> gene to the genes encoding the 41 and 59KDa proteins.	128
7.2.1	Fusing the 41KDa protein encoding gene to <i>lacZ</i> .	128

7.2.2	Inserting the 41KDa-LacZ fusion into the shuttle vector pHV33.	129
7.2.3	Fusing the gene encoding the 59KDa protein to <i>lacZ</i> .	129
7.2.4	Inserting the 59KDa-LacZ fusion into the shuttle vector, pHV33.	130
7.2.5	Transformation of <i>B.subtilis</i> BR151 with the plasmids pHV41-LacZ and pHV59-LacZ.	130
7.3	Analysis of the expression of the 41KDa-LacZ and 59KDa-LacZ fusions in <i>E.coli</i> and <i>B.subtilis</i> .	131
7.4	Analysis, using antibodies of the expression of the 59KDa protein in <i>B.sphaericus</i> .	132
7.5	Preparation of total RNA from <i>B.sphaericus</i> at various stages of growth, and analysis of expression of the 41 and 59KDa protein encoding mRNA.	135
7.6	Summary.	136

## Chapter 8.

### Discussion.

8.1	Introduction.	139
8.2	Identification of toxin proteins encoded by DNA sequence cloned from <i>Bacillus sphaericus</i> .	139
8.3	Analysis of the 125KDa crystal protein of the toxic <i>B.sphaericus</i> strain 1593.	143
8.4	Analysis of the gene encoding the 41KDa protein and the attempted high level expression of this gene.	147
8.5	DNA sequence analysis of the gene encoding the 59KDa protein and the high-level expression of this gene.	149
8.6	Regulation of expression of the toxin encoding genes in <i>E.coli</i> , <i>B.subtilis</i> and <i>B.sphaericus</i> .	154
8.7	Future work.	158

### References.

162

The Lord in his wisdom made the fly,  
And then forgot to tell us why.

Ogden Nash in "The Fly"

## Chapter 1.

### Introduction.

The pathogenic effects of microorganisms upon insects has been known since the nineteenth century, when Augustino Bassi studied *Muscardine*, the silkworm rot disease (Caullery, 1965), and traced the cause to a fungus. This was subsequently identified by G. Basalmo-Crinelli in 1835, and named *Botrytis bassiana* in honour of A. Bassi. Soon after this Louis Pasteur saved the French silkworm industry from collapse by identifying and listing measures to eliminate the *Pebrine* disease of the silkworm, *Bombyx mori*, which was subsequently found to be caused by the protozoan *Nosema bombycis*. In addition, Pasteur traced the cause of the *Flacherie* and *Gattine* diseases of the *Bombyx mori* to two bacteria, *Bacillus bombycis* and *Streptococcus bombycis*, respectively (Dubois, 1951). These observations firmly established the study of disease-causing microorganisms of insects useful to man.

Early in the twentieth century Berliner isolated a bacterium, which he named *Bacillus thuringiensis*, from a diseased moth population in a German granary (Luthy and Ebersold, 1981). This was the first identification of a microorganism causing disease in an insect pest. The isolation of many such insect pathogens or "entomopathogens", after the demise of the boom years of chemical insecticides in the light of environmental side-effects, has established the study of entomopathogenic organisms as an important control mechanism for insect pests.

Environmental consciousness in the last twenty or so years has led to stringent constraints on the use of polluting and persistent insecticides. In addition, the use of chemicals has been associated with the development of resistance by the target organism. This represents a case for the use of biological control agents. Bioinsecticides are biodegradable, being of biological origin, and are therefore non-polluting. In addition, in over fifty years of use, few cases of insect resistance in the field have been documented (McGaughey, 1985). The host range of insect pathogens is diverse, and thus an effective control mechanism for a variety of insects

could be developed from existing pathogens. Finally, and most importantly, these pathogens are extremely specific for their targets, and do not affect non-target organisms under the conditions of their use (Klausner, 1984; Khachatourians, 1986). A selection of insect pathogens are described below.

### 1.1. Insect pathogens.

There are many examples of insect pathogens from classes ranging from viruses to nematodes. The pathogens do not all cause disease by the same mechanism. Some pathogens are opportunistic, infecting hosts that are stressed or compromised in some manner. Others attack healthy insects at specific stages in their life cycle, and still others produce proteinaceous toxins that cause death when the toxins are consumed by susceptible insects.

The number of isolates of entomopathogens exceed one thousand, but only a few of these have been recognized as being true pathogens or have been developed for use as insecticides.

#### 1.1.1. Entomopathogenic viruses.

The number of viruses isolated so far from diseased insects has been reported as being 650 (Khachatourians, 1986), of which 540 were isolated from lepidoptera (butterflies and moths), 90 from hymenoptera (bees), and 20 from orthoptera, coleoptera and diptera (beetles and flies). Several of these viruses are being marketed as biocontrol agents. Although control is very effective, the production of viruses in large quantities is time consuming and expensive because of their requirement for live hosts for growth. On the other hand, some bacteria and fungi can be grown on inexpensive artificial media in large quantities. Some examples of viruses and their targets are: *Heliothis* Nuclear Polyhedrosis Virus (NPV), which infects both *Heliothis zea* (corn earworm) and *H.virescens* (tobacco budworm), *Orygia pseudotsugata* NPV, which infects *O. pseudotsugata* (Douglas fir tussock moth), and *Lymantria dispar* NPV, which infects *L. dispar* (gypsy moth). In addition to being extremely specific for their hosts, most known entomopathogenic viruses belong to the family Baculoviridae

that do not infect vertebrates. Their dissemination does not therefore pose a danger to vertebrates in general (Klausner, 1984). Other viruses isolated from mosquitoes have been classified as Poxviridae, Iridoviridae and Parvoviridae, whilst Reoviridae and Iridoviridae have been reported in blackfly (Lacey and Undeen, 1986).

#### 1.1.2. Fungal pathogens.

Fungi used as insect control agents are entomopathogenic or saprophytic (Khachatourians, 1986). The use of fungi for insecticide production has been facilitated by the immense amount of work involved in the use of fungi for the production of secondary metabolites and biomass. Examples include *Beuveria<sup>a</sup> bassiana*, which targets the Colorado potato beetle, codling moth, European corn borer and pine caterpillar; *Metarhizium anisopliae*, which targets the spittle bug and sugarcane frog hopper; *Verticellum lecanii*, which targets aphids, the coffee green bug, greenhouse whitefly and thrips. Fungi are marketed as formulations of mycelial fragments and conidia or blastospores. The advantages of fungi are that they infect insects on contact, and do not need to be consumed for an infection to occur (Klausner, 1984).

#### 1.1.3. Protozoa and nematodes.

Several protozoa of the genus *Nosema* are used as biocontrol agents, such as *N. locustae*, which targets grasshoppers, *N. pyransta*, which targets the European corn borer, and *N. fumiferanae*, which targets the spruce budworm. Another protozoan, *Veriomorpha necatrix* has a wide host range which includes the cabbage looper, corn earworm and the tobacco budworm (Khachatourians, 1986).

Nematodes have a unique pathogenic mechanism to kill their hosts. Thus, associated with each entomopathogenic nematode is a bacterial symbiont that is released when the nematode enters the insect larval host. The bacteria multiply rapidly inside the larva, causing its death. The nematode then feeds on the bacterium and multiplies in the larval carcass. Subsequently, progeny nematodes emerge, each carrying the bacterium in their guts to complete the cycle. The nematode

therefore acts as a syringe to inoculate the larva with a bacterium, and it is the bacterium that causes the death of the larva (cited in Lysenko, 1985; Aronson et al., 1986). Examples of such nematodes are *Steinernema feltiae* and *Neoplectana carpocapsae*, which harbour the bacterium *Xenorhabdus nematophilus*, and *Heterorhabditis* species, each of which carries a different, specific bacterial symbiont.

#### 1.1.4. Bacterial pathogens.

Bacterial pathogens include obligate pathogens such as *Bacillus popillae* and *Bacillus larvae*, both of which affect coleopteran larvae. In addition, there are facultative pathogens, which cause disease in a stressed and compromised host, such as *Pseudomonas aeruginosa* and *Xenorhabditis* species. Another group contains the crystalliferous sporeformers such as *Bacillus thuringiensis* species and *Bacillus sphaericus*. These organisms cause death through the production of crystals that are toxic to insect larvae upon ingestion (Lysenko, 1985; Khachatourians, 1986). Finally, there are potential insect pathogens such as *Serratia marcescens* (Bucher, 1960), which are capable of causing disease under certain circumstances.

The representative group of entomopathogens examined in this thesis are the crystalliferous sporeformers which are "food-poisoning" organisms and cause the death of the target insect through the production of toxic factors that are consumed as food. Examples of this type of pathogen are the sporeforming Bacilli, of which *Bacillus thuringiensis* and *Bacillus sphaericus* are the most important. These two organisms will be discussed later in greater detail with reference to their mode of pathogenesis. Other sporeforming Bacilli that have been implicated in insect disease are *B. cereus*, *B. alvei*, *Clostridium brevifaciens* and *C. malacosomae* (Davidson, 1981).

### 1.2. The response of insects to pathogens.

The insect's response to infection is often overlooked and is an important factor in pathogenesis. The effectivity of the insect response often dictates the course the pathogenesis will take, from the killing of the pathogen by the insect to the killing of the insect by the pathogen.

Insects produce a variety of small cationic molecules in response to infection that are antibacterial in nature. These "defensins" belong to a family of anti-bacterial peptides induced following infection, that have broad spectrum activity towards Gram positive and Gram negative bacteria (Dimarcq *et al.*, 1990). Hurlbert *et al.* (1985) have documented up to twenty seven proteins produced by the insect host in response to infection, and several of these may have a protective role. In addition, these insects also show a humoral response to infection, encapsulating invading bacteria prior to phagocytosis (Gotz *et al.*, 1987). In reaction to these responses, some successful pathogenic bacteria like *B.thuringiensis* and *S.marcescens*, and nematodes like *Neoplectana* species, produce immune inhibitors which include chitinases and proteolytic enzymes (Dalhammar and Steiner, 1983; Flyg and Xanthopoulous, 1983; Lovgren *et al.*, 1990).

### 1.3. The toxic factors of entomopathogenic Bacilli.

Bacilli are aerobic, rod-shaped, spore forming organisms. *B.thuringiensis* and *B.sphaericus* occur naturally in soil. Entomopathogenic strains of *B.thuringiensis* and *B.sphaericus* have been isolated from insect cadavers and have varying host-specificities. Some *B. thuringiensis* strains are toxic to the larvae of lepidoptera, coleoptera and diptera. *B.sphaericus* strains are toxic only to dipteran mosquito larvae. *B.thuringiensis* and *B.sphaericus* strains have been classified variously and often confusingly, according to their flagellar serotype, crystal serotype and host ranges (de Barjac *et al.*, 1985; Krych *et al.*, 1980).

During the process of sporulation, these Bacilli synthesize proteins that accumulate in a "crystal" that is physically associated with the developing spore. This crystal

has been shown in several cases to be the factor which is toxic for insect larvae (Somerville, 1971; Yamamoto and McLaughlin, 1981; Andrews *et al.*, 1981). The final size of the crystal is usually reached by stage VI of sporulation. Upon spore maturation, the crystal is released from the mother cell together with the spore.

The crystal is an aggregate of proteins, whose size, morphology, number and composition vary between strains (Ibarra and Federici, 1986). In some *B.thuringiensis* strains, the crystal has been shown to be composed of protoxins, which are converted by the action of larval gut enzymes into the toxin protein. The toxin then binds to specific receptors present on gut epithelium cells, causing swelling and lysis of the cells, and finally, paralysis of the larva and death (Schnepf and Whiteley, 1985). The time taken from the ingestion of the crystal by the larva to death can in some cases be as little as four hours. Morphological abnormalities of gut epithelial cells can be seen within a few minutes of toxin addition, indicating a rapid cytolytic mode of action (Schnepf and Whiteley, 1985). By disruption of this gut membrane barrier, the bacteria gain entry to the haemocoel, where they multiply inside the dead larva. Interestingly, the insect haemocoel is one of the few environments where these soil organisms reach sufficiently high densities for genetic exchange by cell mating to occur (Aronson *et al.*, 1986).

The crystal has been shown to be present in entomopathogenic strains of *B.thuringiensis* and *B.sphaericus*. Purification of the crystal from spore-crystal complexes, and its analysis in bioassays led to the identification of the crystal as the toxic factor (Aronson *et al.*, 1986; Baumann *et al.*, 1985). The morphology of the crystal of *B.thuringiensis* can, <sup>in some cases</sup> be related to its insecticidal spectrum. Bipyramidal crystals are toxic to lepidoptera, amorphous multicomponent crystals are toxic to mosquitoes and blackfly, cuboidal crystals contain lepidoptera and mosquito-toxic factors and square or flat crystals are normally toxic to coleoptera (Whiteley *et al.*, 1987). In the case of *B.sphaericus*, toxic factors have also been found associated with the spore-coat (Myers and Yousten, 1980; Myers and Yousten, 1981). The size of the polyhedral crystal varies between *B.sphaericus* strains

from being large in *B.sphaericus* 2297 to small in *B.sphaericus* 1593, 1593M and *B.sphaericus* 2013-4, to having no crystal at all in *B.sphaericus* SSII-1 (Davidson, 1981; Davidson and Myers, 1981; Sgarella and Szulmajster, 1987; Kalfon et al., 1983). For *B.sphaericus*, although strains containing crystals are generally more toxic than strains without inclusions, non-crystal forming strains can also be toxic to some extent (Davidson and Myers, 1981; Myers and Yousten, 1978). The strain SSII-1 is weakly toxic to mosquito larvae with an  $LC_{50} = 2.5 \times 10^8$  cells/mL compared to strain 1593, which has an  $LC_{50} = 2.9 \times 10^2$  cells/mL (Myers et al., 1979). This indicates that the mosquito larvicidal toxin may not necessarily be accumulated into a crystal. The protein composition of crystals from several strains of *B.thuringiensis* and *B.sphaericus* are listed in Table 1.1.

Crystal formation in *B.thuringiensis* has been shown to be triggered when the bacterial cell enters stage II of sporulation (completion of forespore septum; Schnepf and Whiteley, 1985; Somerville, 1971). In *B.thuringiensis* the crystal may, following synthesis, constitute as much as 30% of the dry weight of the cell (Lecadet and Dedonder, 1971). Synthesis of the crystal is associated with an increase in the toxicity of the cell to target larvae (Kalfon et al., 1984; Somerville, 1971). The crystal is always associated with the spore of the bacterium, but in *B.sphaericus*, the crystal and the spore are together contained within a tough exosporeum, which must be ruptured in order to purify the crystal (Kalfon et al., 1984; Baumann et al., 1985).

The protein composition of the crystal of *B.sphaericus* has been the source of considerable contention and confusion, being variously reported as consisting of proteins of 125, 110, 72, 63, 57 and 43KDa, with a minor protein of 37KDa (Baumann et al., 1985) and 15 protein species of 14-100KDa (Tinelli and Bourgouin, 1982). Whilst the amount of the larger proteins varied between different preparations in such studies, Narasu and Gopinathan (1986) reported that the crystal from *B.sphaericus* 1593 contained proteins with sizes of 42.6, 44.1, 50.7 and 51.3KDa, apparently lacking entirely the high molecular weight forms.

Table 1.1    Reported protein composition of crystals of  
B.thuringiensis (B.t.) and B.sphaericus.

In the table are shown the protein compositions of several  
*B.thuringiensis* and *B.sphaericus* strains.

References:

1. Whiteley et al. (1985)
2. Ward and Ellar, (1988)
3. Walfield et al. (1986)
4. Waalwijk et al. (1985)
5. Pfannensteil et al. (1984)
6. Herrnstadt et al. (1987)
7. Hofte et al. (1986)
8. Chak and Ellar, (1987)
9. Chestukhina et al. (1988)
10. Baumann et al. (1985)
11. Narasu and Gopinathan, (1986)

Table 1.1.

Reported protein composition of crystals of *B.thuringiensis* (*B.t.*) and *B.sphaericus*.

<u>Strain</u>	<u>Size(KDa)</u>	<u>Gene type</u>	<u>Ref.</u>
<i>B.t.kurstaki</i> HD73	135	<i>cryIA(c)</i>	1
<i>B.t.thuringiensis</i> HD2	130	<u><i>cryIA(b)</i></u>	1
<i>B.t.kurstaki</i> HD-1 Dipel	135	<i>cryIA(a), (c)</i>	1
<i>B.t.kurstaki</i> HD-1	135,130	<i>cryIA(a), (b), (c)</i>	1
<i>B.t.israelensis</i>	135,130, 72,27		2,3, 4,5
<i>B.t.sandiego</i>	65		6
<i>B.t.berliner</i> 1715	140,130		7
<i>B.t.aizawai</i> HD-133	>1 protein of 130		8
<i>B.t.galleriae</i>	2 proteins of 130		9
<i>B.sphaericus</i> 2362	125,110,92, 72,63,43,28		10
<i>B.sphaericus</i> 1593	51.3,50.7, 44.1,42.6		11

The crystal proteins of *B.thuringiensis* and *B.sphaericus* have also been reported to contain associated carbohydrate. In the case of *B.thuringiensis* subspecies *israelensis*, the carbohydrates have been reported to be covalently associated (Pfannenstiel et al., 1987), and the glycosylated nature of the protein will later be shown to have important implications for the specificity of the toxins. The *B.sphaericus* toxin is also reported to contain 12% associated carbohydrate (Narasu and Gopinathan, 1986), although covalent binding of carbohydrate to protein has not yet been demonstrated. In addition, Watson and Mann (1988) identified at least five peptides in the *B.thuringiensis* subspecies *kurstaki* HD-1 crystal preparation that are subject to phosphorylation. One of these proteins corresponds to the P1 (lepidopteran-specific) protoxin, and a second abundant species has a molecular weight of 25KDa. The functional significance of the phosphorylation is not known.

#### 1.4. The toxin encoding genes.

Although the crystal is generally regarded as being responsible for toxic activity, the identity of the specific component that causes the toxic effect is not very clear from studies on the purification of individual crystal proteins from the *B.sphaericus* cell or the spore/crystal complex. For example, the size of the toxin protein of *B.sphaericus* was variously reported as being 100KDa (Davidson, 1982), 38KDa (Sgarella and Szulmajster, 1987), 43KDa (Baumann et al., 1985), 150KDa (Davidson, 1981) and 25, 43 and 56KDa (Tinelli and Bourgouin, 1982; Davidson, 1983) or 50KDa (Bourgouin et al., 1984). As a further complication, *B.thuringiensis* at least produces other toxins in addition to the crystal toxins. These contribute to opportunistic infections and include a heat-labile alpha-exotoxin, phospholipase C, haemolysins and diarrheagenic and demonecrotizing toxins (Schnepf and Whiteley, 1985). The presence of so many toxins has given impetus to the cloning of the specific toxin encoding genes in order to identify conclusively the nature of individual larvicidal toxins. The study of the cloned genes has led in many cases to the delineation of their protein structure and mode of action. Toxin encoding genes cloned from both *B.thuringiensis* and *B.sphaericus* will be discussed in some

detail in this section.

#### 1.4.1. Genes from *B.thuringiensis* encoding toxicity to insect larvae.

The first report of the cloning of a crystal protein gene from *B.thuringiensis* appeared ten years ago (Schnepf and Whiteley, 1981). Since then the nucleotide sequences of over 42 crystal protein genes have been reported. Several genes are identical, or nearly identical, and thus represent the same gene, or variants of the same gene. Taking this into account 14 distinct genes remain, and 13 of these specify a family of related insecticidal crystal proteins (Cry proteins) (Hofte and Whiteley, 1989). These have been subdivided into four classes based upon structural similarities and insecticidal spectra. These classes, with their representative toxins, are shown in Table 1.2. The remaining one of the 14 genes, *cytA*, from *B.thuringiensis* subspecies *israelensis*, codes for the synthesis of a toxin of 27KDa which exhibits cytolytic activity against a variety of insect and mammalian cells (Thomas and Ellar, 1983a).

Analysis of the toxin genes of *B.thuringiensis* has shown amino acid and nucleotide sequence similarity between toxins from various strains (Aronson et al., 1986; Thorne et al., 1986; Hofte and Whiteley, 1989). A diagrammatic representation of regions of similarity between these toxin genes is shown in Figure 1.1. Within the sequences of the toxin genes, five highly conserved blocks of amino acids can be distinguished, which align with few, or no gaps. These blocks are separated by highly variable sequences of varying lengths. The exceptions to this case are the CryII and CryIVD proteins, which show homology to the other Cry proteins only in the region corresponding to Block I. Also conserved in all but the CryII and CryIVD proteins is the presence of a hydrophobic region within the first 120 N-terminal amino acids which could be involved in membrane binding (Hofte and Whiteley, 1989). Most cry genes encode insecticidal proteins of either 130, 140 or 70KDa, containing a toxic fragment of approximately 50 to 70KDa within the N-terminal region of the protein. The 72KDa CryIVD protoxin, in contrast, is proteolytically converted into a 30KDa active toxin. The C-

Table 1.2 Insecticidal crystal protein genes of B.thuringiensis.

The Table is adapted and modified from Hofte and Whiteley (1989).

a: specified host ranges: L, Lepidoptera; D, Diptera; C, coleoptera; cyt, cytolytic and haemolytic.

b: detailed references can be obtained from Hofte and Whiteley, (1989).

c: references obtained from Hofte and Whiteley, 1989.

d: Hofte, unpublished observations, cited in Hofte and Whiteley, (1989).

References:

- 1: Schenpf *et al.* (1985)
2. Wabiko *et al.* (1986)
3. Adang *et al.* (1987)
4. Brizzard and Whiteley, (1988)
5. Honee *et al.* (1988)
6. Donovan *et al.* (1988)
7. Widner and Whiteley, (1989)
8. Herrnstadt *et al.* (1987)
9. Ward and Ellar, (1987)
10. Chunjatupornchai *et al.* (1988)
11. Thorne *et al.* (1986)
12. Donovan *et al.* (1988)
13. Waaljwick *et al.* (1985)

Table 1.2.

Insecticidal crystal protein genes of *B.thuringiensis*.

Gene type	Host range <sup>a</sup>	Size(KDa)	Other designations <sup>b</sup>	Ref <sup>c</sup> .
<i>cryIA(a)</i>	L	133.5	4.5Kb gene, <i>cryI-I</i>	1
<i>cryIA(b)</i>	L	130.6	5.3Kb gene, <i>cryI-2</i>	2
<i>cryIA(c)</i>	L	133.3	6.6Kb gene	3
<i>cryIB</i>	L	139.5	<i>cryA4</i> , Type B	4
<i>cryIC</i>	L	134.7	Type C, BTVI, <i>Bta</i>	5
<i>cryID</i>	L	132.5		d
<i>cryIIA</i>	L/D	70.9	P2 gene, <i>cryBI</i>	6
<i>cryIIB</i>	L	70.8	<i>cryB2</i>	7
<i>cryIIIA</i>	C	73.1	<i>cryC</i>	8
<i>cryIVA</i>	D	134.5	ISRH3, 130KDa-endotoxin gene, 125KDa protein gene	9
<i>cryIVB</i>	D	127.6	ISRH3, <i>Bt8</i> , 130/135KDa-endotoxin gene	10
<i>cryIVC</i>	D	77.8	ORFI	11
<i>cryIVD</i>	D	72.4	<i>cryD</i>	12
<i>cytA</i>	D/cyt	27.3	27KDa toxin gene	13

Figure 1.1 Comparison of the amino acid sequences of the CryI(A), CryI(B) and CryI(C) entomocidal toxins of *Bacillus thuringiensis*.

The deduced amino acid sequences of the *B.thuringiensis* crystal proteins were aligned and gaps were added for optimal alignments (not indicated). Vertical lines represent amino acids that are conserved for:

- (A) all lepidopteran crystal proteins (CryI);
- (B) all dipteran crystal proteins (CryIV);
- (C) all CryI, CryIII and CryIV crystal proteins except CryIVD.

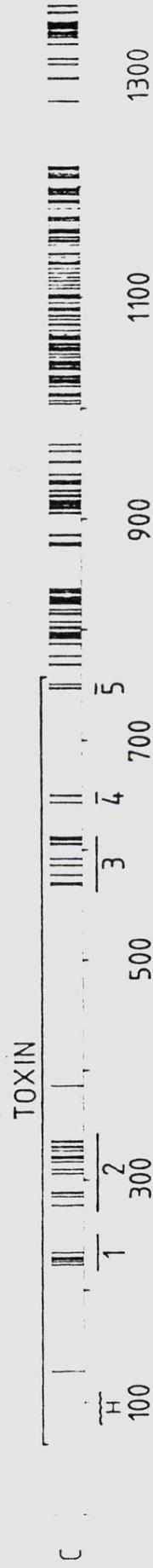
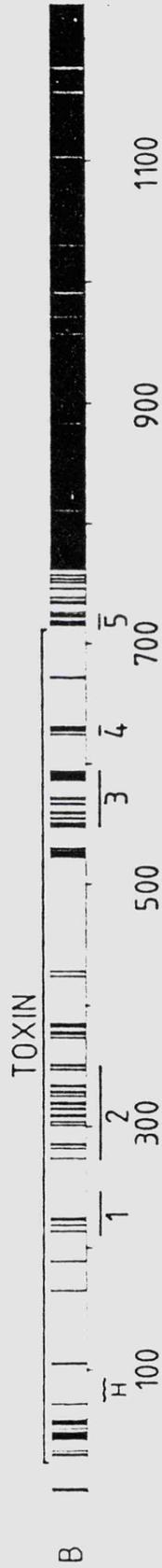
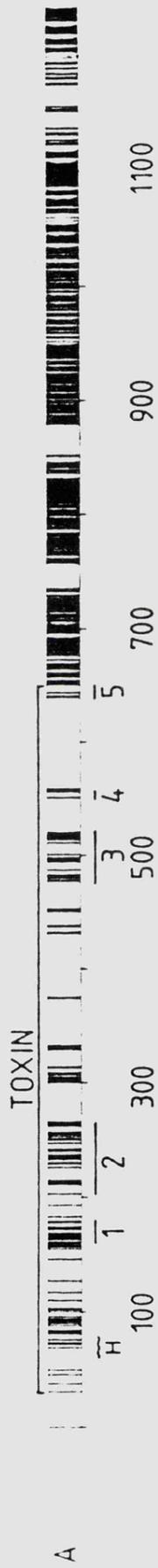
The positions of the five conserved sequence blocks are underlined. The CryII proteins show significant homology only to the sequence of block I. Vertical lines in the C-terminal half (outside the toxin encoding region) represent amino acids conserved for the CryIVA and CryIVB proteins in Panel B, and for all crystal proteins specified by cry genes (except CryII, CryIII and CryIVC and CryIVD) in Panel C.

Abbreviation:

H: hydrophobic transmembrane sequence present in all crystal proteins except CryII and CryIVD.

Numbers refer to positions in the sequence after alignment (amino acid plus gaps).

Figure reproduced from Hofte and Whiteley (1989).



terminus of the Cry proteins, whilst constituting the region of highest conservation, is not essential for toxicity (Delecluse et al., 1988; Aronson et al., 1986; Perlak et al., 1990).

Genes encoding crystal proteins have been found to be located variously on the bacterial chromosome or on large host plasmids. In several cases, more than one toxin encoding gene has been demonstrated for a single strain (Kronstad et al., 1983; Nicholls et al., 1989; Visser et al., 1988). *B.thuringiensis* strains containing plasmids have been shown to contain twice as much diaminopimelic acid (DPA) per spore than plasmid-cured strains. The presence of DPA in the spore in some way renders them more sensitive to ultraviolet light (Benoit et al., 1990; Greigo et al., 1978). It has been documented that insecticide formulations containing *B.thuringiensis* have to be applied more often in the field than those containing *B.sphaericus* (Trisrisook et al., 1990). This lack of maintenance in the environment of toxic *B.thuringiensis* strains has been correlated with the UV sensitivity of the plasmid-carrying strains. In addition, some *B.thuringiensis* strains are known to carry at least one prophage that can be induced by the action of ultraviolet light. Their induction is lethal to the *B.thuringiensis* strain (cited in Benoit et al., 1990).

Analysis of the 75Kb-plasmid present in *B.thuringiensis* subspecies *kurstaki* HD73 and encoding the CryIA(c) toxin, has led to the identification of the inverted repeat sequences IR2150 and IR1750 flanking the toxin encoding genes on host plasmids. Southern hybridization analysis using probes originating from these inverted repeat sequences demonstrated their presence on plasmids of 14 out of 15 strains (Whiteley et al., 1985). These sequences were present in multiple copies on the plasmid and chromosomal DNA in *B.thuringiensis* subspecies *kurstaki* HD73 (Kronstad and Whiteley, 1984). Detailed analysis of the 42MDa plasmid of *B.thuringiensis* subspecies *berliner* 1715 has shown that the crystal gene is located on a 7Kb DNA fragment, flanked by two inverted repeat sequence elements called IR1. The nucleotide sequence of the IR1 element was determined. This consisted of inverted 20bp repeat sequences flanked by two 11bp direct repeat sequences

on both sides. Nucleotide sequence similarities to other sequences in a database indicated that the IRI element encoded a transposase (Klier and Rapaport, 1987). A second sequence, designated "Th", was also flanked by two IRI's. This Th sequence had all the characteristics of a transposon, and has been designated Tn4430 (Klier and Rapaport, 1987). Other analyses have shown that cry genes from different species often have similar upstream and downstream regulatory regions (Hindley and Berry, 1987; Aronson et al., 1986). The presence of transposons flanking these crystal genes together with the fact that these genes are often related (Thorne et al., 1986; Galjart et al., 1987; Donovan et al., 1988; Klier et al., 1985; Garduno et al., 1988), points to a mechanism of transmission between strains, between various plasmids of *B.thuringiensis*, and possibly, and between the plasmid and chromosome (Schnepf and Whiteley, 1985; Thorne et al., 1986). Plasmid recombinational exchanges following mating has been shown to occur between different species of Bacillus (Gonzalez et al., 1982; Minnich and Aronson, 1984). In one case, the transposon Tn4430 present on a *B.thuringiensis* subspecies *thuringiensis* plasmid pOX12, which encodes a crystal protein gene, has been shown to promote cotransfer of plasmids between strains of *Bacillus anthracis* (Green et al., 1989).

#### 1.4.2. Genes from *B.sphaericus* reported to encode toxicity to mosquito larvae.

Several groups in recent years, using diverse methods, have cloned genes which encode the toxins that confer larvicidal activity upon strains of *B.sphaericus*. Since this is the organism from which the genes used in this thesis have been cloned, the genes identified by various groups from different strains will be examined in detail.

##### 1.4.2.1. *B.sphaericus* 1593:

Three groups have reported the cloning of toxin genes from *B.sphaericus* 1593, and 1593M, an isolate of strain 1593 obtained by passages through mosquito larvae. Strain 1593M showed slightly increased toxicity to mosquito larvae ( $1-2 \times 10^{-3} \mu\text{g/mL}$  compared to  $1-5 \times 10^{-3} \mu\text{g/mL}$  for strain 1593; Jamuna et al., 1982; Louis et al., 1984). Each will be considered

separately.

The first report of the cloning of a *B.sphaericus* toxin encoding gene was by Ganesan *et al.* (1983) and a restriction enzyme map of the insert obtained in the resulting recombinant plasmid, pGsp03, is reproduced in Figure 1.2. Maxicell analysis of the products expressed by this clone indicated proteins of 12, 15, 19 and 21KDa. All of these proteins were required for larvicidal activity (Louis and Szulmajster, 1985). Toxicity resulting from the recombinant plasmid expressed in *E.coli*, was detected in the cell extracts in the logarithmic phase of growth. This activity was increased when the *E.coli* cells were sonicated in the presence of the detergent Triton X-100. Dot-blot analysis was used to show that the cloned gene was present in a single copy in strains with low toxicity, and in multiple copies in highly toxic strains (Louis *et al.*, 1984).

Hindley and Berry (1987) cloned and sequenced the gene from strain 1593 encoding a 41.9KDa protein. Recombinant clones containing the gene were identified by probing a *B.sphaericus* total DNA library with oligonucleotides. These were constructed using data obtained from a published N-terminal amino acid sequence of a 43KDa protein present in crystals produced by *B.sphaericus* 2362. The 43KDa protein was initially presumed to be the N-terminus of a larger precursor protein (Baumann *et al.*, 1985). The entire 41.9KDa protein was shown to be encoded on a 1.85Kb *EcoRI-HindIII* restriction enzyme fragment. A restriction enzyme profile of the fragment deduced from the DNA sequence is shown in Figure 1.2. Nucleotide sequencing of this gene revealed a 370 amino acid protein with a calculated molecular weight of 41.9KDa, deduced from an open reading frame of 1.107Kb. The estimated molecular weight corresponds well with the molecular weight of a 43KDa (Baumann *et al.*, 1985), 42.6KDa (Narasu and Gopinathan, 1986), or 41KDa (this work, Chapter 3, Section 3.3) protein associated with the crystal.

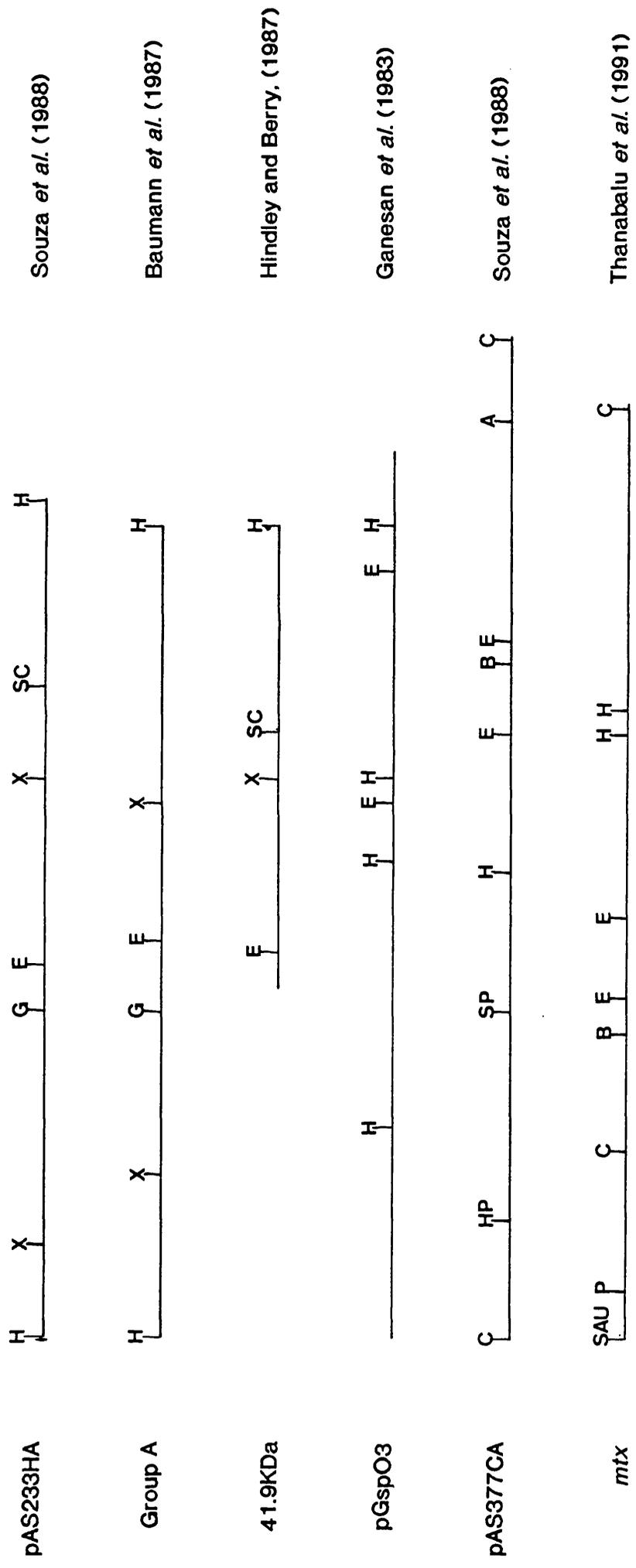
Souza *et al.* (1988) identified two non-identical DNA fragments from strain 1593M encoding toxicity to mosquito larvae in *E.coli* cells, when cloned into the multicopy vector, pBR322, generating pAS233 and pAS377 respectively.

Figure 1.2 Comparison of the restriction enzyme maps of larvicidal toxin genes cloned from *B.sphaericus* strains.

The figure shows the restriction enzyme maps of the DNA inserts encoding proteins with larvicidal activity cloned from several *B.sphaericus* strains (see Section 1.4.2 for details). The Group A insert was cloned from *B.sphaericus* 2362, and the 41.9KDa insert were cloned from *B.sphaericus* 1593, and the inserts of pAS233HA, pGspo3 and pAS377CA were cloned from *B.sphaericus* 1593M and that of the *mtx* gene was cloned from *B.sphaericus* SSII-1. Only the inserts in each case are shown. The references are also indicated on the left of the map.

Abbreviations:

A: *Ava*I; B: *Bam*HI; C: *Cla*I; E: *Eco*RI; G: *Bgl*III; H: *Hind*III;  
HP: *Hpa*I; P: *Pst*I; SAU: *Sau*3A; SC: *Sca*I; X: *Xba*I



1Kb

a. pAS233: A 3.6Kb *Hind*III restriction enzyme fragment was subcloned from a larger fragment of 8.6Kb. This conferred the ability upon recombinant *E.coli* strains to encode larvicidal activity for mosquito larvae in bioassays. The initially reported restriction enzyme map of this 3.6Kb fragment is compared to those of the genes cloned by Baumann *et al.* (1985), Hindley and Berry, (1987) and Louis and Szulmajster, (1985) in Figure 1.2. The restriction map of this fragment is similar to the maps of genes cloned by Baumann *et al.* (1985) and Hindley and Berry (1987), but is different from the inserts of pAS377 and pGsp03 described by Souza *et al.* (1988) and Ganesan *et al.* (1983), respectively.

Maxicell analysis by Souza *et al.* (1988), however, failed to identify any protein products of the 3.6Kb fragment. This fragment was later analyzed in minicells as part of this project, and two protein products of 41 and 59KDa were shown to be encoded by it (Chapter 3, Section 3.3).

b. pAS377: A second DNA fragment was also cloned by Souza *et al.* (1988) that encoded mosquito larvicidal activity in bioassays against *Culex* mosquito larvae, but not against *Anopheles* species. The restriction enzyme map of the 4.3Kb fragment in pAS377CA subcloned from the original insert of 15.7Kb is also shown in Figure 1.2. Thanabalu *et al.* (1991) have recently cloned a gene encoding a 100KDa mosquitocidal protein from *B.sphaericus* SSII-1 whose restriction map is reported to resemble the restriction map of the insert of pAS377CA. Maxicell analysis performed on this clone detected only one protein at 29KDa as a possible gene product (Souza *et al.*, 1988). The presence of this gene in addition to the inserts of the plasmids pGSp03 (Ganesan *et al.*, 1983) and pAS233 (Souza *et al.*, 1988) points to the presence of multiple, unrelated toxin genes in *B.sphaericus*. There is a precedent for such an observation in *B.thuringiensis*, where several strains have been reported to contain more than one toxin gene (Klier and Rapoport, 1987; Hofte and Whiteley, 1989; Aronson *et al.*, 1986).

#### 1.4.2.2. B.sphaericus 2362:

Two groups have cloned and sequenced genes from *B.sphaericus* 2362 that encode larvicidal activity. Baumann et al. (1987) originally isolated three "groups" of recombinant clones by screening a library of *B.sphaericus* 2362 DNA cloned into phage  $\lambda$ gt11 with antibodies raised against purified 43 and 63KDa proteins, isolated from the crystal of this strain. The three groups were delineated by restriction enzyme mapping of the recombinant plasmids and Ouchterlony immunodiffusion experiments with cell extracts of *E.coli* containing the recombinant phage. Groups A and C gave reactions of non-identity with each other, and both gave reactions of partial identity with group B. Of these three groups, only the clones in group A were toxic in bioassays to mosquito larvae (Baumann et al., 1987). A restriction enzyme map of the insert of the Group A clone described by Baumann et al. (1987) is compared in Figure 1.2 to toxin encoding genes cloned from *B.sphaericus* 1593.

Berry and Hindley (1987) cloned a toxin gene from *B.sphaericus* 2362, using oligonucleotide probes designed to the known N-terminal amino acid sequence of a 43KDa protein obtained from the strain 2362 crystal preparation (Baumann et al., 1985). The nucleotide sequence of this gene was determined, and was identical to the sequence of the gene encoding the 41.9KDa protein from the strain 1593 (Hindley and Berry, 1987).

#### 1.4.2.3. B.sphaericus SSII-1:

Recently, Thanabalu et al. (1991) have cloned a gene from *B.sphaericus* SSII-1, which encodes a 100KDa protein toxic to the larvae of *Culex quinquefasciatus*. The restriction map of this fragment is reported to resemble the insert of the plasmid pAS377CA (Souza et al., 1988; Figure 1.2). This discovery presents further evidence of the presence of a multiplicity of genes in larvicidal *B.sphaericus* strains.

Thus, genes similar in their restriction enzyme maps were obtained from *B.sphaericus* strains 1593, 2362 and SSII-1. From comparisons of the restriction enzyme patterns of the

3.6Kb insert of the plasmid pAS233HA (Souza et al., 1988) and that of the 41.9Kb protein encoding gene of Hindley and Berry (1987), it seemed possible that the 41.9KDa protein encoding gene was present on the 3.6Kb insert. Our interest was to confirm this, and to determine whether other proteins encoded by the 3.6Kb insert was important in the toxicity process.

The cloned toxin encoding genes from strain 1593M produced larvicidal effects in *E.coli* cells in bioassays (Chapter 2, Section 2.9) at a 1000-fold higher LC<sub>50</sub> than the parent *B.sphaericus* 1593M strain (Souza, 1987). This could be attributed to the instability of such foreign proteins (Gottesman, 1989) or to the low level expression of Gram positive promoters which are not efficiently recognized by the Gram negative *E.coli* transcription machinery.

It is also conceivable that the presence of more than one toxin in the parent *B.sphaericus* strain from which these genes were cloned, may cooperatively contribute to observed higher toxicity, when compared to *E.coli* cells containing the gene product of a subset of toxin genes. However, studies which involved mixing the cells containing pAS233HA and pAS377CA prior to a bioassay did not lead to any detectable enhancement of larvicidal activity (Souza, A., Rajan, V. and Jayaraman, K., unpublished results). Alternatively, posttranslational modifications, for example, glycosylation, might be important in determining specificity of the wild type toxin, as has been suggested in the case of *B.thuringiensis* toxins (Muthukumar and Nickerson, 1987). Indeed, the crystal proteins of the *B.sphaericus* 1593 strain have been shown to contain associated carbohydrates (Narasu and Gopinathan, 1986) and these may contribute to increased specificity. *E.coli* has not been shown to carry out such posttranslational modifications (Bialy, 1987). Nevertheless, the presumably unmodified protein in *E.coli* was toxic, showing some measure of specificity, being toxic only to larvae at a certain growth stage (Chapter 2, Section 2.9). This suggests that posttranslational modification is not essential for larvicidal activity of the toxins.

### 1.5. Regulation of toxin synthesis.

In *B.thuringiensis* strains, the synthesis of crystal proteins commences at stage II or III of sporulation, after the formation of the forespore septum (Wong et al., 1983; Ward and Ellar, 1986; Aronson et al., 1986). Synthesis of the crystal is complete by stage V, when the spore becomes octanol- and heat-resistant (Lecadet and Dedonder, 1971). Synthesis of crystal in *B.thuringiensis* subspecies *kurstaki* correlates with the presence in the cell of crystal protein mRNA, detected using radiolabelled DNA probes corresponding to crystal protein genes (Wong et al., 1983). Activation of transcription and translation are therefore coupled to crystal protein accumulation in this species. Such growth-stage dependent synthesis is deregulated in *E.coli*, where *B.thuringiensis* toxin synthesis is seen throughout the growth phase (Wong et al., 1983). Although the levels of toxin expressed in *E.coli* are usually very low, there are reports of *B.thuringiensis* toxin accumulating as inclusion bodies in *E.coli* when expressed from certain high level promoters (Chak and Ellar, 1987; Oeda et al., 1989). In this context, it is pertinent to mention that these studies apply to genes cloned from plasmids in *B.thuringiensis*. When toxin genes cloned from chromosomal locations have been expressed in heterologous hosts, only transcription, but not the translation into protein has been observed (Klier et al., 1982; Klier and Rapoport, 1987). The reason for this is not known. Despite such anomalies seen with the expression of the toxin genes in heterologous hosts, most regulation studies have been carried out either in *E.coli* or in *B.subtilis*. The reason is probably the ease of manipulation of these hosts as compared with *B.thuringiensis* or *B.sphaericus*, for both of which transformation protocols have only recently been established.

Mutants of *B.thuringiensis* that are deficient in spore formation (*spo*<sup>-</sup>), which still accumulate insecticidal crystal proteins (*cry*<sup>+</sup>), have been obtained by the action of mutagenic reagents like N'methyl-N'nitro N'nitrosoguanidine (MNNG; Johnson et al., 1980) and ethylene methane sulfonate (EMS; Wakisaka et al., 1982). Other studies have established that crystal synthesis takes place in *B.thuringiensis* mutants blocked after stage II of sporulation (Somerville, 1971; Wong

et al., 1983) but does not occur in mutants blocked at stage 0 (Meenakshi and Jayaraman, 1979).

Trans-acting proteins have been shown in some systems to affect the production of crystal protein. For example, a segment of DNA present 4Kb upstream of the *cytA* gene (encoding the 27KDa haemolytic and cytolytic protein) in *B.thuringiensis* subspecies *israelensis*, encodes a 20KDa protein which acts posttranslationally on the CytA protein, leading to its enhanced stability and accumulation in *E.coli* (McLean et al., 1987). It is speculated that this protein may be a glycosylase or a scaffolding protein in the crystal, acting in an as yet unknown manner to protect the crystal proteins from proteolysis (Adams et al., 1989; Visick and Whiteley, 1991).

In another case, a downstream, positive regulatory DNA element present in *B.thuringiensis* serves to stabilize mRNA in *E.coli* and *B.subtilis*. This has been localized to an 89bp sequence that functions in either orientation, relative to the direction of transcription (Wong and Chang, 1985). This "retroregulator" sequence has been proposed to lead to the formation of a stem-loop structure at the 3' end of the mRNA, that protects the mRNA from exonucleolytic degradation (Wong and Chang, 1986).

The *B.thuringiensis* subspecies *kurstaki* *cryI A(a)* gene has been shown to have two promoters 16bp apart, BtI and BtII, which are activated early in sporulation ( $t_1$  to  $t_2$ ) and midsporulation ( $t_4$  to  $t_5$ ), respectively, where  $t_n$  represents hours after the Bacillus cell has gone into sporulation (Wong et al., 1983). Brown and Whiteley (1990), have isolated a novel sigma ( $\sigma$ ) subunit of 28KDa required for expression of *cryIA(a)* genes from the BtII (midsporulation) transcription start site in *B.thuringiensis*. In contrast, the transcription start site at BtI requires the presence of a 35KDa  $\sigma$  subunit associated with the RNA polymerase (Brown and Whiteley, 1988). The genes encoding the  $\sigma_{35}$  and the  $\sigma_{28}$  subunits have recently been cloned, sequenced, and shown to resemble the  $\sigma_E$  and  $\sigma_K$  subunits of *B.subtilis*, respectively (Adams et al., 1991). The *cryIA(a)* gene has been shown to be negatively regulated in *E.coli* by a region between -87 to -258 relative to the transcription start point of BtI, although this negative

regulator does not function in *B.subtilis* (Schnepf et al., 1987). It is important to add the comment that most of these discoveries of positive and negative regulators have been serendipitous, and no concerted effort has been undertaken to identify such regions in relation to crystal biogenesis in Bacilli.

Conjugation experiments between *B.thuringiensis* and *B.cereus* conducted by Minnich and Aronson (1984), have indicated a role for certain cryptic plasmids in crystal protein synthesis in *B.thuringiensis* subspecies *kurstaki* HD-1. The recipient *B.cereus* strains in the experiments could only synthesize crystals similar to those found in *B.thuringiensis* subspecies *kurstaki* HD-1, when plasmids of 110, 50 and 4.9MDa were present in the cell, in addition to the 44MDa plasmid containing the cryIA crystal protein gene. The role of these regulatory plasmids has not been elucidated.

Very little is known about toxin gene regulation in *B.sphaericus*. Electron microscopy studies by Kalfon et al. (1984) have established that crystal accumulation in *B.sphaericus* 2362 is first detected at stage II of sporulation, and is completed by stage V-VI. The principal increase in toxicity of the cell to *Culex* mosquito larvae occurs at stage IV of sporulation (Kalfon et al., 1984). Crystal accumulation in *B.sphaericus* 2297 was detected earlier than in *B.thuringiensis* in such morphological studies (Yousten and Davidson, 1982; Kalfon et al., 1984). Broadwell and Baumann (1986) also found that crystal biosynthesis was an early event in *B.sphaericus* relative to *B.thuringiensis*, where crystal formation is related to spore formation (Schnepf and Whiteley, 1985).

As indicated above, no studies have been reported which investigate the transcriptional regulation of *B.sphaericus* toxin genes. Hindley and Berry (1987) identified regions upstream of the coding sequence for the 41.9KDa protein cloned from *B.sphaericus* 1593, that had homology to negative regulatory elements upstream of crystal gene sequences from *B.thuringiensis* subspecies *kurstaki* that controlled crystal gene expression in *E.coli*. The involvement of these sequences in controlling the expression of the 41.9KDa protein in

*B.sphaericus* or *E.coli* have not been analyzed.

#### 1.6. Mode of action of the crystal related toxins.

The general physiopathological symptoms associated with the ingestion by a susceptible larva of a *B.thuringiensis* crystal involves in the first instance the conversion of the protoxin into the toxin by proteolysis. This activated toxin causes swelling of the gut epithelial cells followed by their lysis, cessation of feeding by the larva, paralysis and death (Hofte and Whiteley, 1989). Similar swelling and lysis is seen when gut epithelial cells from susceptible organisms in culture are incubated with *in vitro* activated toxin (Thomas and Ellar, 1983a; Murphy *et al.*, 1976). Intoxication can be a very rapid process. Treated cells *in vitro* show abnormalities within one minute of toxin addition (Schnepf and Whiteley, 1985).

The detailed mode of action of the *B.thuringiensis* toxin has been studied using cell cultures. An overview of the mechanism is presented below.

The 27KDa CytA toxin of *B.thuringiensis* subspecies *israelensis* and an immunologically distinct 28KDa peptide in *B.thuringiensis* subspecies *darmstadiensis* 73-E10-2, are cytolytic and haemolytic for a large number of eukaryotic cells (Thomas and Ellar, 1983b). These toxins have been shown to bind strongly to unsaturated fatty acids containing dipolar ionic phospholipid headgroups such as phosphatidyl choline and phosphatidyl ethanolamine (Drobniewski and Ellar, 1988; Drobniewski and Ellar, 1989; Ellar *et al.*, 1985). The CytA protein exerts its toxic effect by binding to such lipids in the membrane. Following binding of the toxins to the membrane, they promote the formation of pores of 0.6-1.0nm radius, causing colloid-osmotic lysis of the cell (Knowles and Ellar, 1987; Drobniewski and Ellar, 1988). In contrast to mammalian and insect membranes, bacterial membranes contain few unsaturated fatty acids and are not therefore bound by the CytA toxin (Ellar *et al.*, 1985; Drobniewski and Ellar, 1989). Recently, the *cytA* gene of *B.thuringiensis* subspecies *israelensis* strain was replaced by *in vivo* recombination with a gene encoding erythromycin resistance. The resultant

*B.thuringiensis* strain did not show alteration of specificity or toxicity, leading to the conclusion that this toxin is not essential for pathogenesis (Delecluse et al., 1991)

In contrast to the CytA toxin which does not have a defined species specificity, most crystal protein toxins produced by *B.thuringiensis* have a very well defined specificity, and the molecular basis of the specificity is now under investigation. The factors that could influence such specificity are the presence of specific toxin binding sites in the membrane of different insects, or a consequence of differential processing of the protoxin to the toxic form. Although in most cases, experiments have suggested that cellular susceptibility is independent of the method of activation of the protoxin, Haider and Ellar (1987a) have shown that the *B.thuringiensis* subspecies *aizawai* protoxin can be differentially processed, with consequent variations in species specificity. Thus, the 130KDa protoxin can be converted into a 55KDa active form, toxic for lepidoptera, by the action of lepidopteran gut proteases. Moreover, this 55KDa protein has been shown by Western blotting analysis to specifically bind lepidopteran gut membrane proteins of 120 and 68KDa (Haider and Ellar, 1987b). On the other hand, the action of *Aedes aegypti* (blackfly) proteases releases a 53KDa diptera-specific form from the 130KDa protoxin which binds a dipteran membrane component of 90KDa (Haider and Ellar, 1987a,b).

The activated 54KDa lepidopteran-specific toxin of *B.thuringiensis* subspecies *kurstaki*, in contrast to the CytA cytolytic toxin from *B.thuringiensis* subspecies *israelensis* and the 28KDa protein from *B.thuringiensis* subspecies *darmstadiensis*, shows no specificity for phospholipids. Toxicity of this protein for susceptible cell lines was neutralized in the presence of the sugars N-acetylgalactosamine and N-acetylneuraminic acid. Further, the incubation of susceptible cell lines with the lectins Wheat germ agglutinin and Soybean agglutinin which bind N-acetylgalactosamine, partially protected the cells from lysis. However, no insect cells so far tested have been shown to contain N-acetylneuraminic acid. These findings therefore led to the hypothesis that the sugar N-acetylgalactosamine was

present as part of a glycoconjugate receptor which might constitute the binding site of the toxin on the target cell (Knowles et al., 1984; Ellar et al., 1985). Subsequently, activated lepidopteran-specific toxin from *B.thuringiensis* subspecies *kurstaki* HD-1 was labelled with  $^{125}\text{I}$  and shown to bind a 146KDa glycoprotein on Western blots of membranes from susceptible lepidopteran cells grown in culture (Knowles and Ellar, 1986).

Pfannenstiel et al. (1987) have demonstrated the presence of 1% neutral- and 1.7% aminosugars in the *B.thuringiensis* subspecies *israelensis* crystal. These sugars are covalently bound to the crystal proteins. Periodate treatment to remove sugars from the crystal proteins, or the incubation of growing *B.thuringiensis* subspecies *israelensis* cells with tunicamycin, a glycosylase inhibitor, led to a 10-40 fold decrease in activity of the crystal proteins in bioassays to *Aedes aegypti* larvae. Independently, the inclusion of the sugar N-acetylglucosamine in the bioassays led to a 7-fold decrease in the activity of the crystal (Muthukumar and Nickerson, 1987). Based on these results, Muthukumar and Nickerson (1987) suggested that the larvicidal toxin binds to a lectin-like receptor in the larval gut which has an affinity for N-acetylglucosamine. This hypothesis is the converse of that of Knowles and Ellar (1986) and Knowles et al. (1984), who suggest that it is the crystal protein that acts as a lectin, and binds to a glycosylated receptor in the larval gut.

The mechanism by which crystal toxins act is not known. The lepidoptera-specific toxin acts very rapidly on target cells. This is in contrast to toxins like the diphtheria toxin, which shows a lag between addition and the effect on the cell. This lag is presumed to be the period taken for the toxin to cross the membrane. Thus the rapid action of the lepidoptera-specific *B.thuringiensis* toxin could imply that the target cell membrane is rapidly made leaky to small ions and larger molecules (Knowles et al., 1984).

The specificity of the *B.thuringiensis* subspecies *kurstaki* toxin has also been correlated with the presence of saturable binding sites on the brush border of susceptible cells and their absence on non-susceptible cells like the rat intestinal

brush border membrane (Hoffmann et al., 1988). Indeed, recently Van Rie et al. (1990) have demonstrated that specific sites define the host range of a toxin using *Plo dia interpunctella* (Indian meal worm), selected for resistance to the *B.thuringiensis* subspecies *kurstaki* crystal, which contains CryIA(b) and CryIII toxins. These *P.interpunctella* strains were also resistant to *B.thuringiensis* subspecies *berliner* 1715, whose crystals contain the CryIA(b) toxin, but were susceptible to *B.thuringiensis* subspecies *entomocidus* HD110, whose crystals contain a CryIC toxin. Thus, a mutation in *P.interpunctella* strains was correlated with the apparent loss in affinity of a binding site for a particular toxin.

Biochemical studies by Sacchi et al. (1986) on isolated brush border membrane vesicles of *Pieris brassicae*, showed that activated toxin from *B.thuringiensis* subspecies *kurstaki* HD-1 and subspecies *thuringiensis* 4412, dissipated the potassium ion gradient by the formation of pores in the membrane (cited in Hofte and Whiteley, 1989). These pores did not appear to inhibit the sodium ion gradient. On the basis of this observation, the pores were concluded to be potassium ion specific. Furthermore, English and Cantley (1986) showed that the addition of these activated toxins at high concentrations (100µg/mL), led to the inhibition of a K<sup>+</sup>-adenosine triphosphatase from insect cells, human erythrocytes and dog kidney. However, the requirement for such high concentrations of toxin make it unlikely that this mechanism plays an important role in insect toxicity (Hofte and Whiteley, 1989).

In contrast to the amount of work devoted to the mode of action of the *B.thuringiensis* toxins, comparatively little work has been carried out on the action of *B.sphaericus* toxins. Davidson et al. (1987) used fluorescein-labelled 43KDa toxin from *B.sphaericus* 2362 to show that the toxin bound strongly to susceptible cells of *Culex quinquefasciatus* *in vitro*, and less strongly to a resistant clone of the *C.quinquefasciatus* cell line. Following binding to the cell membrane, the toxin was internalized in about 30min. Intoxication of the cell lines could be rescued by trypsin treatment up to 15min after the addition of the toxin, demonstrating that the toxin-receptor complex was accessible

to enzymes for a period of time. Incubation of the toxin with lipids extracted from insect and human cells did not affect its binding to cells in culture (Davidson et al., 1987).

In a manner similar to that found for *B.thuringiensis* toxins, prior incubation of the *B.sphaericus* 2362 toxin with the sugar N-acetyl-D-glucosamine and to a lesser extent with N-acetyl-D-galactosamine, decreased toxin binding to susceptible cells (Davidson et al., 1987). Incubation of the cells prior to addition of toxin with Wheat Germ Agglutinin or Concanavalin A, also inhibited toxicity. Thus, Davidson et al. (1987) concluded that the receptor for the *B.sphaericus* 2362 toxin appeared to be a glycoprotein, possibly with N-acetyl-D-glucosamine as the associated sugar.

#### 1.7. Perspectives.

The future of bioinsecticides is promising, not only because of the spectacular success exhibited by commercial preparations in controlling various pests, but because of the potential that genetic engineering holds for the manipulation of the toxin genes to suit particular requirements.

It has been described earlier that most biological agents have clearly defined insecticidal spectra. Analysis of individual genes in the larvicidal bacterium have been successful in defining the toxin that causes the death of a particular organism. Given this knowledge, and knowledge of the regions of the protein determining toxicity and specificity, manipulation of the genes in the pathogenic bacteria to alter or increase the insect host range is possible. A more elementary approach has already been used to clone the 130KDa toxin gene of *B.thuringiensis* subspecies *israelensis*, specifying toxicity to *Aedes aegypti* (blackfly) larvae into the strain *B.sphaericus*. The latter strain persists longer in the environment than *B.thuringiensis*, but is normally poorly toxic to *A.aegypti* larvae. Interestingly the recombinant *B.sphaericus* strain was now highly toxic for *Aedes*, *Culex* and *Anopheles* larvae (Trisrisook et al., 1990).

Other approaches to increase persistence of the toxin proteins in the larval feeding zone have included the cloning

of the toxin genes of *B.sphaericus* 1593M into the aquatic organism *Anacystis nidulans* R2 (deMarsac et al., 1987) and into *Azotobacter vinelandii* (A. Souza, V. Rajan, K. Jayaraman and H. R. Das, unpublished results). Both recombinant organisms expressed the toxin and were larvicidal to mosquitoes.

More elegant procedures have succeeded in the transfer of *B.thuringiensis* toxin genes, using *Agrobacterium* Ti plasmids, to plants that are devastated by agricultural pests. Tomato (Delannay et al., 1989), tobacco (Vaeck et al., 1987) and cotton (Perlak et al., 1990) plants have thus been obtained expressing *B.thuringiensis* crystal proteins. The expression of the genes in the plants causes *in vivo* resistance of the plants to the attacking pest. This is a significant step forward in the protection of plants. So far, the plants that have been protected are dicotyledons that are amenable to transformation by the Ti plasmid. Approaches are now being sought for the transfer of the crystal genes of *B.thuringiensis* into monocotyledonous plants by other methods. This synthesis of approaches could result in the production of plants specifying toxicity to pathogenic insects.

#### 1.8. Aims of this project.

The 8.6Kb DNA fragment of *B.sphaericus* recombined into the vector pBR322 was earlier identified as conferring mosquito larvicidal activity upon the host *E.coli* cell. This insert was later subcloned to a 3.6Kb fragment that still conferred toxicity on recombinant *E.coli* cells (Souza, 1987). The specific protein responsible for the larval toxicity of this fragment was not known. It was therefore important to identify the toxin that was responsible for the larvicidal activity. The precise relationship of specific toxin proteins to the crystal of any *B.sphaericus* strain was also obscure at the outset of this project. Several groups had identified proteins of different sizes isolated from *B.sphaericus* that were reported to be responsible for larvicidal activity (Baumann et al., 1985; Davidson, 1982; Tinelli and Bourguin, 1982; Davidson, 1983). It was also suggested that the lower molecular weight forms in the crystal (43 and 63KDa proteins) were breakdown products of higher molecular weight proteins

(Baumann *et al.*, 1985). Our aim was to identify the toxin protein encoded by the 3.6Kb fragment cloned from *B.sphaericus* 1593M and to demonstrate unequivocally its relationship to the high molecular weight proteins reported to be present in the *B.sphaericus* crystal.

The sequence of a 41.9KDa crystal protein, cloned from *B.sphaericus* 1593 and apparently associated with the crystal, was reported in the early stages of this study (Hindley and Berry, 1987). In the light of this, no attempts were made to sequence the gene encoding the 41KDa protein in this study, but the sequence reported by Hindley and Berry (1987) was used as the basis for a number of methods to investigate attempts to obtain sufficient amounts of this protein to use as antigen. The antibodies would be used for subsequent studies of the regulation or mode of action of the 41KDa protein. These methods included the use of the 23KDa C-terminal signal region of the secreted *E.coli* protein haemolysin fused to the 41KDa protein, to promote the secretion of the protein from *E.coli* cells. Much less was known about other potential *B.sphaericus* toxins and another aim of this project was to identify other possible toxins produced by *E.coli*, and in particular to obtain the sequence of the gene identified as encoding the 59KDa protein. Attempts would also be made to raise antibodies to the 59KDa protein in order to investigate the relationship of this protein also with the crystal.

The regulation of crystal protein production from *B.sphaericus* has received little attention and another objective of my study was to construct  $\beta$ -galactosidase fusions to toxin gene promoters in order to follow expression of these genes in *E.coli* and *Bacillus subtilis*. Finally, a preliminary analysis of the transcriptional regulation of both *B.sphaericus* toxin genes as a function of growth phase would also be carried out.

## Chapter 2.

### Materials and Methods.

#### 2.1. Genetical and molecular methods associated with Bacillus strains.

##### 2.1.1. Strains.

*Bacillus sphaericus* strains 1593, 2362, 2297, 14577, 2013-4 and SSII-1 were obtained from Prof. H. de Barjac, WHO Collaborative Research Centre on Entomopathogenic Bacilli, Institut Pasteur, 28, rue du Dr. Roux, Paris Cedex 15, France (see below).

*Bacillus sphaericus* 1593M is a natural isolate of obtained by passage of strain 1593 through mosquito larvae in Madurai, India. Strain 1593M is identical to the classical strain 1593, but has increased larvicidal activity for *Culex tritaenirhyncus* ( $1-2 \times 10^{-3} \mu\text{g/mL}$  compared to *B.sphaericus* 1593:  $1-5 \times 10^{-3} \mu\text{g/mL}$ ; Ganesan et al., 1983; Louis et al., 1984; Jamuna et al., 1982).

*Bacillus subtilis* BR151 (*trpC2*, *metB10*, *lys3*: Young et al., 1969) was a gift from Mr. Paul Everest.

<u>Strain</u>	<u>Toxicity</u>	<u>Crystal</u>	<u>Sporulation</u>
<i>B.sphaericus</i> 1593	+	+	+
<i>B.sphaericus</i> 2362	+	+	+
<i>B.sphaericus</i> 2297	+	+	+
<i>B.sphaericus</i> 2013-4	+	+	+
<i>B.sphaericus</i> SSII-1	+	-	+
<i>B.sphaericus</i> 14577	-	-	+
<i>B.subtilis</i> BR151	-	-	+

##### 2.1.2. Media, growth and storage.

All media and solutions in this and other sections were sterilized by autoclaving at 15psi for 15min unless otherwise indicated. All *Bacillus* strains were grown with aeration in either Luria broth (see below), or in MSB sporulation medium (Kalfon et al., 1984) at 30°C or 37°C. For analysis of the *lac* phenotype of *B.subtilis*, the cells were plated on McConkey agar (obtained from Oxoid and prepared according to

manufacturer's instructions).

Storage for short term use was on Luria agar plates (Luria broth autoclaved with 1.5% (w/v) agar) or in stabs of 1.5% (w/v) Luria agar. Longer term storage was in Luria broth containing 20% (v/v) glycerol at -80°C. Antibiotics were added as required. Purified spores were stored at -20°C. Inoculation of *B.sphaericus* and *B.subtilis* was carried out by subjecting the spores resuspended in 5mL broth to heat shock at 78°C for 10min (Kalfon et al., 1984), and diluting into the growth medium.

Luria broth:

Tryptone	1% (w/v)
Yeast extract	0.5% (w/v)
NaCl	0.5% (w/v)
pH 7.0	

MSB Sporulation medium:

Tryptose	1% (w/v)
Yeast extract	0.2% (w/v)
CaCl <sub>2</sub>	0.02% (w/v)
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.002% (w/v)
Fe(SO <sub>4</sub> ) <sub>3</sub>	0.002% (w/v)
MnSO <sub>4</sub>	0.002% (w/v)
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.03% (w/v)
pH 7.4	

2.1.2.1. Antibiotics.

Chloramphenicol (dissolved at 25mg/mL in 100% (v/v) ethanol) was used at a final concentration of 5µg/mL.

2.1.3. DNA isolation from Bacillus.

2.1.3.1. Chromosomal DNA extraction from Bacillus.

An overnight culture was inoculated into 100mL Luria broth to an A<sub>600</sub>=0.05. The cells were grown with shaking at 30°C and harvested at an A<sub>600</sub>=1.0 and resuspended in 1.5-2mL TEN (10mM Tris-HCl pH 8.0; 100mM NaCl; 1mM EDTA) containing 20% (w/v) sucrose and 10mg/mL freshly added lysozyme. The cells were incubated with gentle shaking at 37°C for 2 hours. The

cells were then freeze-thawed several times by transfer from room temperature to  $-70^{\circ}\text{C}$ . Sodium sarkosyl was added to 1% (w/v) and the cells were gently stirred to promote lysis. CsCl was added to a concentration of 1g/mL and ethidium bromide (EtBr) to a final concentration of  $250\mu\text{g/mL}$ .

The cell extracts were then distributed into polypropylene ultracentrifuge tubes which were balanced, sealed and centrifuged overnight at  $20^{\circ}\text{C}$  and 45Krpm in a Beckman L5-65 model ultracentrifuge with a VTi65 rotor. The chromosomal DNA was recovered by withdrawing the relevant band under longwave ultraviolet illumination with a large bore (16G) needle attached to a 1mL syringe. The chromosomal DNA was gently extracted with CsCl-saturated isobutanol to remove EtBr and then precipitated with isopropanol in the ratio DNA: distilled water: isopropanol = 0.4: 0.5: 0.54. The DNA was subjected to electrophoresis on an agarose gel to determine purity and molecular weight.

#### 2.1.3.2. Plasmid DNA isolation from *B.subtilis*. (Birnboim and Doly, 1979)

The cells from which plasmid DNA was to be isolated were grown overnight in Luria broth containing  $5\mu\text{g/mL}$  chloramphenicol. Cells were pelleted in an eppendorf tube from 1.5mL of medium by centrifugation for 5min at room temperature at 13Krpm. The cells were incubated for 30min at  $37^{\circ}\text{C}$  in  $100\mu\text{L}$  TEG (50mM glucose; 25mM Tris-HCl; 1mM EDTA, pH 8.0) containing 10mg/mL freshly added lysozyme. The cells were lysed by the addition of  $200\mu\text{L}$  alkaline SDS (1% (w/v) SDS in 0.2M NaOH) and gentle mixing. The cells were placed on ice for 5min, and  $150\mu\text{L}$  of 3M sodium acetate, pH 5.2 was added and mixed well. The supernatant was recovered after centrifugation of the mixture for 10min at room temperature at 13Krpm. The DNA in the supernatant was recovered by ethanol precipitation (Section 2.3.4).

#### 2.1.4. DNA mediated transformation of *B.subtilis*.

##### 2.1.4.1. Preparation of competent *B.subtilis* cells for transformation. (Hardy, 1985)

*B.subtilis* BR151 was grown overnight at 30°C in 4mL of Subtilis Minimal Medium (SMMI; see below) with aeration, and 1.5mL of this culture was diluted into 40mL of SMMI in a 500mL flask, and grown at 37°C with vigorous aeration. The A<sub>450</sub> was monitored and when it was between 1.7-1.8, 20mL of this culture was diluted with an equal volume of pre-warmed Subtilis Minimal Salts (SMS; see below) containing 0.5% (w/v) glucose. Growth was continued for a further 90min and at this stage the cells were "competent".

A volume of 0.9mL of the cells was mixed in a tube with the DNA to be transformed and the mixture was shaken gently at 37°C for 60min. To allow expression of antibiotic resistance to occur, 4mL of pre-warmed Luria broth was then added to the mixture, and incubated for a further 90min at 37°C with vigorous shaking. The culture was plated out on selective medium at 37°C overnight.

##### Subtilis Minimal Salts (SMS):

(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.8% (w/v)
K <sub>2</sub> HPO <sub>4</sub>	5.6% (w/v)
Trisodium citrate	0.4% (w/v)
KH <sub>2</sub> PO <sub>4</sub>	2.4% (w/v)
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.08% (w/v)

##### Subtilis Minimal Medium (SMMI):

4X SMS	100mL
20% (w/v) glucose	10mL
0.8% (w/v) casein hydrolysate	10mL
Sterile distilled water to 400mL	

Each ingredient was autoclaved separately, and mixed aseptically.

##### 2.1.4.2. Preparation of *B.subtilis* cells for transformation by electroporation. (Bone and Ellar, 1989)

*B.subtilis* cells were harvested at mid-exponential stage of growth and cooled on ice for 10min. The cells were

harvested by centrifugation in a refrigerated tabletop centrifuge at 2.5Krpm for 15min. The cells were washed twice in equal volumes of ice-cold sterile distilled water and then washed once in HEPES-glycerol (HG) electroporation buffer (1mM HEPES, pH 7.0; 10% (v/v) glycerol), and resuspended to  $10^9$  cells/mL in HG buffer. For electroporation, 100 $\mu$ L of cells in buffer was mixed with 100ng DNA and transferred to an electroporation cuvette. Electroporation was carried out at 25 $\mu$ F capacitance, 2.5KV and 200  $\Omega$  resistance. 1mL of prewarmed Luria broth was then added to the cells, and incubated at 37 $^{\circ}$ C for 1 hour. Aliquots of the cells were plated out on selective medium.

#### 2.1.5. Preparation of crystal from *B.sphaericus* strains.

Two methods of crystal preparation were used.

##### 2.1.5.1. Crystal preparation using NaBr density gradient centrifugation. (Baumann et al., 1985)

*Bacillus sphaericus* cells were grown to sporulation in Luria broth. At this stage crystal formation is complete (Baumann et al., 1985). The cells were pelleted by centrifugation (6Krpm, 4 $^{\circ}$ C, 20min) in a GS3 rotor. The cells were then sonicated to break open the exosporeum, or alternatively, the cells were ruptured in a French pressure cell by one passage at a setting of 1200psi. Lysis of the cells was monitored under a microscope. The cell lysates were layered on a 48% (w/v) NaBr gradient in water, and centrifuged for 4 hours at 5000xg at 4 $^{\circ}$ C in a swing out HB-4 rotor. The layers banding at different densities obtained after centrifugation, visible through the polysulfone tubes, were drawn off with a Pasteur pipette, pelleted, and analysed under a microscope for the presence of crystals. These are visible as small phase-dark bodies, quite distinct from the large phase-bright spores under a phase-contrast microscope. Fractions judged to be rich in crystals were pooled and recentrifuged as many times as necessary to obtain a homogeneous preparation as judged microscopically. After the final spin the crystals were washed twice with sterile distilled water, pelleted as above and stored in distilled water at -20 $^{\circ}$ C. Proteins in the crystal were analysed on SDS

polyacrylamide gels (Section 2.4.5).

2.1.5.2. Crystal purification by discontinuous sucrose density gradient centrifugation. (Haider and Ellar, 1987a)

The cell pellet from sporulated cultures was obtained as described above (Section 2.1.5.1). The pellets were resuspended in 50mM Tris-HCl pH 7.5; 10mM KCl and subjected to sonication to rupture the exosporeum. The lysates were layered on top of a discontinuous sucrose density gradient (87, 82, 79, 67, 56 and 45% (w/v) sucrose in 50mM Tris-HCl pH 7.5; 10mM KCl). The gradient was centrifuged for 4 hours at 5Krpm at 4°C in an HB-4 swing out rotor. The layers banding at different densities were withdrawn, and analysed microscopically for the presence of crystal. Crystal-rich fractions were pooled and recentrifuged as many times as necessary to obtain a microscopically homogeneous preparation. The crystals were washed twice in sterile distilled water as above, and stored at -20°C. Crystal proteins were analysed on SDS polyacrylamide gels (Section 2.4.5).

2.1.6. Preparation of spores from *B.sphaericus*.

Sporulated cells were grown as described in Section 2.1.5.1. The cells were sonicated to rupture the exosporeum and centrifuged on discontinuous sucrose density gradients as described in Section 2.1.5.2. The fractions with different densities banding on the gradient were withdrawn and analysed under a phase contrast microscope for the presence of refractile spores. Spore-rich fractions were pooled and recentrifuged on gradients as many times as required to obtain microscopically homogeneous spore preparations. The spores were collected by centrifugation, washed twice with sterile distilled water and resuspended in sterile distilled water. A spore count was then performed with a haemocytometer, and this count was confirmed by a viable spore count. This was done by heating an aliquot of spores to 78°C for 10min to kill any non-sporulated cells and to activate dormant spores, and plating dilutions of the suspension on 1.5% (w/v) Luria agar plates. The plates were incubated overnight at 30°C, and a colony count established the number of viable spores in the

preparation. Spores were stored at  $-20^{\circ}\text{C}$ .

## 2.2. Genetical and molecular methods associated with *Escherichia coli*.

### 2.2.1. Strains.

MC4100	K12: $F^{-}$ , $mal^{+}$ , $\lambda^{-}$ , $thi^{-}$ , $araD139$ , $\Delta(lac$ $IPOZYA)$ , U169, $rpsL$ , $relA$
TG1	K12: $\Delta(lac-pro)$ , $supE$ , $thi^{-}$ , $hsd\Delta5$ /[ $F'$ traD36, $proA^{+}B^{+}$ , $lacI^{Q}$ , $lacZ\Delta M15$ ]
DS410"T"	K12: $F^{-}$ , $minA$ , $minB$ , $thi^{-}$ , $ara^{-}$ , $gal^{-}$ , $xyl^{-}$ , $mtl^{-}$ , $tonA$ , $rpsL$
BL21-DE3	B: $hsdS$ , $gal(\lambda, cI_{857}, ind1, Sam7, nin5,$ $lacUV5-T7gene\ 1)$
JM101	K12: $supE$ , $thi^{-}$ , $\Delta(lac-proAB)$ /[ $F'$ , traD36, $proA^{+}B^{+}$ , $lacI^{Q}$ , $lacZ\Delta M15$ ]

### 2.2.2. Media, growth and storage.

All *E.coli* strains were grown in Luria broth (Section 2.1.2.), Minimal medium or Terrific broth (see below). To make plates or stabs, 1.5% (w/v) agar was autoclaved with the broth. In the case of Minimal medium plates, 100mL of Minimal medium was added to 400mL sterile warm Water agar (1.5% (w/v) agar in pH 8.5 water).

#### Minimal Medium:

To 100mL sterile distilled water was added:

10X MS salts (see below) 10mL

Glucose (20% (w/v)) 5mL

CM salts (see below) 1mL

1% (w/v) thiamin (filter sterilized) 0.1mL

10X MS salts: (boiled to dissolve, in the order) 6% (w/v)

$\text{Na}_2\text{HPO}_4$  (anhydrous); 3% (w/v)  $\text{KH}_2\text{PO}_4$ ; 0.5% (w/v) NaCl; 1% (w/v)  $\text{NH}_4\text{Cl}$ .

CM salts: 0.01M  $\text{CaCl}_2$ ; 0.1M  $\text{MgSO}_4$ .

### Terrific broth:

KH <sub>2</sub> PO <sub>4</sub>	2.314g
K <sub>2</sub> HPO <sub>4</sub>	16.43g
Tryptone	12.0g
Yeast extract	24.0g
Glycerol	4.0mL
Distilled water to 1L.	

For short term storage, *E.coli* strains were streaked out on Luria agar plates containing the appropriate antibiotics, grown and stored at 4°C. Longer term storage involved stabbing the cultures into 1.5mL cryotubes containing Luria agar (Luria broth with 1.5% (w/v) agar) with antibiotics and maintaining at room temperature, or by freezing in 20% (v/v) glycerol (overnight cultures made to 20% (v/v) glycerol with sterile 50% (v/v) glycerol, aliquoted into cryotubes and frozen at -70°C).

#### 2.2.2.1. Antibiotics.

Antibiotics were added to the following concentrations:

<u>Antibiotic</u>	<u>Stock</u>	<u>Final conc.</u>
Ampicillin	40mg/mL in water	100µg/mL
Chloramphenicol	10mg/mL in 100% (v/v) ethanol	25µg/mL
Kanamycin	20mg/mL in water	50µg/mL
Tetracycline	5mg/mL in 50% (v/v) ethanol	15µg/mL
Rifampicin	10mg/mL in 1:1 0.1M K <sub>2</sub> CO <sub>3</sub> :100% (v/v) ethanol	25µg/mL

Stocks were stored at 4°C, except rifampicin, which was stored at -20°C.

#### 2.2.3. Transformation of *E.coli*.

Three methods were used. The choice of method depended on the efficiency of transformation desired.

##### 2.2.3.1. Rapid calcium chloride transformation.

The strain to be transformed was scraped off a fairly fresh (not more than one week old) plate with a sterile toothpick and resuspended in 200µL sterile ice-cold 100mM CaCl<sub>2</sub> and incubated on ice for 5min. Plasmid DNA (0.1 to 0.5µg) was

added and the suspension mixed and placed on ice for 40min. The cells were then subjected to heat shock at 42°C for 2min and 1mL Luria broth, prewarmed to 37°C was added. The cells were placed at 37°C for 1 hour to recover and to allow expression, and then plated out on selective medium. The efficiency of transformation by this method was low, usually  $10^2$ - $10^3$  transformants/ $\mu$ g DNA.

#### 2.2.3.2. Rubidium chloride transformation. (Hanahan, 1985)

An overnight culture of cells was used to inoculate 100mL of Luria broth to an  $A_{600}$  of 0.05. The cells were grown with shaking at 37°C until the  $A_{600}$  was 0.4-0.5. The cells were then subjected to cold shock by swirling them in an ice-water bath for 10min and centrifuged to pellet the cells. This was done in 50mL Falcon tubes in a refrigerated tabletop centrifuge at 2.5Krpm for 10min at 4°C. The pellet was resuspended in 2/5 volume filter sterilized TFBI (30mM potassium acetate; 100mM RbCl<sub>2</sub>; 10mM CaCl<sub>2</sub>; 50mM MnCl<sub>2</sub>; 15% (v/v) glycerol, pH to 5.8 with 0.2M acetic acid). The cells were placed on ice for 10min and then pelleted as before. They were then resuspended in 1/25 volume of filter sterilized TFBII (10mM MOPS/PIPES; 75mM CaCl<sub>2</sub>; 10mM RbCl<sub>2</sub>; 15% (v/v) glycerol, pH to 6.5 with KOH). After 10min on ice, the cells were aliquoted into eppendorf tubes (200 $\mu$ L/tube), snap frozen in a dry ice-methanol bath, and stored at -70°C until required.

For transformation, the cells were thawed on ice, the DNA (1-50ng) added, mixed, incubated on ice for 40min and then subjected to heat shock at 42°C for 2min. The cells were then incubated at 37°C with 1mL Luria broth for 1 hour. Aliquots of cells were then spread plated on selective medium. 1ng of pBR322 DNA was included as control. The frequency of transformation was  $10^5$ - $10^6$ / $\mu$ g DNA.

#### 2.2.3.3. Transformation by electroporation. (Dower et al., 1988)

100mL of Luria broth was inoculated to an initial  $A_{600}$ =0.05, and grown at 30°C or 37°C to an  $A_{600}$ =0.6-1.0. The cells were shocked by chilling in an ice-water bath, and left

for 10min. The cells were then centrifuged in sterile Falcon tubes at 4°C, 2.5Krpm, 20min. The pellet was resuspended in an equal volume of ice-cold sterile distilled water, left on ice for 10min and centrifuged as before. Subsequent washes were in 1/2 volume ice-cold distilled water, 1/50 volume ice-cold 20% (v/v) glycerol, and the cells were finally resuspended in 1/500 volume 20% (v/v) glycerol. At this stage, the cells were dispensed in 50µL aliquots into ependorf tubes, snap frozen in a dry ice-methanol bath and stored until use at -70°C.

When required, the cells (50µL) were thawed on ice and upto 50ng salt-free DNA added in a 5µL volume, and mixed gently with a pipette. The cells were then transferred to sterile electroporation cuvettes. A known concentration of pBR322 was included as a separate control to check the transformation frequency. The cells were subjected to a potential of 1.5KV, 25µF capacitance, 1000 Ω resistance in a Bio-Rad Gene-Pulser machine. 1mL of prewarmed SOC (2% (w/v) tryptone; 0.5% (w/v) yeast extract; 10mM NaCl; 2.5mM KCl; 20mM MgCl<sub>2</sub>/MgSO<sub>4</sub>; 20mM glucose) was added, the cells transferred to 15mL glass tubes and shaken vigourously for 1 hour at 30°C or 37°C. Aliquots were spread plated on selective medium for transformants. The transformation frequency was 10<sup>6</sup>-10<sup>7</sup>/µg DNA.

### 2.3. Methods associated with DNA.

#### 2.3.1. Plasmids and phage.

The following plasmids and phage were used:

<u>Plasmids</u>	<u>Reference</u>
pBR322	Bolivar <i>et al.</i> (1977)
pAS233HA	Souza <i>et al.</i> (1988)
pLKC480 series	Tiedeman and Smith (1988)
pJLA504	Schauder <i>et al.</i> (1987)
pLG609 series	Holland <i>et al.</i> (1990)
pLG800-1	Kenny, (1990)
pET3 series	Rosenberg <i>et al.</i> (1987)
p14B1	Bott <i>et al.</i> (1981)
pHV33	Primrose and Ehrlich (1981)

<u>Phage</u>	<u>Reference</u>
M13mp18	Yanisch-Perron et al. (1985)
$\lambda$ rif <sup>d</sup> 18	Kirschbaum and Konrad (1973)

Plasmid pLG800-1 was obtained from Dr. B. Kenny, University of Leicester, pHV33 was a gift from I. Courtney, University of Oxford, pLG609 series was obtained from R. Haigh, University of Leicester, pl4B1 was a gift from Dr. S. Seror, University Paris-Sud, Orsay, France,  $\lambda$ rif<sup>d</sup>18 was obtained from L. Jones, University of Leicester, pLKC480 series was obtained from Dr. J. Smith, Seattle Biomedical Research Institute, Seattle, USA and the pET3 series was obtained from Dr. W. Studier, Brookhaven National Laboratory, Upton, New York, USA.

### 2.3.2. Small scale plasmid isolation by the minipreparation method. (Birnboim and Doly, 1979)

A culture containing plasmid was grown overnight in 2mL of Luria broth containing the appropriate antibiotics. The culture was decanted into sterile 1.5mL eppendorf tubes, centrifuged for 1min at 13Krpm in an MSE microcentrifuge to pellet the cells, and the supernatant withdrawn completely. The cell pellet was resuspended in 100 $\mu$ L sterile TEG (50mM glucose; 25mM Tris-HCl; 1mM EDTA, pH 8.0). The tubes were placed on ice and 200 $\mu$ L of freshly made alkaline SDS (1% (w/v) SDS in 0.2M NaOH) added. The tubes were incubated on ice for no more than 5min and 150 $\mu$ L of 3M sodium acetate, pH 5.2 added. The tubes were vortexed and incubated at -20°C for 30min. The precipitate was pelleted at room temperature by centrifuging the contents of the tube at room temperature for 10min at 13Krpm. The supernatant was decanted into a fresh tube and the DNA precipitated by the addition of 2 volumes of ethanol at -70°C for 1 hour. The DNA was recovered by centrifugation at room temperature for 10min at 13Krpm, washed in 70% (v/v) ethanol in water, vacuum dried and resuspended in 50 $\mu$ L sterile distilled water. A 5 $\mu$ L aliquot of this DNA solution was sufficient for restriction enzyme digestion analysis.

An RNase step was often included between the acid denaturation and ethanol precipitation steps. DNase-free RNase (prepared by heating a 10mg/mL solution of RNase in 10mM

Tris-Cl, pH 7.5; 15mM NaCl at 100°C for 15min and cooling slowly to room temperature) was added to 250µg/mL to the decanted supernatant and incubated at 20min at 37°C. This was followed by phenol-chloroform extraction (Section 2.3.5.), ethanol precipitation (Section 2.3.4.), 70% (v/v) ethanol wash and vacuum drying.

### 2.3.3. Large scale plasmid DNA extraction and CsCl purification. (Maniatis et al., 1982)

Plasmid harbouring strain was grown overnight in 200mL of Terrific or Luria broth containing the appropriate antibiotics. The culture was centrifuged to pellet the cells at 6Krpm, for 15min at 4°C in a GS3 rotor. The supernatant was thoroughly removed after a second spin. The cells were resuspended in 5mL TEG (Section 2.3.2.). Two volumes of freshly made alkaline SDS (Section 2.3.2.) were added and mixed gently. The tubes were placed on ice for 5min and then 1.5 volumes of 3M sodium acetate, pH 5.2 was added. The tube contents were mixed vigorously and the precipitate pelleted as before. The nucleic acid in the supernatant was precipitated with ethanol (an alternative precipitation step using an equal volume of propan-2-ol was often employed), the precipitate was recovered by centrifugation, washed with 70% (v/v) ethanol, and vacuum dried. The DNA precipitate was resuspended in a solution of 1.1g/mL CsCl in water in 20mL sterilin tubes. 50µL of a 10mg/mL solution of EtBr was added (EtBr is a proven mutagen and was handled with extreme care). The solution was placed in the dark for 20min at room temperature, and then centrifuged for 5min in a non-refridgerated table-top centrifuge at 2.5Krpm to precipitate the flocculated protein. The clear CsCl-EtBr solution was transferred using a syringe into 2mL or 4mL sealable polyallomer ultracentrifuge tubes. The tubes were sealed using a Beckman Tube Sealer, and centrifuged overnight (~16 hours) in a TL-100 Beckman Table-top Ultracentrifuge with a TL100.2 fixed angle at 100Krpm at 20°C, or in a VTi65 rotor in a Beckman L5-65 Ultracentrifuge at 55Krpm at 20°C. Before stopping the TL-100 centrifuge, the nucleic acid bands were relaxed with a 1 hour spin at 70Krpm.

The tops of the tubes were cut off with a heated scalpel,

and the plasmid DNA band was visualised under longwave ultraviolet transillumination. The DNA was withdrawn using a needle attached to a syringe and was transferred to an eppendorf tube, extracted several times with an equal volume of CsCl-saturated butan-1-ol, following which the DNA was precipitated as described in Section 2.3.4. The pellet was washed with 70% (v/v) ethanol, dried under vacuum, and resuspended in distilled water. The amounts of DNA were quantified approximately on a 1% (w/v) agarose gel against  $\lambda$  (*HindIII*) markers. The DNA was finally resuspended to 0.1 $\mu$ g/mL in distilled water and stored at -20°C.

#### 2.3.4. Ethanol precipitation of DNA. (Maniatis et al., 1982)

To the solution containing the DNA, 2 volumes of 100% (v/v) ethanol (or an equal volume of propan-2-ol) were added together with 0.1 volume of sodium acetate, pH 5.2. The solution was mixed well, and chilled at -70°C for 1 hour. The DNA was then recovered by centrifugation and the precipitate washed with 70% (v/v) ethanol to remove salt.

#### 2.3.5. Phenol-chloroform extraction of DNA. (Maniatis et al., 1982)

The solution containing the DNA was mixed well with an equal volume of phenol-chloroform (equal volumes of 24:1 chloroform:isoamyl alcohol and phenol equilibrated with 0.1M Tris-HCl, pH 8.0, and containing 0.1% (w/v) 8-hydroxyquinoline). The extraction was continued until there was no protein at the interphase. The aqueous phase was then precipitated with ethanol.

#### 2.3.6. Digestion of DNA with restriction enzymes. (Maniatis et al., 1982)

A total of 0.1 to 0.5 $\mu$ g of plasmid DNA was suspended in 19 $\mu$ L of the appropriate REact buffer (supplied as 10X concentrate by the manufacturer), and 1 $\mu$ L of the restriction enzyme (5 to 10Units, manufacturer's definition) was added and incubated at the recommended temperature for a minimum of 1 hour. If a larger quantity of DNA was to be digested, the volume for digestion was increased to 50 or 100 $\mu$ L, the quantity

of restriction enzyme doubled and the incubation time increased until digestion was complete as monitored on an agarose gel. Chromosomal DNA was digested in a similar manner and digestion monitored on an agarose gel.

2.3.7. Agarose gel electrophoresis and fragment recovery.  
(Southern, 1979)

DNA was digested with the appropriate restriction enzyme and the fragments separated by agarose gel electrophoresis. The concentration of agarose in the gels varied according to the size of the fragments expected and was usually between 0.8 and 1.2% (w/v). The gels were made by dissolving SeaKem HGT agarose in 1X TAE buffer (40mM Tris base; 5mM sodium acetate; 2mM EDTA, pH 8.0) which was also the running buffer used. The gels contained 0.5µg/mL ethidium bromide. **Ethidium Bromide is a powerful mutagen and was handled with care.** The gels were placed in horizontal agarose gel tanks with the gels submerged in the buffer. The DNA to be separated was resuspended in 1X agarose sample buffer and pipetted into slots made at the top of the gel with a slot former. λ DNA digested with *HindIII* was used as size markers. Voltage was applied across the electrodes to mobilize the DNA. When separation was sufficient as monitored by the mobility of the dye-front, the voltage was switched off and the gel removed for visualisation under longwave ultraviolet illumination on a transilluminator. Photography was performed using Kodak Tmax-100 film.

10X agarose sample buffer.

0.25M Tris-HCl, pH 6.8	10mL
100% (v/v) Glycerol	4mL
EDTA	5mM
Orange C/bromophenol blue as dye	
Distilled water to 40mL	

2.3.7.1. DNA fragment recovery using "Glass milk".  
(Vogelstein and Gillespie, 1979)

For DNA fragment recovery a slice of the agarose gel containing the DNA fragment was excised with a scalpel under long wave ultraviolet transillumination. The agarose was transferred to an eppendorf tube and dissolved by the addition

of 500 $\mu$ L saturated sodium iodide (181.4g sodium iodide dissolved in 995mL warm water; a pinch of sodium sulphite added as preservative). 30-50 $\mu$ L (depending on the amount of DNA) of glassmilk (finely ground glass powder from scintillation vials resuspended in distilled water and autoclaved) was added, mixed and the tube incubated at room temperature for 5min. The glass was recovered by centrifugation at 13Krpm in a microfuge for 5min at room temperature. The pellet was washed four times with 500 $\mu$ L of freshly made washing solution (2:1:1 of 100% (v/v) ethanol, distilled water, and 2X TNE. 2X TNE is 0.02M Tris-HCl, pH 7.2; 0.2M NaCl; 2mM EDTA). The supernatant was then withdrawn completely and the glass pellet resuspended in 30-50 $\mu$ L distilled water and incubated at 45-55 $^{\circ}$ C for 2min. The glass was removed by centrifugation as before and the supernatant, which now contained the DNA was saved, and an aliquot analysed on an agarose gel to estimate recovery and concentration. Recovery was usually 75-80%.

#### 2.3.7.2. Fragment recovery from Low Gelling Temperature agarose gels

DNA fragments were separated on Low Gelling Temperature (SeaKem, Ltd) agarose gels for use in DNA fragment labelling with  $^{32}$ P-dCTP. The agarose slice containing the DNA fragment was excised from the gel and 1.5mL of water per gram of the gel was added. The gel slice was boiled for 7min, and then transferred to 37 $^{\circ}$ C for 10min before use. If the fragment was to be used following storage at -20 $^{\circ}$ C, it was boiled for 3min, and placed at 37 $^{\circ}$ C for 10min before use. The DNA concentration in the subsequent labelling reaction was calculated assuming 100% recovery from the gel.

#### 2.3.7.3. Fragment recovery by the "Death-Wish" method. (Maniatis et al., 1982)

Electrophoresis was conducted in the dark, and the position of the desired DNA fragments monitored with a portable longwavelength ultraviolet lamp. Following electrophoresis, the gel was sliced laterally alongside the fragment to be recovered and also longitudinally from the ends of the first cut towards the bottom of the gel. A piece of

dialysis tubing boiled for 2min in TE (10mM Tris-HCl; 1mM EDTA, pH 8.0) buffer and cut 5mm wider than the lane or trough containing the DNA fragment was inserted into the lateral cut. The DNA fragment was electrophoresed onto the membrane at 100V, and the membrane was rapidly transferred into an eppendorf tube using two pairs of Millipore forceps, and without switching off the current. One corner of the membrane was trapped in the cap of the tube and the DNA collected by centrifugation for 1min in a microcentrifuge. The membrane was washed with TE and centrifuged again to recover any DNA on the membrane. The membrane was then discarded, and the DNA concentrated by ethanol precipitation (Section 2.3.4).

### 2.3.8. Filling in of recessed ends and digestion of protruding ends of DNA fragments. (Maniatis et al., 1982)

In such cases where a recessed end of DNA had to be blunt-ended for ligation, and depending on the direction of the overhang, the following methods were used.

#### 2.3.8.1. Filling in of 3' recessed ends.

The Klenow fragment of DNA Polymerase I was used to fill in the recessed end of the DNA fragment. The reaction mixture contained:

DNA (in distilled water)	17 $\mu$ L
5X ligase buffer	6 $\mu$ L (supplied by the manufacturer)
T. M. buffer	3 $\mu$ L
0.1M spermidine	1.2 $\mu$ L
0.25mM dNTP's	2 $\mu$ L
Klenow fragment	1 $\mu$ L (2 Units)
Total volume	30 $\mu$ L

The Klenow fragment was added last and the tube contents were mixed gently with a pipette. The tube was incubated for 15min at room temperature and the reaction stopped with 1 $\mu$ L of 0.5M EDTA. The DNA was extracted with phenol-chloroform and precipitated with ethanol before subsequent steps were carried out.

5X ligase buffer is 0.25M Tris-HCl, pH 7.8; 50mM magnesium chloride; 100mM DTT; 5mM ATP; 250 $\mu$ g/mL BSA.

T.M. buffer is 100mM Tris-HCl, pH 8.0; 100mM magnesium chloride. Stored at -20°C.

0.25mM dNTP's were prepared in 10mM Tris-HCl, pH 8.0; 0.1mM EDTA.

Spermidine is 0.1M in 10mM Tris-Cl, pH 8.0.

#### 2.3.8.2. Digestion of 5' recessed ends.

5' recessed ends of DNA molecules cannot be filled in with the Klenow fragment of DNA polymerase I and the 3' overhang had to be removed by polymerases with 3'-5' exonuclease activity. The enzyme used in this case was T4 DNA polymerase.

The reaction mixture contained:

DNA (in distilled water)	9 $\mu$ L
10X T4 buffer	2 $\mu$ L
0.25mM dNTP's	8 $\mu$ L
T4 DNA polymerase	1 $\mu$ L (1 Unit)

The tube contents were pipetted up and down to allow thorough mixing and incubated at 37°C for 15min. The reaction was stopped by transfer to 70°C for 5min, and the DNA was extracted with phenol-chloroform (Section 2.3.5) and precipitated with ethanol (Section 2.3.4) before further manipulations were carried out.

10X T4 buffer is 0.33M Tris-acetate, pH 7.9; 0.6M potassium acetate; 0.1M magnesium acetate; 5mM DTT; 1mg/mL BSA.

0.25mM dNTP's were prepared in 10mM Tris-HCl, pH 8.0; 0.1mM EDTA.

#### 2.3.9. Alkaline phosphatase treatment of DNA. (Maniatis et al., 1982)

The DNA was digested to completion with the relevant restriction enzyme, extracted with phenol-chloroform, precipitated with ethanol, washed with 70% (v/v) ethanol and dried before use. The DNA was resuspended in 1X CIP buffer (see below) and 1 Unit of calf intestinal phosphatase was added and mixed gently. For DNA with protruding 5' termini, the reaction mixture was incubated at 37°C for 30min. Since alkaline phosphatase has a short half-life, a second aliquot was then added and the incubation continued at 37°C for a further 30min. In the case of protruding 3' termini, incubation was at 37°C for 15min. The tube was then transferred to 56°C for 15min, and this was repeated following the addition of a second aliquot of alkaline phosphatase.

Both reaction mixtures were extracted with phenol-chloroform (Section 2.3.5) to stop the reaction, and the DNA was precipitated with ethanol (Section 2.3.4), washed with 70% (v/v) ethanol and dried before use in ligation reactions.

10X CIP buffer is 0.5M Tris-HCl, pH 9.0; 10mM magnesium chloride; 1mM zinc chloride; 10mM spermidine.

#### 2.3.10. Ligation of DNA fragments. (Maniatis et al., 1982)

Ligation reactions were usually conducted overnight in 1X ligase buffer (supplied as 5X concentrate by the manufacturer; Section 2.3.8.1) in a 10 $\mu$ L volume using T4 DNA ligase. For plasmid recircularizations, the amount of DNA used was between 10-20ng, and for bimolecular ligations a 1:5 or 1:10 ratio of vector to insert DNA was used. Ligations of DNA with 3' or 5' extensions were incubated at 15°C, and those with blunt ends at 4°C. Ligation reactions that were to be used for transformation of DNA into *E.coli* by the electroporation method (Section 2.2.3.3) were precipitated with ethanol (Section 2.3.4) in the presence of 5 $\mu$ g tRNA as co-precipitant, washed with 70% (v/v) ethanol and resuspended in distilled water before use.

#### 2.3.11. DNA fragment labelling with <sup>32</sup>P-dCTP. (Feinberg and Vogelstein, 1984)

The DNA fragment to be labelled for use in Southern and Northern hybridization procedures, was isolated from low melting gels (Section 2.3.7.2). 10-25ng of DNA was used per reaction. The reaction was carried out in the following steps:

1. The labelling reaction: the following reagents were added at room temperature:

Oligo labelling buffer (OLB)	6 $\mu$ L
BSA	1.2 $\mu$ L
DNA	upto 25ng (boiled to denature)
<sup>32</sup> P-dCTP	3 $\mu$ L
Klenow fragment	0.5 $\mu$ L (1 Unit)
Distilled water	to a total of 30 $\mu$ L

The labelling reaction was allowed to proceed for 3 hours, or overnight, at room temperature. Smaller amounts of DNA take longer than 3 hours to label.

The reaction was stopped by the addition of distilled water to 100 $\mu$ L, 425 $\mu$ L ethanol, 20 $\mu$ L 3M sodium acetate, pH 5.2, and 9 $\mu$ L 10mg/mL heat denatured herring testis DNA with chilling when required. The DNA was spooled out with a silicanized Pasteur pipette, transferred to a fresh tube, and washed with 70% (v/v) ethanol. The DNA was then resuspended in 50 $\mu$ L Tris-HCl, pH 7.5.

Oligo labelling buffer was composed of solutions A, B, and C mixed in the ratio 2:5:3. This stock is stable for 3 months at -20°C with repeated freezing and thawing.

Solution A is 625 $\mu$ L 2M Tris-HCl, pH 8.0; 25 $\mu$ L 5M magnesium chloride; 350 $\mu$ L distilled water; 18 $\mu$ L 2-mercaptoethanol; 5 $\mu$ L dATP; 5 $\mu$ L dTTP; 5 $\mu$ L dGTP (each dissolved in 3mM Tris-HCl; 0.2mM EDTA at a concentration of 0.1M). Storage was at -20°C.

Solution B is 2M HEPES titrated to pH 6.0 with sodium hydroxide. Storage was at 4°C.

Solution C is hexadeoxyribonucleotides (Pharmacia PL Cat. No. 27-2166XX) evenly suspended (this does not completely dissolve) in 3mM Tris-HCl; 0.2mM EDTA, pH 7.0 at 90 A<sub>260</sub> Units/mL.

Bovine serum albumin (BSA) is 10mg/mL enzyme grade.

The <sup>32</sup>P-dCTP was from Amersham, PB10205, 3000Ci/mM, 10 $\mu$ Ci/ $\mu$ L.

### 2.3.12. Southern transfer of DNA fragments.

#### 2.3.12.1. DNA fragment transfer. (Southern, 1975)

The fragments of DNA were first separated by agarose gel electrophoresis at a low voltage, preferably overnight.  $\lambda$  DNA digested with *Hind*III was used as size markers and were included asymmetrically to orient the gel. After separation was completed, the fragments were visualized under ultraviolet illumination and photographed to provide a record. The

agarose gel was then subjected to a series of steps to denature the DNA:

1. 2x7min wash in 0.25M hydrochloric acid.
2. 2x15min in 0.5M sodium hydroxide; 0.5M Tris-HCl.
3. 2x15min wash in 3M sodium chloride; 0.5M Tris-HCl, pH 7.4.

The gel was rinsed with distilled water between each step. The gel was then transferred to a blotting apparatus that consisted of a glass plate laid across a tray half-filled with transfer buffer (10X SSC). The plate was covered with a 3MM Whatman filter paper with wicks dipping into the reservoir. The denatured gel was laid slot side down on the 3MM paper and a sheet of nitrocellulose membrane, cut to the same size and previously soaked in 6X SSC, was laid over it. Care was taken at each step described to avoid trapping bubbles. Two pieces of Whatman 3MM paper cut to the same size and dipped in 6X SSC were laid over the nitrocellulose sheet. The edges of the gel in contact with the Whatman 3MM paper below were sealed with parafilm, to avoid short-circuiting of the transfer buffer to paper towels laid over the gel to draw up the transfer buffer through the gel. The DNA fragments are transferred to the nitrocellulose membrane by capillary action. The paper towels were weighed down with a glass plate topped with a 500g weight. Wet towels at the bottom of the pile were changed every 5min for the first 30min, and then after 2 hours. The blot was then allowed to proceed overnight. Care was taken not to disturb the gel or the nitrocellulose membrane when changing the towels. Following transfer, the positions of the slots were marked on the nitrocellulose membrane with a soft pencil and the nitrocellulose membrane was rinsed with 2X SSC, blotted dry and baked for 2 hours in a vacuum oven at 80°C. When Hybond-N was used in place of nitrocellulose membrane, the DNA was fixed by wrapping the membrane in Saran Wrap, followed by exposure of the DNA to longwave ultraviolet illumination for 4min.

The gel was then examined under ultraviolet light to confirm transfer of the DNA fragments. The nitrocellulose membrane or Hybond-N membrane could be stored between sheets of Whatman 3MM paper at 4°C until use.

20X SSC (Saline sodium citrate).

NaCl                      3.0M  
Trisodium citrate      0.3M  
pH 7.0

2.3.12.2. Colony transfer. (Buluwela et al., 1989)

In the case of DNA ligations with blunt ended fragments where the efficiency of ligation was low, colony transfer followed by screening with the insert as probe was used to identify recombinant colonies.

The ligation mix was transformed into bacteria (Section 2.2.3.3) and the transformants plated at the required colony density between 200-500 colonies/plate. When the colonies were still fairly small, a disc of Hybond-N was laid over the colonies to transfer some cells onto it. The sheet was orientated, stripped off and transferred to a fresh plate colony side up, and incubated until the colonies had grown. The original (master) plate was also incubated until the colonies were visible. The Hybond-N membrane was transferred, colony side up, to a sheet of Whatman 3MM paper soaked in 2X SSC; 5% (w/v) SDS for 5min. The Whatman paper and the Hybond-N were then transferred to a microwave oven and subjected to 2.5min at full setting. This treatment lysed the cells and also fixed the DNA to the Hybond-N membrane. The membrane could be stored at room temperature until required.

2.3.13. Hybridization with radiolabelled probe DNA.

Nucleic acid hybridizations were carried out in thick-walled leakproof perspex boxes. The Hybond-N sheet or the nitrocellulose membrane were cut to fit the box, taking care to orient them first with respect to each other. The membranes were dipped in 1X SSC for 15min. They were then treated in the following manner with all washes at 62°C:

1. 50mL 1X Denhardt's solution for 30min
2. 50mL 1X CFHM (Denhardt's solution containing 50µg/mL heat denatured herring sperm DNA) for 30min
3. 50mL 1X CFHM; 6% (w/v) PEG6000 (degassed) for 30min
4. 20mL 1X CFHM; 6% (w/v) PEG6000 (degassed) containing heat denatured probe for a minimum of 4 hours. Hybridizations were

usually conducted overnight.

The probe solution was discarded and the membranes were washed with 1-2L of 1X SSC; 0.1% (w/v) SDS; 10µg/mL denatured herring sperm DNA. Washes were carried out at 62°C, taking care not to let the membranes to dry out at any stage. The membranes were then dried at room temperature between 3MM sheets, covered in aluminium foil, and exposed with an intensifying screen to X-ray film at -70°C. If hybridization with a second probe was desired, Hybond-N membrane was used because of its greater strength, and the membranes were not dried before autoradiography, but were wrapped in clingfilm and exposed as usual.

A second method was used for colony hybridizations with the Boehringer Mannheim non-radioactive DNA hybridization kit. However, the result was not satisfactory due to the higher background. This method is recommended for genomic blots but not for colony blots.

#### 2.3.14. Stripping radiolabelled probe DNA from Hybond-N membranes.

When it was necessary to hybridize nucleic acid on a Hybond-N membrane to a second DNA probe, the first probe was removed by incubation for 30min at 45°C in 0.4M NaOH, followed by a similar incubation in 0.1X SSC; 0.1% (w/v) SDS; 0.2M Tris-HCl, pH 7.5. Removal of radioactive isotope was confirmed by autoradiography.

#### 2.3.15. Oligonucleotide directed *in vitro* mutagenesis of DNA.

The Oligonucleotide directed *in vitro* mutagenesis kit Version 2.0 was obtained from Amersham International plc. (Code: RPN 1523) and the procedure followed was exactly as described in the manual. Oligonucleotides incorporating the desired nucleotide changes were synthesized in the Department of Biochemistry, University of Leicester by Mr. J. Keyte.

## 2.4. Methods associated with proteins.

### 2.4.1. Preparation and labelling of minicells. (Stoker et al., 1984)

Plasmids which were used in minicell assays were introduced into the *E.coli* strain DS410"T" by the rapid calcium chloride method (Section 2.2.3.1). Transformants were selected and plasmid DNA was isolated by the minipreparation method (Section 2.3.2). Plasmid identity was confirmed by restriction enzyme digestion analysis (Section 2.3.6).

The strains containing plasmid DNA were then grown overnight in 5mL antibiotic containing medium and then used as inoculum for 400mL sterile Terrific broth (Section 2.2.2) in 2L baffled flasks. The flasks were shaken vigourously overnight at 37°C. The cells were harvested in a GS3 angle rotor with an initial clearing spin at 4°C to remove the majority of whole cells. The minicell-enriched supernatant was transferred to fresh GS3 bottles and harvested by centrifugation at 6Krpm for 15min at 4°C. The pellets were drained well and resuspended thoroughly in 7mL minimal medium (Section 2.2.2) and layered on a previously prepared sucrose gradient in 35mL clear polysulfone tubes. The gradient was made by dissolving 22% (w/v) sucrose in minimal medium. The solution was aliquoted into polysulfone tubes and frozen at -70°C for a minimum of 1 hour, and thawed slowly overnight at 4°C. The tubes were kept upright at all times. After layering the sucrose gradients with the minicell-enriched medium, the tubes were centrifuged in a HB-4 swing out rotor for 20min at 5Krpm at 4°C. At the end of the spin, the minicells could be seen as a dense band in the middle of the tube. The top two-thirds of this band was carefully withdrawn using a Pasteur pipette and transferred to clean polycarbonate tubes, diluted 1:1 with minimal medium and the minicells pelleted by centrifugation at 10Krpm for 10min at 4°C. The pellet was again resuspended in 7mL minimal medium, layered on sucrose gradients and the minicells isolated as before, The process was repeated until there was no pellet on the bottom of the sucrose gradient tubes, indicating that no whole cells were present. In addition the minicells were checked for the absence of whole cells under the microscope (less than one whole cell per field indicates a clean preparation). The

cells were pelleted and resuspended to a final concentration of 10 A<sub>600</sub> Units/mL.

The minicells were transferred to an eppendorf tube and the volume measured. 4μL of a 50mg/mL solution of cycloserine in water was added per 100μL of minicells. The tube contents were mixed and incubated at 37°C for 1 hour to kill growing whole cells. Then, 2μL of <sup>35</sup>S-methionine (800Ci/mMol) was added per 100μL of minicells and gently mixed and incubated at 37°C for 1 hour to allow labelling of cells. To terminate labelling and to allow completion of amino acid chains, 5μL of a 30mg/mL solution of L-methionine was added for 5min. The minicells were harvested by centrifugation in an eppendorf centrifuge at room temperature, washed once with 10mM Tris-HCl, pH 7.5, resuspended to the original volume in B buffer (For 1L: 3g KH<sub>2</sub>PO<sub>4</sub>; 7g anhy. Na<sub>2</sub>HPO<sub>4</sub>; 4g NaCl; 0.1g MgSO<sub>4</sub>) containing 20% (v/v) glycerol and stored at -20°C until required. All minicells were used within 2-3 months of preparation.

An aliquot of labelled minicells was analysed by SDS polyacrylamide gel electrophoresis (Section 2.4.5) in order to check the level of labelling and background.

#### 2.4.1.1. Fractionation of minicell proteins.

An aliquot of minicells prepared as described in Section 2.4.1 was washed with ice-cold B buffer (Section 2.4.1) to remove the glycerol and the cells were pelleted for 3min at room temperature in an MSE microcentrifuge. The cells were immediately resuspended in 100μL 10mM Tris-HCl, pH 7.8; 0.3M sucrose; 1mM EDTA containing 1mg/mL lysozyme. The suspension was gently shaken at 37°C for 1 hour in order to disrupt the outer membrane. The spheroplasts were then pelleted by centrifugation for 5min at room temperature in an MSE microcentrifuge. The supernatant containing the periplasmic proteins were transferred to a fresh tube. The solution was made up to 10% (v/v) with 50% (v/v) Trichloroacetic acid (TCA) and placed on ice for 30min. The tubes were centrifuged at 13Krpm for 10min at room temperature, and the supernatant discarded. The pellet containing the periplasmic proteins was centrifuged again and all traces of TCA were removed by

aspiration.

The spheroplast pellet from the earlier step were osmotically shocked to release the cytoplasm by resuspending them in water. The supernatant (cytoplasm) and pellet (inner and outer membranes) were then separated by centrifugation as above. The supernatant was transferred to a fresh tube and the proteins concentrated by TCA precipitation as described above. The various fractions were resuspended in SDS loading buffer. Pellets obtained by TCA precipitation were neutralized by the addition of SDS loading buffer (Section 2.4.5) containing 0.1 volume saturated Tris solution.

#### 2.4.2. In vitro coupled transcription-translation assays. (Pratt, 1984)

The plasmid DNA used in this method had to be RNase free, and was prepared as described in Section 2.3.3.

The following method was used to label the proteins. A cocktail was made containing solutions A:B:C=75:35:50, and maintained on ice:

Solution A (LMM) : 56.4mM Tris-acetate, pH 8.2; 1.76mM DTT; 1.22mM ATP; 0.85mM GTP; 0.85mM UTP; 27mM phosphoenolpyruvate; 0.35mM amino acid mixture B (55mM total concentration); 1.9% (w/v) PEG6000; 34.6µg/mL folinic acid; 0.64mM 3'5'cAMP; 0.17mg/mL *E.coli* tRNA; 36mM ammonium acetate; 72mM potassium acetate; 9.7mM calcium acetate).

Solution B: magnesium acetate, 0.1M

Solution C: S-30 extracts from *E.coli* Sp<sup>R</sup> (RNase<sup>-</sup> cells) or *E.coli* LC137 (*lon*<sup>-</sup> cells) were made according to Pratt (1984) and used from the laboratory stock.

To 12µL of plasmid DNA (1.5µg) in an eppendorf tube was added 16µL of the cocktail. A control without added DNA was included. The solutions were mixed gently with a pipette and incubated for 10min at 37°C to degrade endogenous mRNA. After this, 2µL of <sup>35</sup>S-methionine were added to the mixture to label proteins. The tube contents were mixed by pipetting and incubated at 37°C for 1 hour. Chase (5µL of a 30mg/mL solution of L-methionine) was added to allow completion of polypeptide chains for 5min. If pulse chase experiments were carried out,

labelling was continued for specific periods, and chased as long as required. Antibiotics or protease inhibitors were added 5min following the addition of the chase mixture. The reaction tube contents were finally centrifuged at 13Krpm for 10min at room temperature to remove ribosomes and the proteins in the supernatants were heated to 100°C in SDS loading buffer and analysed by SDS polyacrylamide gel electrophoresis (Section 2.4.5).

### 2.4.3. Labelling of whole cells of *E.coli*.

Labelling of whole cells of *E.coli* were carried out in minimal medium containing any growth requirements, as rich media containing methionine led to poor labelling efficiencies. The cells were grown overnight in minimal medium supplemented with the necessary amino acids and diluted into the same medium to an  $A_{600}=0.05$ . The cells were then grown to an  $A_{600}=0.2$ . An aliquot of 10mL was transferred to a fresh flask (Experimental flask) and the original flask was used to monitor the optical density of the culture. This flask is called the Sampling flask. 5 $\mu$ L of  $^{35}$ S-methionine per  $A_{600}$  Unit of the culture was added to the Experimental flask. Incubation was continued for 1 hour. If required, the cells were induced for the synthesis of inducible proteins by transfer to 42°C (for  $\lambda$   $P_R$ ,  $P_L$ -based promoters) or by the addition of isopropylthio- $\beta$ -D-galactopyranoside (IPTG; for *lac*-based promoters). The Sampling flask was used to monitor the  $A_{600}$  values. 5 $\mu$ L of chase solution (30mg/mL L-methionine) was then added to the Experimental flask per  $A_{600}$  Unit of the culture for 5min. The culture was centrifuged to collect the cells in an eppendorf tube at 13Krpm for 10min at room temperature. The cells were washed with 10mM Tris-HCl, pH 7.5 to remove excess radioactivity. Cells were stored frozen at -20°C in B buffer (Section 2.4.1) containing 20% (v/v) glycerol until required. Cells were used within 3 months of preparation.

#### 2.4.4. Preparation of cell and supernatant samples of *E.coli*.

*E.coli* cells were grown from an initial  $A_{600}=0.05$  to 0.4 at 37°C. The cells were then allowed to grow for a further 5 hours, and pelleted at 10Krpm in an HB-4 swing out rotor for 20min at 4°C. The supernatant was transferred to a fresh tube taking care not to disturb the cell pellet. The proteins in the supernatant were precipitated by the addition of 10% (v/v) final concentration TCA and incubation on ice for 30min. The proteins in the supernatant were recovered by centrifugation at 10Krpm, 4°C for 20min. The TCA was removed completely by aspiration. The proteins in the supernatant and pellet were analysed by SDS polyacrylamide gel electrophoresis after heating to 100°C for 3min in SDS loading buffer (Section 2.4.5). Solubilization of the TCA precipitated proteins in SDS loading buffer was facilitated by the addition of 0.1 volume saturated Tris.

#### 2.4.5. SDS polyacrylamide gel electrophoresis. (Laemmli, 1970)

SDS polyacrylamide gel electrophoresis was used for the separation of proteins. The solutions used were:

Buffer A: 0.75M Tris-HCl, pH 8.8; 0.2% (w/v) SDS

Buffer B: 0.25M Tris-HCl, pH 6.8; 0.2% (w/v) SDS

Acrylamide: Acrylamide:bis-acrylamide in the ratio 44:0.8 (incubated overnight with activated charcoal and filtered before use) and stored at 4°C. Acrylamide is a cumulative neurotoxin and was handled with sufficient precautions.

Ammonium persulphate (APS): 10mg/mL in distilled water, freshly prepared.

Tetraethylmethylethylenediamine (TEMED): Obtained from Sigma Chemical Company, used undiluted. TEMED is toxic and was handled with care.

SDS running buffer: 0.025M Tris-HCl, pH 8.3; 0.192M glycine; 0.19% (w/v) SDS

2X SDS loading buffer: 0.625M Tris-HCl, pH 6.8; 0.08% (w/v) SDS; 10% (v/v) glycerol; 0.72M 2-mercaptoethanol; 0.04g bromophenol blue. Mercaptoethanol is very toxic and was handled with caution. For non-denaturing gels, SDS and 2-mercaptoethanol were omitted.

Gel preparation: The gel was prepared as described in the table below.

Separating gel:	5%	8.5%	11%	15%
Buffer A(mL)	13.5	13.5	13.5	13.5
Acrylamide(mL)	3.0	5.3	6.8	9.2
Distilled water(mL)	9.8	7.5	6.0	3.6
APS(mL)	0.95	0.95	0.95	0.95
TEMED(mL)	0.075	0.075	0.075	0.075

<u>Stacking gel:</u>	2.5%	5%	7%
Buffer B(mL)	10.0	10.0	10.0
Acrylamide(mL)	1.2	2.4	3.3
Distilled water(mL)	8.8	7.6	6.7
APS(mL)	0.5	0.5	0.5
TEMED(mL)	0.04	0.04	0.04

The gels were poured in BRL Protean gel kits or in homemade minigel kits. The protein samples were heated in 1X SDS loading buffer at 100°C for 3min before loading for electrophoresis. Protein markers used were obtained from Sigma Chemical Co. or from BRL. They were Myosin (200KDa),  $\beta$ -galactosidase (116KDa), phosphorylase-a (97KDa), bovine serum albumin (68KDa), ovalbumin (43KDa), chymotrypsin (29KDa), and lysozyme (18KDa). Radioactively labelled <sup>14</sup>C markers were obtained from BRL and were myosin (200KDa), phosphorylase-b (100, 92KDa), bovine serum albumin (68KDa), ovalbumin (43KDa), chymotrypsin (29KDa).

#### 2.4.6. Visualizing proteins separated by SDS polyacrylamide gel electrophoresis.

Proteins were visualized by staining with coomassie blue (25% (v/v) methanol; 10% (v/v) acetic acid; 0.125% (w/v) coomassie blue G-250). Destaining was carried out with 25% (v/v) methanol; 10% (v/v) acetic acid. Radioactively labelled proteins were visualized by fluorography. Thus, the gels were subjected to 2 x 30min washes in DMSO, 1x 1 hour wash in DMSO-PPO (DMSO containing 20% (w/v) PPO), and 1x 30min wash in water to precipitate PPO in the gel. The gel was dried and exposed to FUJI X-ray film at room temperature. DMSO-PPO is harmful and should be handled with appropriate precautions.

#### 2.4.7. Inclusion body preparation. (Marston, 1987)

*E. coli* cells (1g) containing inclusion bodies were resuspended in 3mL Lysis buffer (50mM Tris-HCl, pH 8.0; 1mM EDTA; 100mM NaCl) at or below 10°C. 8µL of 50mM phenylmethylsulfonylfluoride (PMSF) freshly dissolved in methanol was then added followed by 80µL lysozyme (freshly prepared in Lysis buffer). The suspension was incubated on ice for 20min with occasional stirring. 4mg deoxycholic acid was then stirred in and the suspension was transferred to 37°C with gentle mixing. When the suspension became viscous, 20µL of DNaseI (freshly made, 1mg/mL in Lysis buffer) was added and stirred. The suspension was transferred to room temperature until no longer viscous. It was centrifuged at 12000 x g for 5min at 4°C. The pellet was resuspended in 9 volumes of Lysis buffer containing 0.5% (v/v) Triton X-100 and 10mM EDTA. This mixture was incubated for 5min at room temperature and centrifuged again as before. The pellet was analysed by SDS polyacrylamide gel electrophoresis (Section 2.4.5). This Triton X-100/EDTA washing procedure was sufficient to remove adsorbed proteins from the inclusion bodies and further treatments, for example, with urea were considered unnecessary.

#### 2.4.8. Electroelution of protein from SDS polyacrylamide gels.

This procedure was used to prepare proteins for amino acid sequencing. The proteins were separated on a 5% separating, 5% stacking SDS polyacrylamide gel and the proteins visualized by staining with aqueous coomassie blue stain (300mL 50mM Tris-HCl, pH 7.5; 25% (v/v) methanol; 0.25% (w/v) coomassie blue G-250). This stain does not fix proteins as it does not contain acetic acid. Destaining was carried out in water. The 125KDa crystal associated protein in this case was seen as an opaque region against a clear background. This protein band was carefully excised from the gel and sliced into small pieces with a sharp scalpel. The slices were transferred into a homemade electroelution chamber placed in a horizontal electrophoresis apparatus. The chamber consisted of a slot covered on the near side with a permeable membrane (Schleicher and Shuell BT2 BioTrap Membrane) and on the far side with a

non-permeable dialysis membrane. The gel slices were loaded such that they were in contact with the permeable membrane. The buffer used was SDS running buffer (Section 2.4.5). A voltage of 16V was applied overnight to draw the protein in the gel slices into the slot across the permeable membrane where they were trapped. The slot was accessed through a small pore above the slot using a drawn out Pasteur pipette. The protein solution was dialysed extensively against 0.005% (w/v) SDS in MilliQ<sup>TM</sup> pH 7.0 water at 4°C. The protein was quantified by the Lowry method against BSA standards. 100µg quantities of protein were aliquoted into eppendorf tubes and lyophilized.

#### 2.4.9. N-terminal amino acid sequencing of the 125KDa protein.

N-terminal amino acid sequencing was carried out by Dr. J. N. Keen at the University of Leeds. The protein was dialysed against 0.001% SDS in MilliQ<sup>TM</sup> water at 4°C over several days with a final step against water and then lyophilised. The freeze dried protein was dissolved in 35µL 2% (v/v) N-methylmorphine; 0.1% (w/v) SDS with warming and sonication. The sample was dried at 56°C for 30min onto a diisothiocyanate-activated membrane disc (Sequelon-DITC<sup>TM</sup>) to covalently attach protein for solid phase sequence analysis. Analysis was carried out following automated Edman degradation on a Milligen-Biosearch 6600 ProSequencer with on-line HPLC.

#### 2.4.10. Lowry estimation of protein concentration. (Lowry et al., 1951)

Solutions A and B (see below) were mixed 1:1 to give solution C. Folin's phenol reagent was separately mixed with 1M HCl in a ratio of 3:4 to give solution D.

BSA standards (0-200µg/mL) were prepared in 0.4mL volume of water in triplicate. The samples were also prepared in water. To each sample and standard tube, 1mL of C mix was added and the tube contents were mixed and allowed to stand at room temperature for at least 10min. This was followed by the addition of 0.1mL D mix, with immediate mixing. The tubes were allowed to stand at room temperature for at least 30min

for the colour to develop. The readings were taken on a spectrophotometer at 500nm.

A mix: 4% (w/v)  $\text{Na}_2\text{CO}_3$  in 0.2M NaOH

B mix: 0.02% (w/v)  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in 0.04% (w/v) sodium potassium tartarate

## 2.5. Nucleotide sequence analysis of DNA.

### 2.5.1. Preparation of single stranded (ss) DNA from M13 phage.

The DNA fragment for the sequence analysis was subcloned into the phage M13mp18 as the source of single stranded DNA. Replicative form M13 was obtained from Pharmacia plc. The DNA was digested with the appropriate restriction enzymes at the multiple cloning site. The DNA fragment was prepared and ligated into the M13 phage genome. The ligation mixture was transformed into *E.coli* TG1 by the rubidium chloride (Section 2.2.3.2) or the electroporation method (2.2.3.3). The *E.coli* cells were plated out in 3mL Luria top agar (Luria broth containig 0.6% (w/v) agar) on minimal agar plates (Section 2.2.2) containing the chromogenic indicator X-gal (25 $\mu$ L of 25mg/mL stock made in dimethylformamide) and IPTG (25 $\mu$ L of 25mg/mL stock made in water). 0.3mL of an overnight culture of *E.coli* TG1 cells were used as plating cells. The plates were incubated overnight. Wild-type plaques were blue, and recombinant phage gave rise to colourless plaques.

Recombinant phage were picked using sterile toothpicks into 1mL  $\lambda$  buffer (For 12L: 72mL 1M Tris, pH 7.2; 29.5g  $\text{MgSO}_4$ ; 0.6g gelatin) in eppendorf tubes. These stocks could be stored for upto 6 months. 100 $\mu$ L of these stocks were diluted into 2mL of Luria broth containing a 1:100 dilution of an overnight culture of *E.coli* TG1. The cells were grown with vigourous shaking at 37°C for 4-6 hours. If single stranded phage were to be prepared, cells were grown for less than 6 hours in order to avoid any cell lysis, which would lead to contamination of phage DNA with RNA. A non-infected control was normally included. 1.5mL of the cells were transferred into eppendorf tubes and were pelleted at 13Krpm for 5min at room temperature and then twisted through 180° and centrifuged again. This was to ensure that no cells stayed in the

supernatant. 0.8mL of this supernatant was transferred to a fresh tube and the phage in the supernatant were pelleted by addition of 10% (w/v) PEG6000; 2.5M NaCl for 15min at 15°C followed by centrifugation at 13Krpm for 10min at room temperature. The supernatant was thoroughly removed and the mouth of the tube wiped with a tissue. The phage pellet was resuspended in 100µL of distilled water and the suspension was extracted with phenol-chloroform (Section 2.3.5) until no precipitate at the interphase was seen. The tube was then transferred to a fresh tube and the DNA precipitated with ethanol (Section 2.3.4). The ssDNA obtained was quantified spectrophotometrically. An A<sub>260</sub> value of 1.0 corresponds to 40µg of ssDNA. The cell pellet from the previous step was used to prepare the replicative form of the phage for restriction enzyme digestion analysis (Section 2.3.2).

## 2.5.2. DNA nucleotide sequencing.

2.5.2.1. Sequencing of ssDNA. (Sanger *et al.*, 1977; modified by A. J. Jeffreys, Univ. of Leicester)

Sequencing was carried out as described below:

1. The ssDNA were incubated at 60°C for 10min, spun and left until use at room temperature.

2. The following solutions were mixed together:

2µg/mL 17mer primer	7.2µL
TM buffer (see below)	18.0µL
Distilled water	64.0µL

This is the primer mix.

3. 5µL of ssDNA (1-1.5µg) was added to 5µL primer mix and incubated at 60°C for 30min, briefly spun to mix and incubated for a further 30min at 60°C for the annealing reaction to occur.

4. 4 tubes labelled "A", "T", "C" and "G" were prepared for each sample to be sequenced.

5. The following solutions were added to the tubes at room temperature:

	T	C	G	A
"T" mix (see below)	2µL			
"C" mix (see below)		2µL		
"G" mix (see below)			2µL	
"A" mix (see below)				2µL
Annealed clone DNA	2µL	2µL	2µL	2µL

6. Immediately prior to use, the following solutions were mixed on ice in a lead pot:

Distilled water	117.0 $\mu$ L
Klenow fragment	3.3 $\mu$ L
$^{35}$ S-dATP	10.0 $\mu$ L (400Ci/mol, 10mCi/mL)

This is the Klenow mix.

7. 2 $\mu$ L of the Klenow mix was pipetted into each tube, mixed gently, and incubated at 37 $^{\circ}$ C for 20min.

8. 2 $\mu$ L of sequence chase mix (see below) was added to each tube, mixed and incubated 37 $^{\circ}$ C for 20min.

9. 4 $\mu$ L of formamide dye mix (see below) was added to each tube and the tubes stored at room temperature until use. If the samples were not to be run the same day, the samples were stored without dye at -70 $^{\circ}$ C, and the dye was added immediately prior to use.

10. The tubes were heated to 100 $^{\circ}$ C for 2min and the samples were loaded on a sequencing gel.

TM buffer: 100mM Tris-HCl, pH 8.0; 100mM magnesium chloride. Stored at -70 $^{\circ}$ C.

dNTP mixes for sequencing: all values in  $\mu$ L

(prepared in M13TE buffer: 10mM Tris-HCl, pH 8.0; 0.1mM EDTA)

	"T"mix	"C"mix	"G"mix	"A"mix
0.5mM dTTP	12.5	250	250	250
0.5mM dCTP	250	12.5	250	250
0.5mM dGTP	250	250	12.5	250
10mM ddTTP	6.2			
10mM ddCTP		4		
10mM ddGTP			8	
10mM ddATP				1.2
TE buffer	500	500	500	500

Formamide dye mix:

Deionized formamide	10mL
Xylene cyanol	10mg
Bromophenol blue	10mg
0.5M EDTA, pH 8.0	200 $\mu$ L

Sequence chase mix: 0.25mM dATP; 0.25mM dCTP; 0.25mM dTTP; 0.25mM dGTP in M13TE buffer (10mM Tris-HCl, pH 8.0; 0.1mM EDTA).

2.5.2.2. Sequencing double stranded (ds) DNA. (Zhang *et al.*, 1988)

Plasmid DNA used for this purpose was prepared by the CsCl density gradient centrifugation method described in Section 2.3.3. The plasmid (4-5 $\mu$ g) was precipitated with ethanol (Section 2.3.4) and resuspended in 50 $\mu$ L freshly prepared 0.2M NaOH; 0.2mM EDTA at room temperature for 5min. The solution was neutralized by the addition 5 $\mu$ L of 2M ammonium acetate, pH 4.5. The DNA was precipitated with ethanol and washed with 70% (v/v) ethanol. The DNA was dried under vacuum and used immediately for sequencing. The method used was exactly as described in the Amersham Sequenase<sup>TM</sup> sequencing protocol. The primers used were prepared at the Department of Biochemistry at the University of Leicester by Mr. J. Keyte.

2.5.3. Preparation of the sequencing gel.

2.5.3.1. Preparation of the gel mould.

To prepare the gel mould, one large and one small glass plate were washed well and wiped with ethanol. The smaller plate was repelcoated with dimethyldichlorosilane (2% (v/v) in 1,1,1, trichloroethane). The plate was washed and dried. Both plates were placed face up on the bench. Spacers were placed down the sides of the larger plates and covered with the smaller plate. The sides and bottom of the plates were sealed with yellow tape, and the plates were clamped together with bulldog clips. The open end could be covered with clingfilm. These moulds could be stored indefinitely.

2.5.3.2. Pouring the sequencing gel.

Stocks were made, filtered and stored at 4°C.

0.5X stock:

Acrylamide	17.1g
Bis-acrylamide	0.9g
Urea	150g
10X M13TE buffer (Section 2.5.2.1)	15mL
Distilled water to 300mL with warming	

### 2.5X stock:

Acrylamide	2.28g
Bis-acrylamide	0.12g
Urea	20g
Sucrose	2g
10X M13TE buffer (Section 2.5.2.1)	10mL

0.025g bromophenol blue

Distilled water to 40mL with warming

Each stock was filtered through Whatman 3MM paper using a Buchner funnel and vacuum pump.

### Gel preparation:

150mL 0.5X stock, 700 $\mu$ L 10% (w/v) ammonium persulphate and 48 $\mu$ L TEMED were mixed together in a clean beaker (Solution I). In a second beaker was mixed 20mL 2.5X stock, 175 $\mu$ L ammonium persulphate and 12 $\mu$ L TEMED (Solution II). Working quickly, 8mL of Solution I was drawn up in a 25mL pipette followed by 12mL of Solution II. The gel solutions were poured into the gel mould and the gel mould was filled to the top with Solution I. Any bubbles that formed were tapped out. A 0.4mm sequencing gel comb was inserted flat side first into the gel mould. The mould was placed at an angle of 15 $^{\circ}$  on the table to set. After the gel had set, the comb was withdrawn, washed with electrophoresis buffer and reinserted with the shark tooth side first into the gel. The gel was assembled into the apparatus. The top reservoir was filled with 0.5X electrophoresis buffer (see below) and the bottom buffer was filled with 2.5X electrophoresis buffer. The gel was loaded with the samples and electrophoresed between 1250-2000V, keeping the gel temperature within the range 55-60 $^{\circ}$ C, until the bromophenol blue dye reached the bottom. The gel was fixed for 30min in 20% (v/v) methanol; 20% (v/v) acetic acid, dried and exposed to X-ray film.

### 10X Electrophoresis buffer:

Tris base	109g
Boric acid	55g
EDTA	9.3g

Distilled water to 1L. It should not be necessary to adjust the pH, which should be 8.3. Store at room temperature and remake if cloudy.

## 2.6. Immunological methods.

### 2.6.1. Preparation of antigen for immunization.

The antigen used for immunizing rabbits to both the 125KDa crystal protein and the 59KDa toxin protein was prepared from SDS polyacrylamide gels. The sample containing the protein of interest (crystal preparation or induced cell sample) were electrophoresed as described in Section 2.4.5. The proteins in the gel were visualized by staining with coomassie blue. The gel was then soaked in 1M Tris-HCl, pH 7.5. The gel was then equilibrated in 1X PBS for 2 days to remove all traces of acetic acid. The pH was checked and when it was 7.2, the protein band was excised with a flamed scalpel. The gel was sliced into small fragments and placed in an eppendorf tube taking care to handle everything aseptically. An approximately equal volume of Complete Freund's Adjuvant was added and the gel fragments sonicated. Unsonicated fragments were removed by centrifugation at 13Krpm for 10sec at 4°C. 100µL of this sample was injected subcutaneously into adult New Zealand White rabbits. Incomplete Freund's Adjuvant was used for the preparation of booster injections every 4 weeks. Test bleeds were taken to determine the serum titre. When the sample was adequate, the rabbit was heart bled and the serum prepared.

### 2.6.2. Serum preparation.

Serum from the blood of the immunized rabbits was prepared by allowing the blood to clot overnight at 4°C or for 2 hours at room temperature. The supernatant after centrifugation at 2.5Krpm at 4°C for 10min was transferred to a fresh tube. Sodium azide was added to 10mM as preservative. Serum was stored at 4°C.

### 2.6.3. Western Immunoblotting of proteins. (Towbin et al., 1979)

Proteins separated on SDS polyacrylamide gels were transferred electrophoretically to nitrocellulose membrane. The transfer buffer was Tris-glycine (9.96g Tris; 43.2g glycine in 3L distilled water). The blotting step was conducted overnight at 16V or at 80V for 3 hours in a home

made apparatus. After blotting the transferred proteins were visualized by staining with 0.1% (w/v) PonceauS in 5% (v/v) acetic acid. This treatment also served to fix the proteins to the nitrocellulose paper. The positions of the marker proteins were marked and non specific binding sites on the membrane were blocked by incubation overnight in either 5% (w/v) Marvel milk in 1X phosphate buffered saline (PBS) or in 10% (v/v) newborn calf serum; 1% (w/v) casein; 0.5% (w/v) bovine serum albumin in 1X Tris buffered saline (TBS). The blot was then washed with 1X TBS or 1X PBS and the antibodies added at the required concentration in 1% (v/v) blocking solution and incubated for 1 hour at room temperature. The antibodies were poured off, the blot rinsed 4 times with 1X TBS or 1X PBS. According to the level of sensitivity desired, the blots were developed directly or by the "Sandwich method" (see below).

For direct development, 1:5000 or 1:10000 horseradish peroxidase conjugated goat anti-rabbit antibodies were added and incubated for 1 hour at room temperature. In the sandwich method, sequential incubations in 1:1000 goat anti-rabbit antibodies and 1:1000 rabbit peroxidase antiperoxidase antibodies were carried out. The blots were washed and developed by flooding the blot with 4-chloro-1-naphthol dissolved in 10mL methanol mixed with 50mL 50mM Tris-HCl, pH 7.5 and 30 $\mu$ L H<sub>2</sub>O<sub>2</sub>. The reaction was stopped by washing the blot in water.

10X Phosphate buffered saline (PBS):

NaCl	80.0g
KCl	2.0g
Na <sub>2</sub> HPO <sub>4</sub>	14.4g
KH <sub>2</sub> PO <sub>4</sub>	2.4g
Distilled water to 1L, pH to 7.2	

10X Tris buffered saline (TBS):

Tris base	30.285g
NaCl	43.83g
Distilled water to 500mL, pH to 7.5	

2.6.4. Reprobing Western immunoblots. (Kaufmann et al., 1987)

If the Western immunoblot was to be reprobed with a second primary antibody, the first antibody was removed by heating the blot to 70°C for 30min in 3% (w/v) SDS in 1X PBS. The SDS was removed by two rinses in 1X PBS. The blot was blocked overnight as before (Section 2.6.3), and the second antibody was added and development carried out as before. Care was taken not to let the blot dry out at any stage.

2.6.5. Immunoprecipitation. (Rees, 1986)

Samples used for immunoprecipitation were minicells, *in vitro* transcription-translation products or whole cells labelled as described in Sections 2.4.1, 2.4.2 and 2.4.3, respectively. 0.5mL of the labelled cells or 10 A<sub>600</sub> Units of the minicells sample were washed once in 10mM Tris-HCl, pH 7.5 to remove excess radioactivity. Whole cells and minicells were resuspended in 0.5mL of 1% (w/v) SDS; 10mM Tris-HCl, pH 8.0; 1mM EDTA. They were boiled for 2min to lyse the cells. The tubes were centrifuged for 5min to remove debris. The supernatants were transferred to a fresh tube and 0.65mL of Triton mix (2% (v/v) Triton X-100; 50mM Tris-HCl, pH 8.0; 150mM NaCl; 1mM EDTA) was added. In the case of *in vitro* transcription-translation products, 0.65mL Triton mix was added directly to the translation products. 5-10µL antibody was added, mixed gently and allowed to incubate with gentle shaking for 90min on ice or overnight at 4°C.

Protein-A conjugated Sepharose beads (Sigma Chemical Co.) were used to specifically bind the IgG fraction in the mixture. The beads were resuspended at 10mg/mL in NET (50mM Tris-HCl, pH 7.4; 150mM NaCl; 5mM EDTA) and incubated at 4°C for 20min. The beads were recovered by centrifugation at 6Krpm for 30sec at room temperature in an MSE microcentrifuge. The beads were resuspended to give a 10% (w/v) suspension in NET. 25µL of this suspension was added to each immunoprecipitation sample and incubated on ice for 1 hour with gentle shaking. The beads were then recovered by centrifugation at 6Krpm as above, and washed three times with NET. They were then resuspended in 30µL of Urea mix (6M urea; 4% (w/v) SDS; 0.5M 2-

mercaptoethanol). An equal volume of SDS loading buffer was added and the samples were heated to 100°C for 3min and the proteins separated by SDS polyacrylamide gel electrophoresis (Section 2.4.5). The gel was fluorographed and exposed to X-ray film (Section 2.4.6).

## 2.7. Tissue culture methods.

### 2.7.1. Cell lines.

The cell lines used were:

*Culex quinquefasciatus* C2

*Anopheles gambiae*

Both cell lines were established from trypsinized larval tissue and were gifts from Dr. D. J. Ellar, Department of Biochemistry, University of Cambridge, Cambridge, U. K.

### 2.7.2. Growth, storage and handling.

The cells were grown in 50mL Sterilin flasks in Mitsushashi and Maromorosch medium (GIBCO BRL) containing 10% (v/v) foetal calf serum (GIBCO BRL) and 50µg/mL gentamycin (Sigma Chemical Co.). Cells were grown at 28±1°C. Carbon dioxide incubation was not necessary. Cells were handled in a Laminar flow containment cabinet.

Cells were subcultured upon confluence, at an average of 4-5 days. The medium was aspirated off and the cells were washed and resuspended in 2mL Mitsushashi and Maromorosch (MM) medium without foetal calf serum. The cells were shaken loose from the flask and 5 drops were transferred with a sterile Pasteur pipette to a fresh flask containing 10mL Mitsushashi and Maromorosch medium and 10% (v/v) foetal calf serum. The flasks were incubated flat until the cells were again confluent.

For storage, the cells were decanted after washing in medium free of foetal calf serum into a sterile centrifuge bottle and centrifuged to pellet the cells at 1.5Krpm for 5min at room temperature. The cells were resuspended in Mitsushashi and Maromorosch medium without foetal calf serum to 2-5 x 10<sup>6</sup> cells/mL counted by a haemocytometer. The cells were placed on ice to cool. Sterile, cooled DMSO was then added to a

final concentration of 10% (v/v). The tubes were placed immediately on ice and the cells aliquoted into cryotubes (1-1.5mL ampoules). The cells were allowed to freeze slowly in liquid nitrogen vapour overnight. The cells were then transferred to metal inserts and immersed in liquid nitrogen. Cells were checked for viability after 2 days.

### 2.7.3. In vitro toxicity assays.

The cells were grown to confluence and then washed in Mitsunashi and Martomrosch medium without foetal calf serum. The cells density was then adjusted to  $2 \times 10^6$  cells/mL as described (Section 2.7.2) and 50 $\mu$ L of cells were transferred to the wells of the microtitre plates and placed for 30min at room temperature. The solubilized *Bacillus sphaericus* crystal samples were then added and the cells incubated for upto 2.5 hours. Morphological changes, eg. swelling, were monitored under a microscope. Cell viability staining with trypan blue was not carried out. Cells were photographed using an Olympus Camera.

### 2.8. In vivo toxicity assays.

*In vivo* toxicity assays using *E.coli* strains harbouring various plasmids were carried out using second and third instar *Culex quinquefasciatus* mosquito larvae collected from the wild. The larvae were washed in tap water, resuspended in tap water and starved for 6 hours. 10 larvae were then resuspended in 10mL tap water in sterile scintillation vials. Different concentrations ( $A_{600}$ ) of *E.coli* cells were added to the vials and toxicity was monitored over 24 hours. Controls included larvae starved for the period of the assay and larvae to which equal volumes of distilled water, as used in the experiments, was added. Mortality was defined as the lack of response to tactile stimulation using a toothpick or by swirling.

### 2.9. RNA methods.

All solutions and materials that were anticipated to come into contact with the RNA prepared by treatment with 0.01% (v/v) diethylpyrocarbonate (DEPC) overnight and autoclaved in

order to render them RNase free. Tris solutions must not be treated with DEPC. In this case solutions are made using a fresh stock of Tris base.

2.9.1. Preparation of total *B.sphaericus* and *E.coli* RNA.  
(Chomczynski and Sacchi, 1987)

*B.sphaericus* and *E.coli* cells were pelleted by centrifugation in an eppendorf tube. If the  $A_{600}$  values of the cells was below 5, the cells were resuspended in a 100 $\mu$ L of Solution D (4M guanidium thiocyanate; 25mM sodium citrate, pH 7.0; 0.5% (w/v) sodium sarkosyl; 0.1M 2-mercaptoethanol). Cell suspensions with an  $A_{600}$  value greater than 5 were resuspended in 500 $\mu$ L of Solution D. The cells were vortexed and 10/50 $\mu$ L of 2M sodium acetate, pH 4 was added to the tubes. The solutions were mixed by vortexing. An equal volume of phenol-chloroform was added to the tubes and vortexed vigourously. The tubes were then placed for 15min at 60°C with occasional vortexing. The tubes were cooled by transfer to room temperature for 5min and then centrifuged to separate the phases. Phenol extraction was carried out three times and the RNA precipitated from the aqueous phase with 0.6 volumes of isopropanol at -20°C for 1 hour. The pellet was then resuspended in 30 $\mu$ L of Solution D and the RNA again precipitated by the addition of 0.6 volumes of isopropanol and the RNA collected by centrifugation at 13Krpm for 10min at room temperature. The pellet was washed with 70% (v/v) ethanol, the pellet dried under vacuum and resuspended in water. The amount of RNA in the preparation was determined by spectrophotometry at 260nm. The  $A_{260}/280$  value is 2 for pure RNA. An  $A_{260}=1$  corresponds to 40 $\mu$ g RNA.

For long term storage of RNA, 0.33 volume of 3M sodium acetate, pH 6.0 and an equal volume of isopropanol were added to the RNA stored at -20°C. The RNA was recovered by centrifugation and resuspended in water as required.

## 2.9.2. Analysis of RNA by transfer onto nylon membranes and hybridization using radioactive probes.

### 2.9.2.1. Northern blot filter hybridization.

To 4.8 $\mu$ L of 1mg/mL RNA the following reagents were added in the order given: 10 $\mu$ L deionized formamide, 2 $\mu$ L 10X MOPS buffer (0.2M MOPS; 0.05M sodium acetate; 0.01M EDTA, pH 7.0) and 3.2 $\mu$ L formaldehyde. The sample was incubated at 65°C for 5min, cooled immediately on ice and 0.1 volume of loading buffer (25% (w/v) Ficoll; 0.001% (w/v) Orange C; 200mM EDTA; 0.001% (w/v) bromophenol blue) and 1 $\mu$ L of 1mg/mL EtBr were added. The samples were then loaded onto a 1% (w/v) agarose gel made by dissolving the agarose in MOPS buffer containing 17.9% (v/v) formaldehyde. Electrophoresis was at 20V overnight with circulation of the buffer. The RNA was then transferred directly to Hybond-N as described for DNA (Section 2.3.12.1), except that the transfer buffer used was 20X SSC. Following transfer, the RNA was bound to the membrane by longwave ultraviolet transillumination for 4min.

To provide molecular weight markers, 10 $\mu$ g of  $\lambda$  DNA previously digested with the restriction enzyme *HindIII* was denatured by incubation for 5min at room temperature in 0.15M NaOH; 0.01M EDTA, pH 8.0. Loading buffer and EtBr were added to the single-stranded  $\lambda$  DNA. These markers were loaded well to one side of the gel. Following electrophoresis, the lane containing the markers was excised stained with EtBr and photographed.

### 2.9.2.2. "Slot-blot" analysis of RNA.

Samples containing the RNA were diluted to 50 $\mu$ L in distilled water and aliquoted onto Hybond-N membrane previously soaked in 10X SSC. A slot blot apparatus under vacuum was used. The filters were irradiated with longwave ultraviolet light to fix the RNA to the membrane.

## 2.9.3. Hybridization of RNA with labelled DNA probes.

Hybridization with DNA probes was carried out as described earlier for Southern blot hybridization analysis (Section 2.3.13). The probes used were radioactively labelled by the

oligolabelling method. (Section 2.3.11). If hybridization to a second probe was desired, the filters were not dried prior to autoradiography. Filters were exposed to FUJI X-ray film at  $-70^{\circ}\text{C}$  with intensifying screens. Stripping probes from membranes was carried out as described in Section 2.3.14.

#### 2.9.4. Densitometric analysis of autoradiographs.

Profiles of autoradiographic images of RNA filters were obtained by scanning the developed X-ray films with an LKB Ultrascan XL Laser densitometer. In order to give a linear response to radioactive decay, the X-ray film was made sensitive to low-level exposure to light prior to autoradiography as described in Section 2.4.6.

#### 2.10. Computing.

Analysis of sequencing data and protein comparisons were done using programmes in the University of Wisconsin Genetics Computer Group (UWGCG) Molecular Biology package of the University of Leicester VMS/VAXCluster. The plasmid diagrams were drawn using Corel Draw! software run on an IBM/PS2 computer. This thesis was written using the Microsoft Word wordprocessing programme, Version 5.0 (Microsoft Corporation) run on an Amstrad PC2086 computer.

#### 2.11. Source of materials.

Reagents and chemicals were of analytical grade and most were supplied by Fisons plc. or Sigma Chemical Co. Ltd, U. K. Chemicals obtained from other sources are listed below.

Chemical	Source
Radionucleotides	Amersham, England
Acrylamide	BDH Ltd., England
HGT agarose	FMC BioProducts, USA
Restriction enzymes	GIBCO BRL Ltd., Scotland
T4 DNA Ligase	"
T4 DNA kinase	GIBCO BRL Ltd., Scotland
Deoxynucleoside triphosphates	Pharmacia Ltd., England
Klenow fragment	"
IPTG	Novo Biochem

X-gal	Novo Biochem
Rabbit PAP	TAGO, USA
Tryptone	Difco, U. K.
Tryptose	"
Yeast extract	"
Hybond-N	Amersham, England
Nitrocellulose paper	Schleicher and Schuell, Germany

## 2.12. Containment and safety.

All experiments conducted in the work described in this thesis were done with reference to the Genetic Manipulation Advisory Group (UK) guidelines on safety and containment conditions for such work. All procedures fall within the category of good microbial practice.

## Chapter 3.

### Identification of the toxin gene products from *B.sphaericus* 1593M.

#### 3.1. Introduction.

The genes used in the investigation reported in this thesis were originally cloned from *B.sphaericus* 1593M as described by Souza et al. (1988) and by Souza (1987). *B.sphaericus* 1593M is a derivative of strain 1593 obtained following passages through mosquito larvae (Jamuna et al., 1982). In brief, for the cloning of the toxin genes, the *Bacillus sphaericus* 1593M chromosome was partially digested with the restriction enzyme *EcoRI*. The resulting DNA fragments were cloned directly into the vector pBR322, which had previously been digested with *EcoRI* and the 5' extensions dephosphorylated. Ampicillin resistant transformants of *Escherichia coli* were then tested qualitatively for toxicity to the larvae of the mosquito *Culex quinquefasciatus*. The toxicity analysis consisted of resuspending the *E.coli* cells containing recombinant plasmids in 10mL of distilled water containing 10 test larvae (Chapter 2, Section 2.8). The ability of the cells to kill the larvae was monitored over a 48 hour period. *E.coli* strains containing the vector pBR322 showed no toxicity. Of 2000 recombinants screened, four transformants were identified as expressing larvicidal activity when whole cells were fed to the larvae. Three of these four recombinant plasmids were found to contain an apparently identical 8.6Kb insert when examined by restriction enzyme digestion analysis. The other recombinant plasmid contained a 15.7Kb insert. These two types of plasmid are referred to as pAS233 and pAS377, respectively (Figure 3.1).

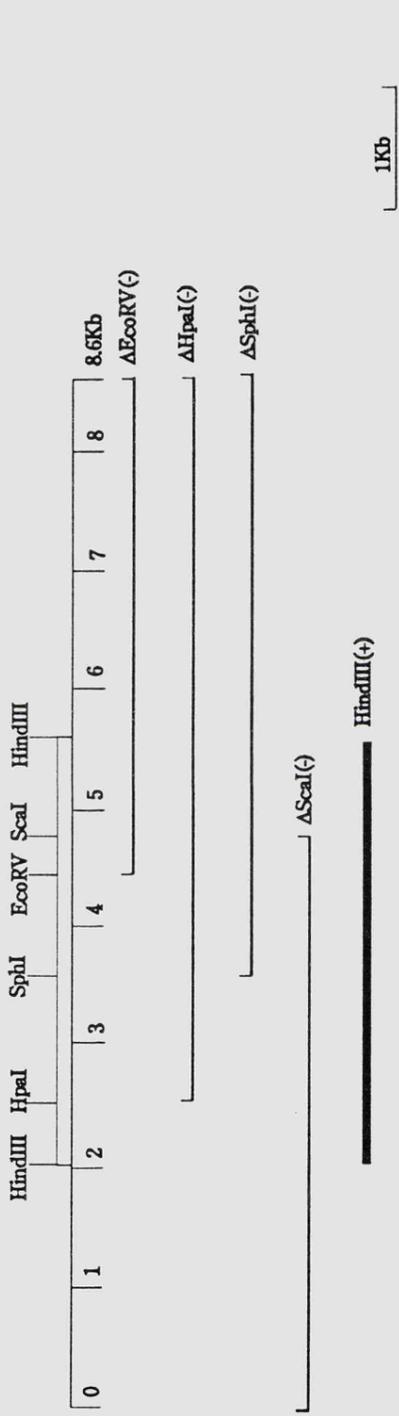
In order to determine more precisely which regions of the recombinant plasmids were necessary for toxicity towards mosquito larvae *in vivo*, several deletions of portions of the 8.6Kb insert, using existing restriction enzyme sites, were constructed (Souza, 1987), and also tested for larvicidal activity. In Figure 3.1, the deletions that led to loss of toxicity are marked (-), and those in which the deletion of a part of the insert did not affect toxicity are indicated by a (+). In this way, a 3.6Kb *HindIII* fragment internal to the

Figure 3.1 Deletion analysis of the plasmids pAS233 and pAS377.

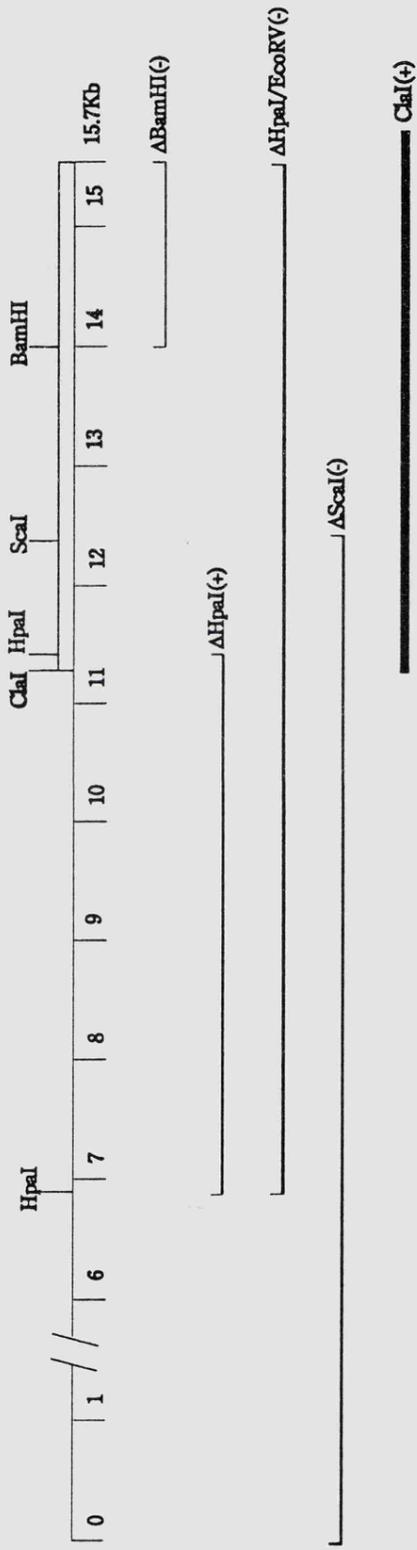
The inserts of the plasmids pAS233 (Panel A) and pAS377 (Panel B) were subjected to deletion analysis using existing restriction enzyme sites to determine the location of the gene encoding the larvicidal protein. Demarcated regions beneath the maps show the portion of the insert deleted. A "+" to the left indicates the retention of the ability of the insert to confer larvicidal activity for mosquitoes on recombinant *E.coli* in bioassays, and a "-" indicates the lack of larvicidal activity of recombinant *E.coli* cells. Only the insert is shown in each case.

Figure adapted from Souza (1987).

A. Insert of pAS233



B. Insert of pAS377



8.6Kb fragment (Figure 3.1A), was identified, which was likely to encode toxicity, and was subcloned into the *Hind*III site of the vector, pBRAT. pBRAT was previously constructed by digestion of the vector pBR322 with the restriction enzymes *Pvu*II and *Eco*RV followed by religation, resulting in the loss of tetracycline resistance of the plasmid (Souza, 1987). The pAS233 *Hind*III subclone is referred to as pAS233HA (*Hind*III, Amp<sup>R</sup>), (Figure 3.4).

Similarly, Souza (1987) subcloned portions of the pAS377 insert into pBR322, and *E.coli* cells harbouring the recombinant plasmids were tested for toxicity to mosquito larvae as above. Using the data shown in Figure 3.1B this allowed the identification of regions that were necessary for the larvicidal activity of the insert. The 15.7Kb fragment of pAS377 was then subcloned to the smallest fragment retaining toxicity, a *Cla*I fragment of 4.3Kb (Figure 3.1B).

The deletion analysis described above indicated that the 3.6Kb *Hind*III fragment from pAS233HA might encode all of the toxin gene, and indeed this fragment was shown to be associated with larvicidal activity when cloned in either orientation in pBRAT, and transformed into *E.coli* (Souza, 1987). This result also indicated that toxin production in this recombinant might be controlled by its own promoter, which was functional in *E.coli*. However, *E.coli* maxicell analysis performed by Souza *et al.* (1988) failed to detect any protein product encoded by this fragment, although the maxicell strain in question was toxic to mosquito larvae after transformation with the plasmid pAS233HA.

This chapter deals with the identification and analysis of the gene products encoded by the recombinant plasmid, pAS233HA.

### 3.2. Screening for the presence of the 3.6Kb insert in other strains of *Bacillus sphaericus*.

It was considered important at the outset of this study to discover whether the 3.6Kb DNA fragment cloned from *B.sphaericus* 1593M was present in other larvicidal strains of *B.sphaericus*, and also whether it was present in poorly toxic

and non-toxic strains of *B.sphaericus*. The presence of the DNA fragment in the latter would indicate that the gene was not sufficient for the larvicidal activity of the toxic strains, or that the gene in non-toxic strains was rendered non-functional in some manner.

An ideal way to infer the presence of the gene in the genome of the test strains was to conduct a DNA-DNA hybridization (Southern hybridization) analysis, using the 3.6Kb *HindIII* fragment as the radioactively labelled DNA probe. Five strains of *B.sphaericus* were used in the analysis, of which three, 1593, 2297 and 2013-4 (de Barjac et al., 1988), are known to be highly toxic, SSII-1, which is poorly toxic (Myers et al., 1979), and 14577, which is non-toxic (Davidson, 1981).

Chromosomal DNA was isolated from these strains by CsCl-density gradient centrifugation (Chapter 2, Section 2.3.3). The chromosomal DNA from each of the strains was digested separately with the restriction enzymes *EcoRI* and *HindIII*, and the DNA fragments separated on a 1% (w/v) agarose gel (Figure 3.2A). Since the assay was qualitative, it was not necessary to load equal amounts of DNA in each lane of the gel. The size-separated fragments were transferred to nitrocellulose paper by capillary action as described in Chapter 2, Section 2.3.12.1. The DNA was fixed to the nitrocellulose paper by baking at 80°C under vacuum for 2 hours. Hybridization was performed using the 3.6Kb *HindIII* fragment isolated from the plasmid pAS233HA as a radioactively labelled DNA probe. All washes were performed at 62°C, exactly as described in Chapter 2, Section 2.3.13.

A small quantity (0.1ng) of  $\lambda$  DNA digested with the restriction enzyme *HindIII* was added to visualize the  $\lambda$  DNA size markers on the nitrocellulose paper. In a separate experiment, I had previously shown that there was no cross-hybridization of  $\lambda$  DNA with the *B.sphaericus* DNA. Thus, endonuclease digested and single-stranded chromosomal DNA from the different strains was dotted onto nitrocellulose paper and hybridized with radioactively labelled  $\lambda$  DNA previously digested with *HindIII*.  $\lambda$  DNA did not hybridize with *B.sphaericus* DNA in Southern hybridization analysis. Single

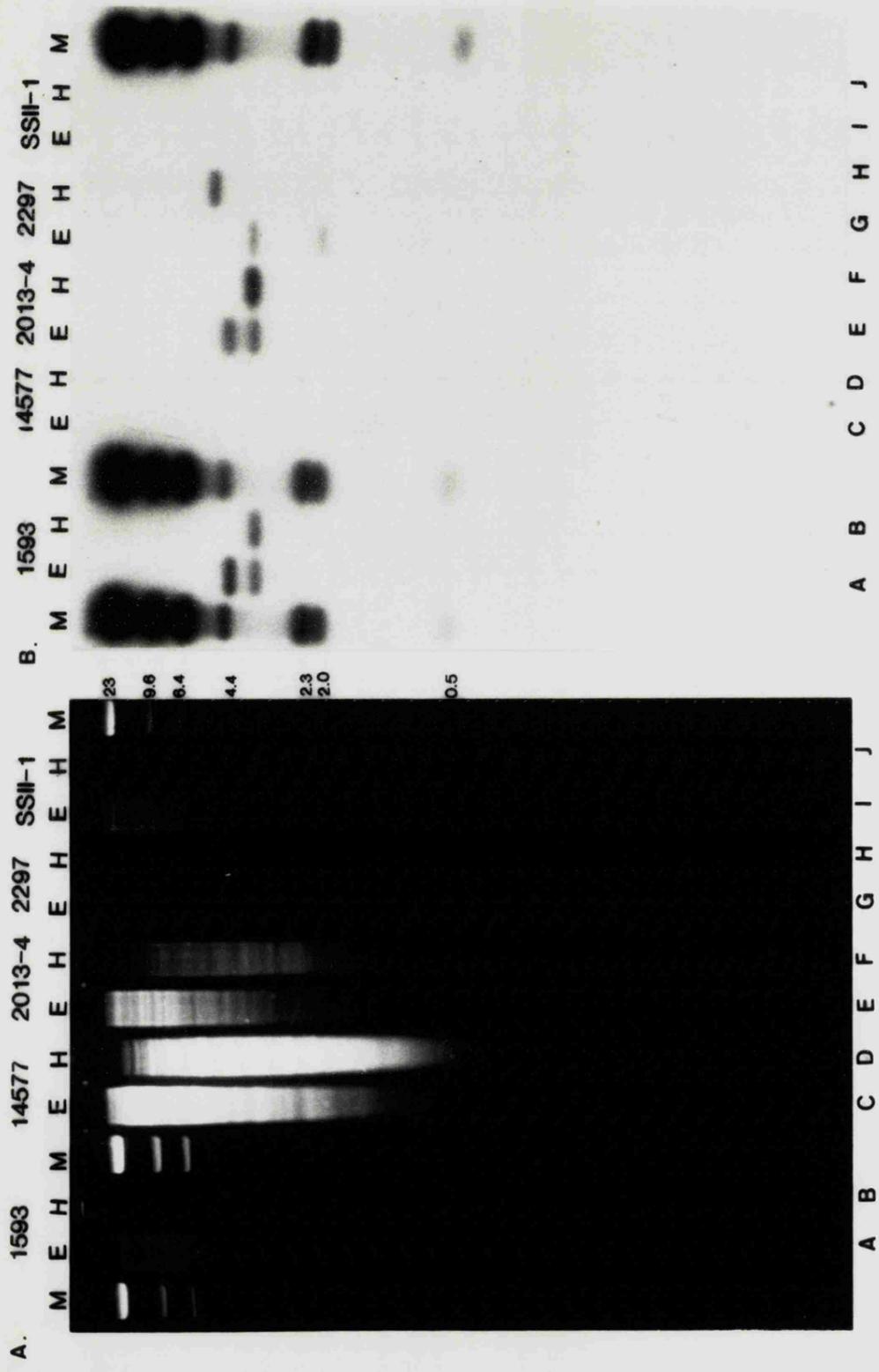
Figure 3.2. Screening for the presence of the 3.6Kb insert in *B.sphaericus* strains by Southern hybridization.

Chromosomal DNA was isolated from *B.sphaericus* strains 1593, 14577, 2013-4, 2297 and SSII-1 as described in Chapter 2, Section 2.3.3. 10 $\mu$ L of each DNA preparation was digested overnight with 10Units (manufacturer's definition) of the restriction enzymes *Eco*RI and *Hind*III. The fragments were electrophoretically separated overnight by applying 10Volts across a 1% (w/v) agarose gel. The DNA in the gel was denatured and transferred to nitrocellulose paper as described in Chapter 2, Section 2.3.12.1. The nitrocellulose paper was subjected to hybridization conditions as described in Chapter 2, Section 2.3.13. Washes were conducted at 62 $^{\circ}$ C using 1X SSC; 0.1% (w/v) SDS; 10 $\mu$ g/mL denatured herring sperm DNA. The DNA probe used was the 3.6Kb *Hind*III fragment of pAS233HA, radiolabelled as described in Chapter 2, Section 2.3.11. and also containing 0.1ng of  $\lambda$  DNA digested with *Hind*III. Figure 3.2A refers to the DNA in the agarose gel stained with ethidium bromide prior to transfer to nitrocellulose. Figure 3.2B shows the autoradiograph obtained after hybridization.

Lanes indicated are chromosomal DNA from the strains 1593, 14577, 2013-4, 2297 and SSII-1 digested with the enzymes *Eco*RI (E) or *Hind*III (H). The DNA size markers in lane M show  $\lambda$  DNA digested with *Hind*III. DNA fragment sizes are indicated in Kb.

Data summarizing sizes of hybridized fragments obtained.

Strain	Restriction enzyme	DNA fragment sizes (Kb)
1593	<i>Eco</i> RI	4.1; 3.3
	<i>Hind</i> III	3.4
2013-4	<i>Eco</i> RI	4.3; 3.4
	<i>Hind</i> III	3.5
2297	<i>Eco</i> RI	3.5; 2.0
	<i>Hind</i> III	4.9



Ethidium bromide stain

3.6Kb DNA probe

stranded  $\lambda$  DNA was also included as a control, and this alone hybridized with the  $\lambda$  DNA probe (data not shown).

From Figure 3.2B, it is evident that there is a positive hybridization signal in the *B.sphaericus* strains 1593, 2297 and 2013-4 with the toxin gene probe. Since these three strains are highly larvicidal, the observation lends credence to the possibility that these strains are toxic by virtue of possessing the same, or closely related gene(s). The fact that the genes are not necessarily identical can be seen from the different hybridization pattern produced by *B.sphaericus* 2297 compared to that of strains 1593 and 2013-4 (Figure 3.2B). The pattern for strain 2297 indicates a shift in the positions of the *EcoRI* and *HindIII* restriction enzyme sites (see Legend to Figure 3.2 for DNA fragment sizes). Significantly, the 3.6Kb DNA probe hybridized neither to the poorly toxic strain SSII-1, nor to the non-toxic strain 14577. This indicates that the gene contained on the 3.6Kb fragment probably confers high larvicidal activity on strains containing this DNA.

### 3.3. Identification of the toxin gene products.

The use of *E.coli* "minicells" allows the identification of proteins encoded by genes present on high copy number plasmids (Stoker *et al.*, 1984). In this procedure, a strain of *E.coli* carrying a cell division mutation, *minB* (Adler *et al.*, 1967) is transformed with the plasmid of interest (preferably in high copy). The *minB* mutation causes asymmetrical cell division to take place at high frequency, leading to the formation of "minicells" which lack the chromosome, but which do contain plasmid molecules partitioned non-specifically with the cytoplasm. Purification of minicells followed by radioactive labelling of proteins allows the selective labelling of plasmid-encoded proteins.

Plasmid pAS233HA (Figure 3.4) was accordingly transformed by the rapid  $\text{CaCl}_2$  procedure (Chapter 2, Section 2.2.3.1) into the *E.coli* minicell strain DS410"T" (Dougan and Sherrat, 1977), which contains the transposon Tn10 carrying the tetracycline marker in the chromosome. The parent plasmid pBRAT was also transformed into strain DS410"T" to be used as

a negative control. Transformants were plated out on Luria agar containing ampicillin to select for the presence of the respective plasmids. Plasmid DNA was then prepared from the transformants by the minipreparation method (Chapter 2, Section 2.3.2) and its identity verified by restriction enzyme digestion analysis.

The DS410"T" colonies carrying the appropriate plasmids identified in this way were grown overnight in Luria broth in the presence of tetracycline and ampicillin. The overnight culture was used as inoculum for a 400mL culture of Terrific broth containing ampicillin, from which the minicells were isolated as described in Chapter 2, Section 2.4.1. Addition of tetracycline to these larger cultures was found to lower the yield of minicells, and was avoided in these experiments. The purified minicells were labelled for 1 hour at 37°C with <sup>35</sup>S-methionine, the cells lysed by heating at 100°C for 3min in SDS loading buffer, and the proteins then separated on an SDS polyacrylamide gel. The gel was fluorographed and exposed to X-ray film in order to visualize labelled proteins (Chapter 2, Section 2.4.6). The profile seen in Figure 3.3A, lane c shows the result obtained.

The analysis revealed the presence of two primary protein products apparently encoded by the 3.6Kb *Hind*III fragment of *B.sphaericus* DNA, of sizes 41KDa and 59KDa, respectively. The 31 and 29KDa products also seen in the protein profile correspond in all probability to the pre- and mature  $\beta$ -lactamase proteins, and are also present in the analysis of the vector control (Figure 3.3A lane a). The clear cut nature of the results obtained indicated that the 41 and 59KDa proteins are probably encoded by distinct genes, perhaps expressed from *B.sphaericus* promoters capable of being recognized by the *E.coli* transcription machinery. The results, however, did not exclude the possibility that a single protein expressed from the *B.sphaericus* DNA was being cleaved or processed by *E.coli* enzymes, and that expression in fact emanated from a vector promoter.

Figure 3.3A Minicell analysis of the plasmid pAS233HA and the deletion derivatives, pHAΔEI and pHAΔEV.

Minicells were prepared from the *E.coli* strain DS410"T" transformed with the plasmids pAS233HA, pHAΔEI and pHAΔEV as described in Chapter 2, Section 2.4.1. Plasmid-encoded proteins were selectively labelled by incubating minicells in minimal medium containing <sup>35</sup>S-methionine. Minicell proteins were separated following heating to 100°C for 3min in SDS loading buffer on an 11% separating, 5% stacking SDS polyacrylamide gel. 0.5 A<sub>600</sub> Units of minicells were used in each lane. The gel was subjected to fluorography. An image of the X-ray film is shown in the figure.

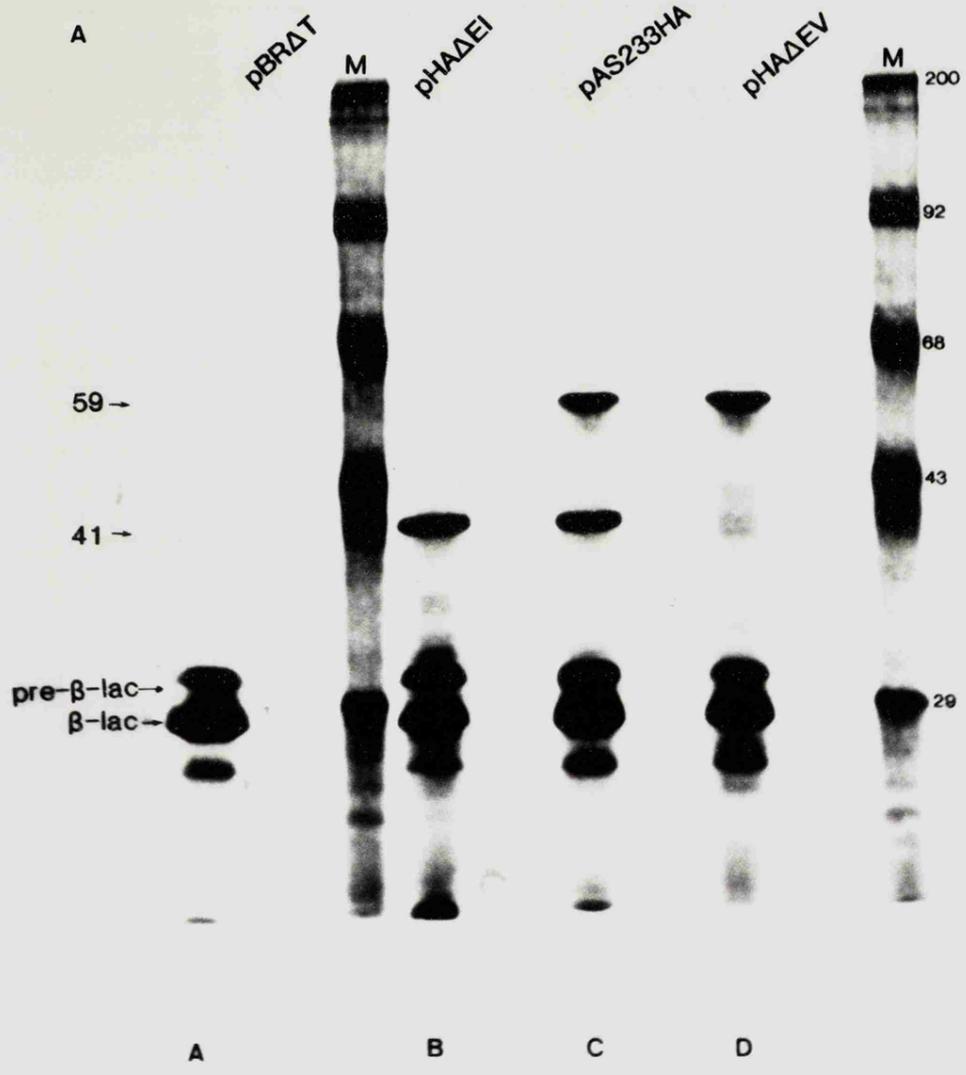
Lanes:

- A, minicells containing the vector control plasmid pBRAT
- M, Protein standards, with sizes indicated in KDa
- B, minicells containing the plasmid pHAΔEI
- C, minicells containing the plasmid pAS233HA
- D, minicells containing the plasmid pHAΔEV.

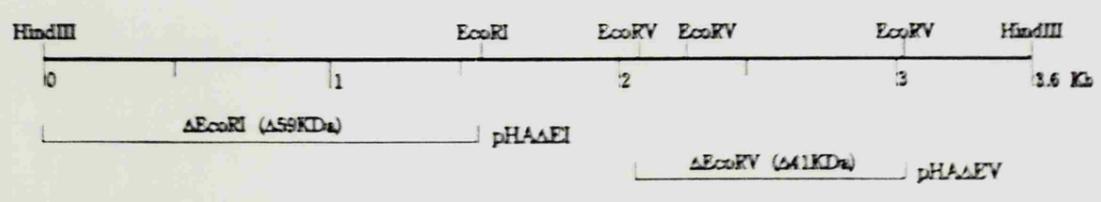
The sizes of proteins obtained in the minicells are shown. "Pre-β-lac" and "β-lac" indicate the positions of the vector encoded pre- and mature β-lactamase proteins.

Figure 3.3B Restriction enzyme map of the 3.6Kb insert showing positions of the deletions giving rise to the plasmids pHAΔEI and pHAΔEV.

The regions of the 3.6Kb insert of pAS233HA that were deleted in order to create the plasmids pHAΔEI and pHAΔEV are shown in relation to the restriction enzyme map of the insert.



**B**



### 3.3.1. Analysis of deletions of the plasmid pAS233HA in minicells.

In order to test the possibility of cleavage or processing of a larger precursor protein leading to the production of 41 and 59KDa proteins, deletions were made within the 3.6Kb *HindIII* insert of pAS233HA prior for further analysis in minicells. The first deletion removed the 1.6Kb fragment between the *EcoRI* site in the vector pBRAT and the *EcoRI* site in the insert, creating pHAΔEI (Figure 3.3B; see Figure 3.4 for plasmid construction). A second, independent deletion was obtained by cutting the plasmid pAS233HA at the proximal and distal *EcoRV* sites in the 3.6Kb insert, creating pHAΔEV (Figure 3.3B, and Figure 3.4 for plasmid construction).

When these constructs were transformed into the minicell strain DS410"T" as described above and analyzed by radioactively labelling the purified minicells, the profiles seen in Figure 3.3A, lanes B and D, were observed. The protein profile of the plasmid pHAΔEV (Figure 3.3A, lane D) compared with the protein profile of the parent plasmid pAS233HA, revealed that the 59KDa band was retained whereas the 41KDa band was no longer visible. This indicates that the *EcoRV* deletion either disrupted the sequence coding for the 41KDa protein, or that it removed the promoter. Similarly, comparison of the profile for pHAΔEI (Figure 3.3A, lane B) with the pAS233HA profile (Figure 3.3A, lane C), showed that the 41KDa protein band was present, whilst the 59KDa protein band was absent. This indicated that the *EcoRI* deletion had either disrupted the sequence coding for the 59KDa protein or interfered with its production, whilst not affecting the 41KDa protein in any way.

From this analysis it can be inferred that the sequence coding for the 59KDa protein is located on the left of the 3.6Kb insert, or spanning the *EcoRI* site, and that of the 41KDa protein is located on the right, as it was disrupted by the *EcoRV* deletion (Figure 3.3B). This analysis also indicated that there were two discrete protein products encoded by the 3.6Kb insert of pAS233HA.

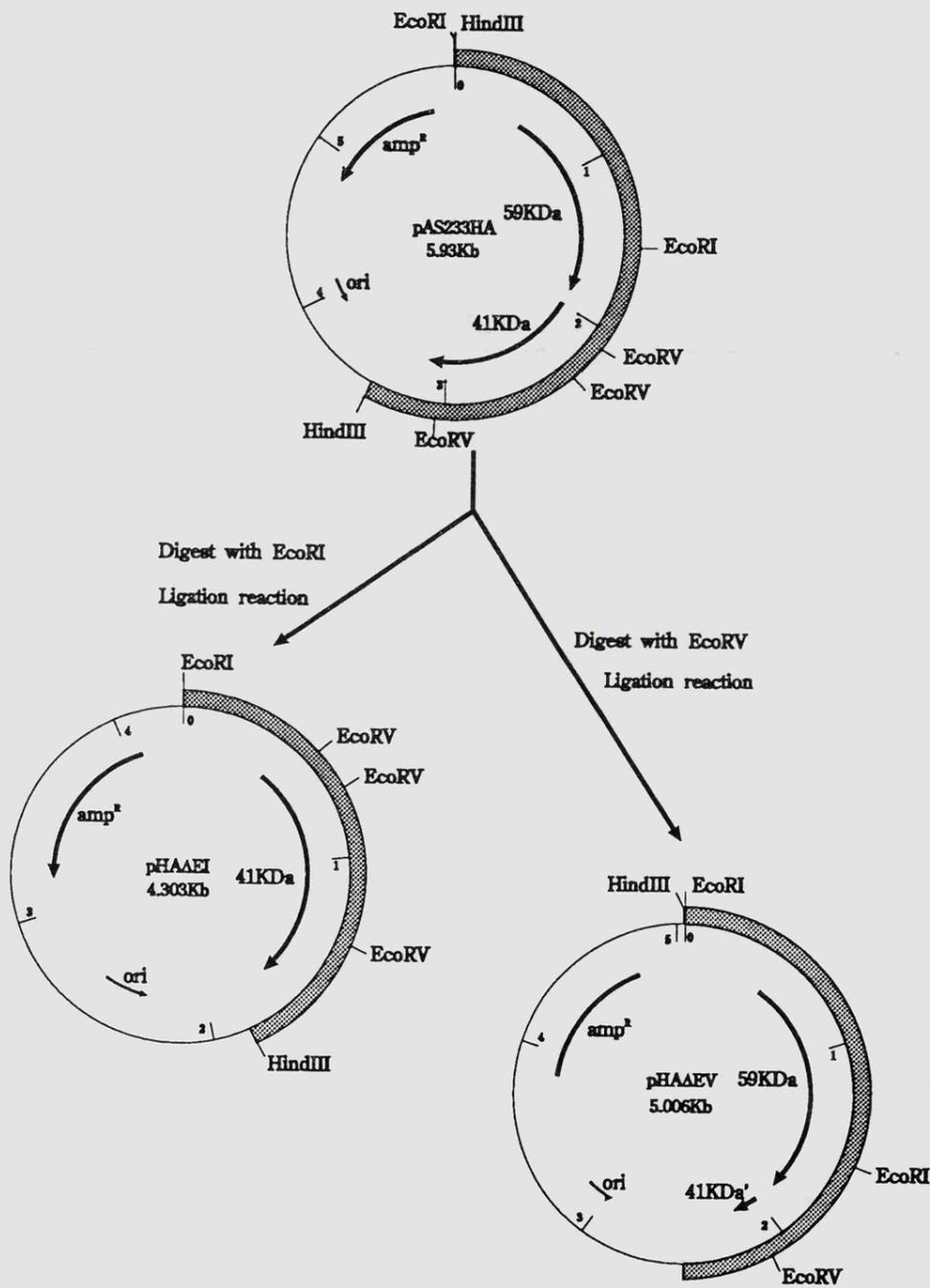
The above conclusions were confirmed using the same

Figure 3.4. Plasmid map for the construction of pHAΔEI and pHAΔEV.

The plasmid pAS233HA was constructed by subcloning a 3.6Kb *Hind*III restriction enzyme fragment from the 8.6Kb insert of the plasmid pAS233, which conferred mosquito larvicidal activity on *E.coli* (Section 1.1; Souza, 1987).

To create the deletion derivative pHAΔEI, the plasmid pAS233HA was digested with the restriction enzyme *Eco*RI and the plasmid religated at low DNA concentrations. The ligation mixture was transformed into *E.coli* by the rubidium chloride method (Chapter 2, Section 2.2.3.2). Digestion of plasmid DNA isolated from ampicillin resistant colonies with *Eco*RI and screening for the presence of a single *Eco*RI site led to the identification of a colony containing pHAΔEI.

pHAΔEV was obtained in a similar manner as pHAΔEI, by the digestion of the plasmid pAS233HA with *Eco*RV, religation of the plasmid at low DNA concentrations, and screening plasmid DNA isolated from *E.coli* transformants for the presence of a single *Eco*RV site. Plasmids are not shown to scale.



plasmids to direct synthesis of proteins *in vitro* in a coupled transcription-translation system (Pratt, 1984). The results obtained for each plasmid is shown in Figure 3.5A and was identical to that observed previously in minicells.

#### 3.4. The 41 and 59KDa proteins are localized to the cytoplasmic fraction of minicells.

It will be described later (see Section 3.6) that the 41 and 59KDa proteins are together required for larvicidal activity in bioassays. This observation could indicate a variety of means by which toxicity is effected through, for example, specific interactions of the two proteins. I decided therefore to fractionate minicells containing the plasmid pAS233HA to determine the localization of the 41 and 59KDa proteins. For example, association of one of the proteins with the *E.coli* membrane could indicate a strongly hydrophobic protein, and could have implications for the binding of the protein to larval cell membranes. Specifically, if one of the proteins was membrane bound, and the other was not, this would limit the forms of possible complexes of the two proteins. Binding of proteins to the membrane could also indicate that subsequent attempts to overexpress the protein in *E.coli* might prove toxic to the cell.

Minicells containing the plasmid pAS233HA encoding both proteins were purified and labelled with <sup>35</sup>S-methionine (Chapter 2, Section 2.4.1). The minicells were washed in bacterial (B) buffer (Chapter 2, Section 2.4.1) to remove excess radioactivity, and then in B buffer containing 20% glycerol. The cells were finally resuspended to 10 A<sub>600</sub> Units/mL and stored at -20°C.

An aliquot of the minicells used for the fractionation analysis was then again washed with ice-cold B-buffer to remove glycerol. The cells were pelleted for 3min at room temperature in an MSE microcentrifuge, and immediately resuspended in 10mM Tris-Cl, pH 7.8; 0.3M sucrose; 1mM EDTA containing 1mg/mL lysozyme. The suspension was gently shaken at 37°C for 60min in order to disrupt the outer cell wall and membrane. The sphaeroplasts were then pelleted by centrifugation for 5min at room temperature in an MSE

Figure 3.5A *In vitro* transcription-translation expression of polypeptides encoded by the plasmid pAS233HA and its deletion derivatives, PHAΔEI and PHAΔEV.

1.5μg of CsCl-purified plasmid DNA was used in each transcription-translation assay as described in Chapter 2, Section 2.4.2. A negative control lacking DNA was also included (lane not shown). The samples shown in lanes designated PHAΔEI and PHAΔEV were separated on a second gel run to the same distance.

Plasmid-encoded proteins were labelled with <sup>35</sup>S-methionine and the proteins separated, following heating to 100°C for 3min in SDS loading buffer, on 11% separating, 5% stacking SDS polyacrylamide gels. The gels were fluorographed, dried and exposed to X-ray film.

Lanes:

- A, negative control, vector pBRΔT alone
- M, protein standards with sizes shown in KDa
- B, proteins directed by the plasmid pAS233HA
- C, proteins expressed from the plasmid PHAΔEI
- D, proteins expressed from the plasmid PHAΔEV.

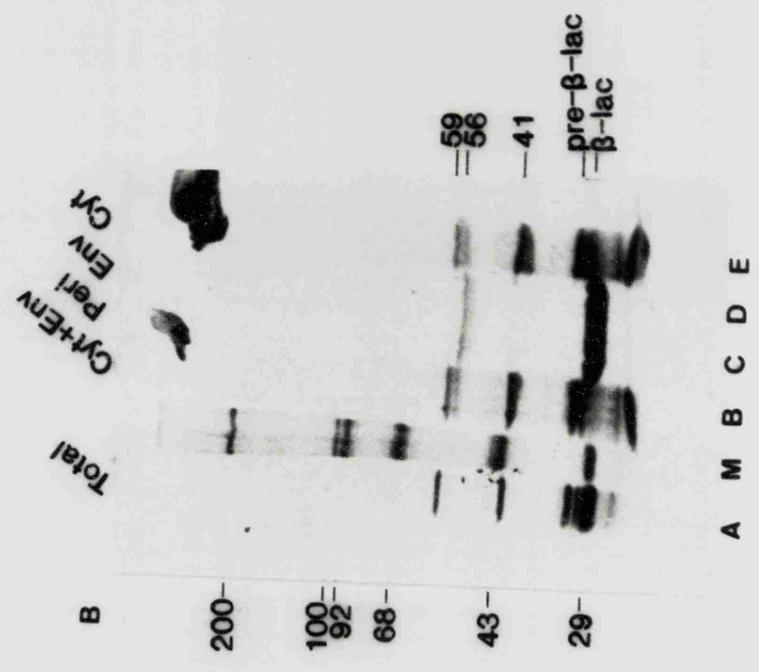
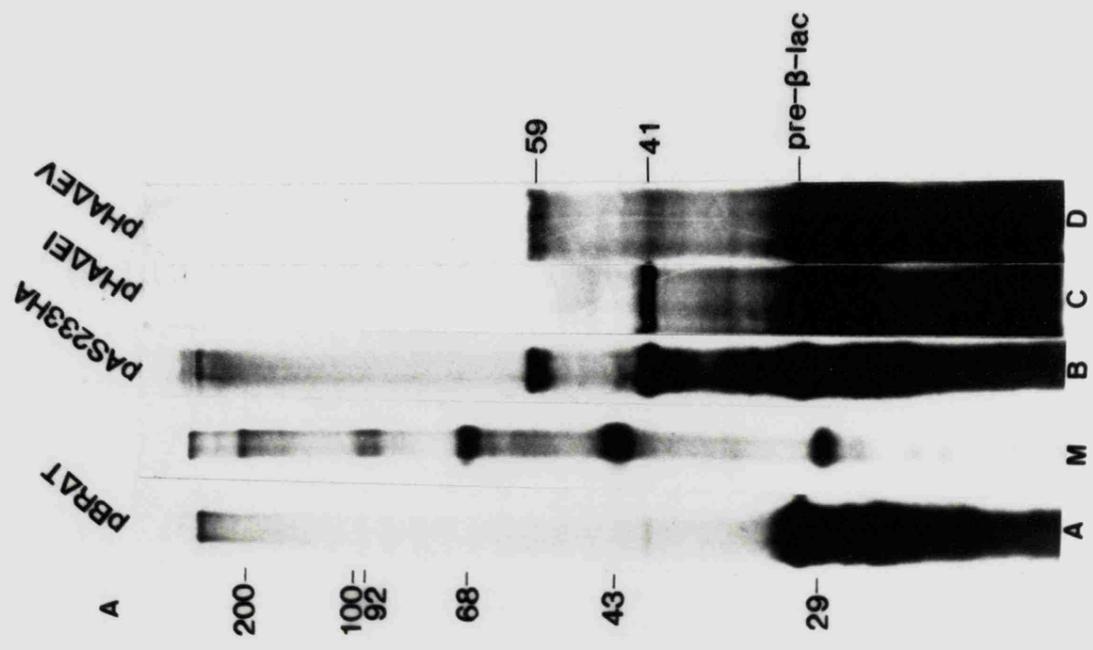
Figure 3.5B Fractionation of minicells containing the plasmid pAS233HA.

Minicells from *E.coli* DS410"T" cells containing the plasmid pAS233HA were prepared as described in Chapter 2, Section 2.4.1. and fractionated as described in Chapter 2, Section 2.4.1.1. The different fractions of the minicells were separated, following heating to 100°C for 3min in SDS loading buffer, on 8.5% separating, 5% stacking SDS polyacrylamide gels. The gels were fluorographed, dried and exposed to X-ray film.

Lanes:

- A, untreated control of labelled minicells (0.1 A<sub>600</sub> Units);
- M, protein standards with sizes indicated in KDa
- B, cytoplasm + envelope fraction (0.1 A<sub>600</sub> Units)
- C, periplasm (0.5 A<sub>600</sub> Units)
- D, envelope of inner and outer membranes (0.1 A<sub>600</sub> Units)
- E, cytoplasm (0.08 A<sub>600</sub> Units).

The sizes of the standards and major proteins are indicated in KDa. "Pre-β-lac" and "β-lac" refer to the pre- and mature β-lactamase proteins.



microcentrifuge. The supernatant containing periplasmic proteins was transferred to a fresh tube and subjected to TCA precipitation to concentrate the protein. The sphaeroplasts in the pellet were osmotically shocked to release the cytoplasm by resuspending them in distilled water. The supernatant (cytoplasm) and pellet (inner and outer membranes) were then separated by centrifugation in an MSE microcentrifuge for 10min at room temperature. The proteins in the cytoplasmic fraction were concentrated by TCA precipitation. The various fractions were mixed with SDS loading buffer and separated on an 8.5% SDS polyacrylamide gel. Following electrophoresis, the gel was subjected to fluorography, dried and exposed to an X-ray film to visualize labelled protein. The result obtained is shown in Figure 3.5B.

The whole minicell profile in lane A of Figure 3.5B shows the 59, 41 and pre- and mature  $\beta$ -lactamase proteins. In lane B, the lysozyme treated pellet containing the cytoplasmic and envelope fractions (sphaeroplasts) contain the majority of the unprocessed form of the  $\beta$ -lactamase protein, indicating satisfactory fractionation. In addition to the 41 and 59KDa proteins, there is a small quantity of a 56KDa protein present in this fraction. Figure 3.5B, lane C shows proteins present in the TCA precipitated supernatant following lysozyme treatment consisting of the proteins in the periplasmic fraction. The major protein in this case is the mature form of the  $\beta$ -lactamase protein that is known to be localized to the periplasm (Broome-Smith and Spratt, 1986). In addition, there is a protein present at 56KDa that corresponds to the protein seen in the cytoplasmic and envelope fractions following lysozyme treatment. Figure 3.5B, lane D shows the proteins in the envelope fraction following osmotic shock of sphaeroplasts, and the profile seen in this case largely resembles that seen in the periplasmic fraction indicating the affinity of the  $\beta$ -lactamase for the membrane under these conditions. The profile in Figure 3.5B, lane E shows the proteins present in the cytoplasm of the minicell. Here again, the  $\beta$ -lactamase precursor protein is present in large quantities. The 41KDa and 59KDa proteins are entirely cytoplasmic, together with small quantities of the 56KDa protein.

Thus, the fractionation of the minicells indicated that the 41KDa protein and the 59KDa protein appeared to be entirely cytoplasmic. The 56KDa protein could be a processed form of the 59KDa protein, and its presence in every fraction could indicate that it may be "sticky" and associate with membranes in a manner similar to  $\beta$ -lactamase. However, the presence of the 56KDa protein could not be reproduced satisfactorily in subsequent experiments and is probably an artefact of this particular experiment.

### 3.5. Attempts to set up an in vitro assay system using cultured mosquito cells.

In order to test the toxic activity of various combinations of the 41 and 59KDa toxins seen in the *E.coli* minicell assay system, we wished to establish an *in vitro* assay using cultured cells of the mosquitoes *Anopheles gambiae* and *Culex quinquefasciatus* C2. These cells were gifts from Dr. D. J. Ellar, University of Cambridge.

In order to set up such an assay system, crystal preparations from various strains of *B.sphaericus* were used to monitor cytolysis of cells *in vitro*. Davidson (1986) had reported that, although protease treatment increased the activity of the toxins of *B.sphaericus* 2362, such activation was not necessary for cytolytic activity of the toxin on the cells. In the experiment described below, the crystals were solubilized in 50mM sodium carbonate buffer, pH 10.5 for 90min at 37°C and added, after centrifugation to remove unsolubilized crystal, to either *A.gambiae* or *C.quinquefasciatus* C2 cells in the wells of a microtitre plate. In each assay,  $2 \times 10^5$  cells were used.

Prior to the addition of the crystal, the cells used in the assay were grown to confluence, washed in Mitsunashi and Maromorosch medium lacking Foetal calf serum, and allowed to adhere to the plate by standing for 30min at room temperature. 50 $\mu$ L of the solubilized crystal at different concentrations, ranging from 0.2 $\mu$ g to 0.001 $\mu$ g was added to each well, and cytolysis monitored microscopically over a 2.5 hour period. The control cells incubated with sodium carbonate buffer

showed no morphological changes compared to the untreated control over the assay period. Figure 3.6A shows control cells of *A.gambiae* incubated in sodium carbonate buffer. Figure 3.6B shows the same cells treated with 0.1µg of *B.sphaericus* 2297 crystal after a period of 2.5 hours. Although cell swelling is evident, no cytolysis was detected. This was typical of results seen with crystals from other strains of *B.sphaericus* incubated with *A.gambiae* cells.

In the case of *C.quinquefasciatus* C2 cells, no swelling was evident in the untreated control cells. When cell free lysates of *E.coli* containing plasmids encoding either the 41KDa (pHAAEI) or the 59KDa (pHAAEV) proteins were used in the assay, all the cells lysed within 5min (data not shown). In controls comprising *E.coli* cell free lysates containing plasmids encoding both the 41 and 59KDa proteins (pAS233HA) or even with the control vector (pBRAT) alone, the treated cells again showed rapid lysis. Apparently, the addition of *E.coli* cell lysates is cytolytic to these cells in culture (D. J. Ellar, personal communication), and this approach, using cell lysates had to be abandoned.

In my hands crystals not activated with proteases did not cause cytolysis of larval cells in culture as reported by Davidson (1986). In these experiments, however, cell death using a vital stain was not monitored. Consequently, exhaustive protein purification to obtain the 41 and 59KDa toxins free from contaminating *E.coli* proteins would be required for tests on cultured cells, and the establishment of such an assay system would therefore be time consuming. I decided therefore to use instead *in vivo* assays to monitor the effect on mosquito larvae of the addition of the 41 and 59KDa proteins in *E.coli* whole cells. An advantage of *in vivo* assays is that they are representative of the reaction of the whole organism to the toxin, whereas cell lines in culture are composed of a heterogeneous population of cells exhibiting varying levels of sensitivity to the toxin (Davidson, 1986). Moreover, in some cases, lines of cells are derived from whole organisms with no guarantee that the gut epithelial cells to which a toxin binds are represented (Knowles and Ellar, 1986; Hofte and Whiteley, 1989). Further toxicity assays were accordingly performed *in vivo* using larvae of

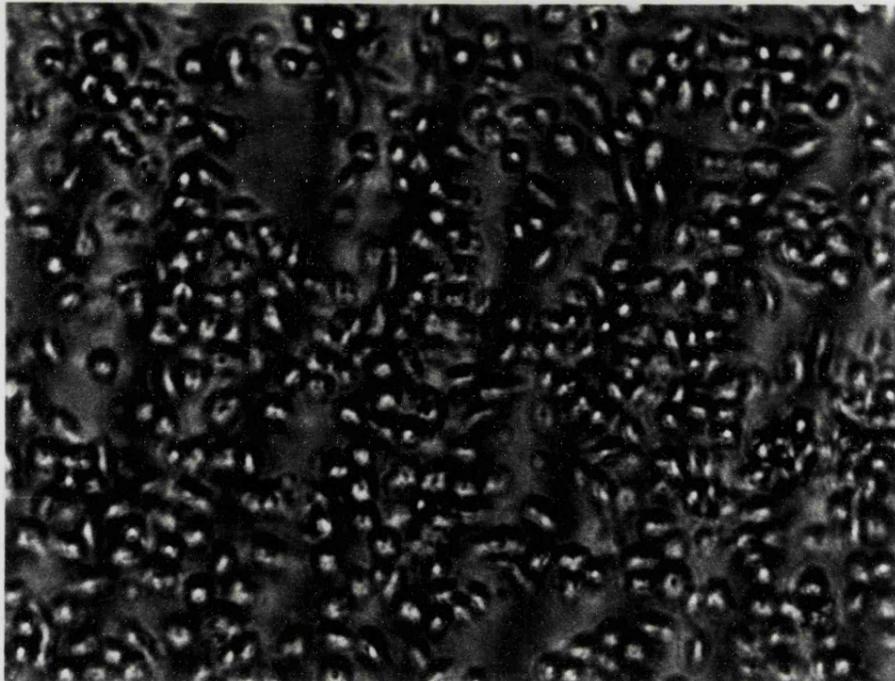
Figure 3.6 Treatment of *Anopheles* cells grown *in vitro* with a crystal preparation obtained from the *Bacillus sphaericus* strain 2297.

*Anopheles gambiae* cells were first grown *in vitro* in Mitsunashi and Maromarosch medium containing 10% foetal calf serum as described in Chapter 2, Section 2.7.2. The cells were grown to confluence and washed in Mitsunashi and Maromarosch medium lacking foetal calf serum. The cells were then shaken loose from the bottle and resuspended in Mitsunashi and Maromarosch medium lacking foetal calf serum to  $1 \times 10^6$  cells/mL. 200 $\mu$ L ( $2 \times 10^5$  cells) of the cell suspension was placed in the wells of a microtitre plate and allowed to stand at room temperature for 30min for the cells to bind to the walls of the well.

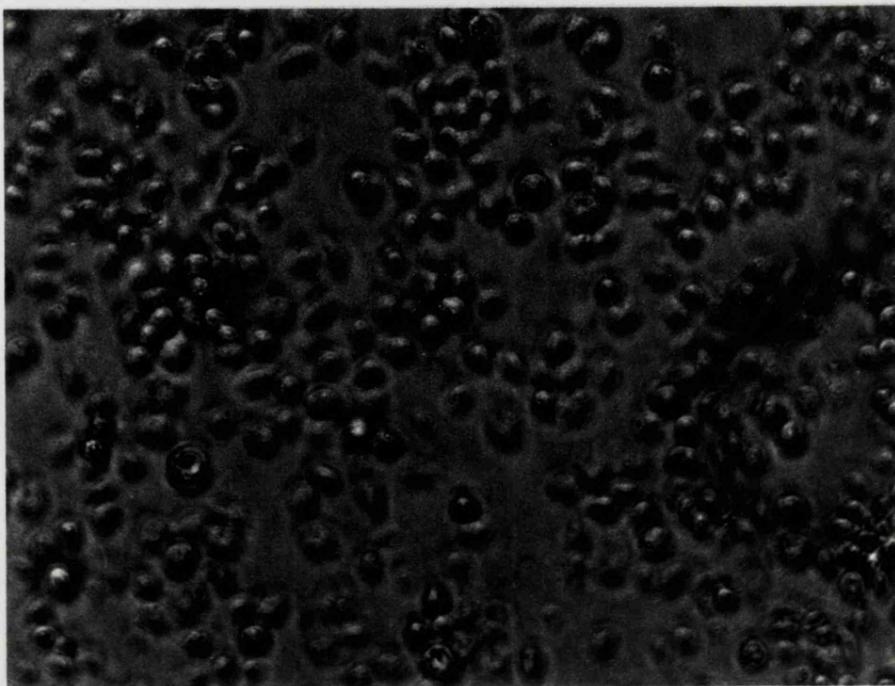
Crystal was prepared from *B.sphaericus* 2297 by the sucrose density gradient method (Chapter 2, Section 2.1.5.2) and resuspended at different concentrations (0.001 $\mu$ g to 0.2 $\mu$ g) in alkaline sodium carbonate, pH 10.5 for 90min at 37°C. This step was required to solubilize the crystal. The sample was centrifuged at 13 Krpm at room temperature for 5min, and the supernatant (50 $\mu$ L) added to the cells in the microtitre plate. Adequate buffer controls and untreated controls were also maintained.

The cells were monitored under a light microscope for 2.5 hours to detect gross morphological changes. Figure 3.5A shows cells of *Anopheles gambiae* treated with alkaline sodium carbonate buffer, pH 10.5. Figure 3.5B shows *A.gambiae* cells treated with 0.1 $\mu$ g of *B.sphaericus* 2297 crystals. The magnification in both cases is x10.

**A**



**B**



*C. quinquefasciatus* collected from the wild. These assays were performed in the laboratory of Prof. K. Jayaraman at the Department of Biotechnology, Anna University, Madras, India.

### 3.6. Analysis of larvicidal activity of pAS233HA and the deletion derivatives in vivo.

The constructs made in Section 3.3.1, together with the parent plasmid pAS233HA and the vector control, were tested for larvicidal directing activity *in vivo*, by feeding whole *E. coli* MC4100 cells containing the relevant plasmids to larvae of *Culex quinquefasciatus*, as described in Chapter 2, Section 2.8. The results obtained are presented in Table 3.1.

Although *B. sphaericus* 1593 is toxic for *C. quinquefasciatus* cells at a concentration of  $2.9 \times 10^2$  cells/mL (Myers *et al.*, 1979), the *E. coli* cells harbouring the plasmid pAS233HA were toxic for mosquito larvae at an  $LC_{50}$  of  $0.355 \times 10^7$  to  $1.4 \times 10^8$  cells/mL at 24h (Table 3.1). Although low, this value was obtained reproducibly and is substantially higher than the value for the *E. coli* cell containing the vector alone (100% survival at  $1.4 \times 10^8$  cells/mL at 36h). The  $LC_{50}$  value represents the concentration of cells in the medium required to kill 50% of the larvae. The deletion derivatives of pAS233HA, pHA $\Delta$ EI and pHA $\Delta$ EV in plasmids expressing either the 41 or the 59KDa proteins alone, were not toxic even at concentrations of  $1.4 \times 10^8$  cells/mL. This would suggest that the two proteins of 41 and 59KDa are required together to produce larvicidal activity. Interestingly, the plasmids pHA $\Delta$ EI and pHA $\Delta$ EV caused mortality when cells containing these constructs were mixed together and fed to the larvae. However, under these conditions, the effect was less than the use of the single plasmid strain (pAS233HA). This shows that whereas the insert of pAS233HA contains all the information required to encode larvicidal activity, the individual deleted plasmids do not.

Table 3.1

Toxicity of *E.coli* MC4100 cells harbouring plasmids derived from pAS233HA.

Toxicity assays were carried out using second- or third-instar larvae of the mosquito *Culex quinquefasciatus*. The larvae were collected from the wild, washed once in tap water and starved for 6 hours. 10 larvae were resuspended in 10mL of tap water in a sterile scintillation vial. Two sets of vials (total 20 larvae) were used at each concentration.

*E.coli* cells containing the plasmids, whose effect was to be assayed, were grown overnight and resuspended in distilled water to known concentrations of cells ( $A_{600}=1.4=10^9$  cells/mL; Miller, 1972). Different concentrations of these cells were added to the scintillation vials containing the test larvae. Unfed larvae and larvae to which an equal aliquot of distilled water as used in the assays was added, were also included in the control. The assays were carried out at 20-24°C. At the end of the test period at 24 hours, no mortality was seen in the unfed and distilled water controls. Mortality was defined as lack of response to tactile stimulation (Chapter 2, Section 2.8).

Table 3.1.

Toxicity of *E.coli* strain MC4100 harbouring plasmids derived from pAS233HA.

<u>Plasmids(protein)</u>	<u>No. of cells/mL</u>	<u>% survival</u>	
		<u>24 hours</u>	<u>36 hours</u>
pBR322 (none)	1.4 x 10 <sup>8</sup>	100%	100%
pHAΔEI (41)	1.4 x 10 <sup>8</sup>	100%	94.45%
pHAΔEI (41)	0.71 x 10 <sup>7</sup>	100%	100%
pHAΔEI (41)	0.355 x 10 <sup>7</sup>	100%	100%
pHAΔEV (59)	1.4 x 10 <sup>8</sup>	100%	100%
pHAΔEV (59)	0.71 x 10 <sup>7</sup>	100%	100%
pHAΔEV (59)	0.355 x 10 <sup>7</sup>	100%	100%
pAS233HA (41/59)	5.6 x 10 <sup>8</sup>	26.3% <sup>a</sup>	10.5% <sup>a</sup>
pAS233HA (41/59)	2.8 x 10 <sup>8</sup>	20% <sup>a</sup>	0%
pAS233HA (41/59)	1.4 x 10 <sup>8</sup>	47.4% <sup>a</sup>	10.5% <sup>a</sup>
pAS233HA (41/59)	0.71 x 10 <sup>7</sup>	55%	40%
pAS233HA (41/59)	0.355 x 10 <sup>7</sup>	100%	60%
pAS233HA (41/59)	0.71 x 10 <sup>6</sup>	84.2%	73.7%
pAS233HA (41/59)	0.355 x 10 <sup>6</sup>	100%	100%
pHAΔEI/pHAΔEV (41/59)	2.8 x 10 <sup>8</sup>	76%	38%
pHAΔEI/pHAΔEV <sup>b</sup> (41/59)	1.4 x 10 <sup>8</sup>	85%	45%
pHAΔEI/pHAΔEV <sup>b</sup> (41/59)	0.71 x 10 <sup>7</sup>	100%	52.4%
pHAΔEI/pHAΔEV <sup>b</sup> (41/59)	0.355 x 10 <sup>7</sup>	45%	35%
Crude crystal <sup>c</sup>	1.5μL	0%	0%
Crude crystal <sup>c</sup>	1.0μL	0%	0%
Crude crystal <sup>c</sup>	0.5μL	0%	0%
Crude crystal <sup>c</sup>	0.1μL	31%	26%

a: survivors were morbid

b: the number of cells shown from each strain was added

c: crystal isolated from *B.sphaericus* 1593 (approximately 1μg/μL)

### 3.7. Summary.

The experiments described in the preceding chapter deal with the identification of the proteins encoded by the 3.6Kb insert of the plasmid pAS233HA. This insert confers larvicidal activity on *E.coli* for the larvae of the mosquitoes *Culex quinquefasciatus* and *Anopheles gambiae*. Moreover, it was demonstrated that this 3.6Kb *Hind*III insert of the plasmid pAS233HA was present in highly toxic *B.sphaericus* strains such as 1593, 2297 and 2013-4, and absent from the weakly toxic and non-toxic strains, SSII-1 and 14577.

Using minicell analysis and *in vitro* transcription-translation assays, I was able to clearly show that the plasmid pAS233HA, which contains the 3.6Kb *Hind*III insert, encoded two proteins, a 41KDa and a 59KDa species. The approximate positions of the corresponding genes were located on the insert by deletion analysis. This was the first unequivocal identification of the proteins from *B. sphaericus* involved in larvicidal activity for mosquitoes. Fractionation analysis performed with minicells containing the plasmid pAS233HA showed that both the 41KDa and 59KDa proteins are exclusively localized to the cytoplasm of the minicell.

Although assays could not be performed on cultured mosquito cells *in vitro*, due to problems with cytolysis in the presence of *E.coli* cell proteins, *in vivo* assays using *C.quinquefasciatus* mosquito larvae showed that both the 41 and 59KDa proteins had to be present in the assay system in order to produce a larvicidal effect. However, the toxicity of the plasmid pAS233HA expressing both the 41 and 59KDa proteins in *E.coli* was significantly lower than that of the parent *B.sphaericus* 1593 strain, but was comparable to that of the weakly toxic *B.sphaericus* strain SSII-1, which has an LC<sub>50</sub> of  $2.5 \times 10^8$  cells/mL for *C.quinquefasciatus* larvae (Myers et al., 1979). This result indicated that the level of expression of the toxin in *E.coli* was very low, that some posttranslational activation step was absent in *E.coli*, or even that additional *B.sphaericus* proteins were required for full larvicidal activity.

The discovery of the 41 and 59KDa proteins as toxic,

larvicidal factors raised the question of their association with the "crystal", which is considered to contain the "toxic principle" in sporulated cells of *B.sphaericus* (Davidson, 1981; Baumann et al., 1985). This question is addressed in the next chapter.

## Chapter 4.

### Analysis of the 125KDa crystal protein of *B.sphaericus* 1593.

#### 4.1. Introduction.

It has been shown (Chapter 3) that the 3.6Kb insert of the plasmid pAS233HA encodes two proteins of 41 and 59KDa, both of which are essential for toxicity of *E.coli* for larvae of the mosquito *Culex quinquefasciatus*. However, the relationship of these two proteins to the sporulation-associated crystal of *B.sphaericus*, which has been advanced as the toxic principle (Baumann *et al.*, 1985), was not clear.

Baumann *et al.* (1985) reported that a crystal with larvicidal properties could be isolated from *B.sphaericus* 2362, and that when analyzed on SDS polyacrylamide gels, polypeptides which migrated at 125, 110, 98, 63, 57 and 43KDa, with a minor band at 37KDa, were observed. Solubilization of the crystal with alkali prior to electrophoresis led to the disappearance of all but the 43 and 63KDa proteins. Nevertheless, larvicidal activity of the alkali-treated crystal was retained. However, it was further shown in the same study, using antibodies to the 43 and 63KDa proteins purified from crystal preparations, that the 98, 110 and 125KDa proteins in the crystal preparations contained antigenic determinants shared by both the 43 and 63KDa proteins. From this study it was concluded that the large proteins in the crystal were high molecular weight precursors or oligomeric forms of the two smaller species.

Following larval-toxicity assays with purified proteins obtained from crystal preparations, Baumann *et al.* (1985) also reported that the 43KDa species purified from *B.sphaericus* 2362 crystals, alone was toxic. In contrast, both the 43 and 63KDa proteins were required for toxicity for mosquito larvae, when synthesized in recombinant *E.coli* cells (Baumann *et al.*, 1987; Broadwell *et al.*, 1990a). These latter results raised some doubts about the validity of the earlier studies. The similarity in size of the 43 and 63KDa crystal associated proteins, as identified by Baumann *et al.* (1987), with the 41 and 59KDa proteins identified in minicells in this study, taken together with the fact that both were required for

larvicidal activity, suggested that the two sets of proteins might be identical, despite their origin from different strains. I next attempted therefore, to demonstrate the relationship between the 41 and 59KDa proteins encoded by pAS233HA to the components of the crystal produced by *B.sphaericus* 1593.

#### 4.2. Isolation and dissociation analysis of the 125KDa crystal protein produced by *B.sphaericus* 1593.

As indicated above, Baumann et al. (1985) reported that the crystal from *B.sphaericus* 2362 purified by NaBr density gradient, could be dissociated simply by boiling with SDS loading buffer and separated on SDS polyacrylamide gels into several constituent protein species. These migrated at 125, 110, 98, 63, 57 and 43KDa, with the 125KDa species the major constituent. However, solubilization with NaOH and the use of antibodies raised to the 43 and 63KDa proteins isolated from the crystal after gel separation, suggested that the higher molecular weight proteins were the precursors or oligomers of the 43 and 63KDa proteins (Baumann et al., 1985). This conclusion, however, was not consistent with the results obtained in this study with minicells, which indicated that the 41 and 59KDa toxic proteins were apparently primary gene products. In addition, Hindley and Berry (1987), had reported the DNA sequence of a 41.9KDa protein, whose restriction enzyme map corresponded closely to that region of pAS233HA expressing the 41KDa protein in minicells (Figure 1.2). This result confirmed that the 41KDa protein was indeed a primary gene product. In order to establish the relationship between the crystal protein(s) and the 41 and 59KDa proteins, I attempted to purify the crystal produced by *B.sphaericus* 1593, and dissociate it into its constituent proteins. My aim was then to raise antibodies to one of the presumed high molecular weight oligomeric forms in the crystal, the 125KDa protein, and to determine whether such antibodies recognized the 41 and 59KDa proteins seen in minicells.

Isolation of the crystal from sporulating cells of *B.sphaericus* 1593, and three other strains, 2297, 2013-4 and 2362 was carried out as described in Chapter 2, Section 2.1.5. The crystals were purified on either 48% NaBr gradients

(Baumann *et al.*, 1985; Chapter 2, Section 2.1.5.1) or on discontinuous sucrose density gradients (Haider and Ellar, 1987a; Chapter 2, Section 2.1.5.2), washed several times in distilled water, resuspended in distilled water and stored at  $-20^{\circ}\text{C}$ . In Figure 4.1A are shown the SDS polyacrylamide gel profiles of such a crystal preparation isolated by discontinuous sucrose density gradients from the four strains of *B.sphaericus* and stained with Coomassie blue. The results showed the presence of a major molecular weight band migrating at approximately 120KDa in some crystal preparations (1593, 2362, 2013-4). In addition, smaller amounts of a 135KDa protein were seen but this was not always reproducible (see Figure 4.1B, lane B). In addition to the major 120-130KDa proteins, there were proteins migrating at 103, 62 and 43KDa in the preparations. The proportions of these proteins varied from preparation to preparation. Moreover, the crystal from strain 2297 did not contain any 120KDa protein as shown in Figure 4.1A, lanes E and F, but contained the 62 and 43KDa proteins seen in the other strains. In complete contrast, strain 2013-4 appeared consistently to lack the 62 and 43KDa proteins in any discernible quantity (Figure 4.1A, lanes G and H).

The 62 and 43KDa proteins detected in the crystal preparations shown in Figure 4.1A may correspond to the 63 and 43KDa proteins described by Baumann *et al.* (1985). They could, for example, represent posttranslationally modified forms of the 41 and 59KDa proteins expressed by pAS233HA, that have been shown to be toxic for mosquito larvae. Indeed, Baumann *et al.* (1985) sequenced the N-terminus of the 43KDa protein isolated from the crystal and this sequence was used by Hindley and Berry (1987) to design oligonucleotide probes leading to the isolation of a gene encoding a 41.9KDa protein. These results strongly suggest that the 43KDa protein in the crystal and the 41KDa protein identified in minicells containing pAS233HA are the same. However, the relationship of the 59KDa protein seen as a product of the 3.6Kb insert of pAS233HA in minicells to the 62/63KDa form seen in crystals remained to be established. Similarly, the nature of the higher molecular weight proteins at their possible relationship to the other constituents required investigation. At this point I shall also clarify that although the major

Figure 4.1A: Isolation of crystals from *B.sphaericus*.

Crystals were prepared from *B.sphaericus* strains 1593, 2362, 2297 and 2013-4 by sucrose density gradient centrifugation of sonicates of sporulated cells (Chapter 2, Section 2.1.5.2). An aliquot of purified crystals was heated to 100°C in SDS loading buffer for 3min, and the proteins separated on 11% separating, 5% stacking SDS polyacrylamide gels. The proteins in the gel were visualised by staining with Coomassie blue.

The figure shows the analysis of crystal proteins isolated from the strains 1593, 2362, 2297 and 2013-4 in lanes A&B, C&D, E&F and G&H, respectively. Lanes A, C, E and G contain a 5µL loading, and lanes B, D, F and H contain a 10µL loading. Lane M contains protein standards

Figure 4.1B: Dissociation of crystals from *B.sphaericus* 1593.

The crystals used in the study were purified on NaBr gradients (Chapter 2, Section 2.1.5.1). They were subjected to various dissociation treatment, and unless otherwise indicated, were each then heated to 100°C for 3min in SDS loading buffer before gel electrophoresis. The proteins were separated on 11% separating, 5% stacking SDS polyacrylamide gels and visualised by Coomassie blue staining. The 62 and 43KDa proteins are very faintly visible, and their positions are indicated.

Lanes:

A, 2.5µL crystal suspension treated with 50mM Na<sub>2</sub>CO<sub>3</sub>; 10mM DTT, pH 10.5 for 1 hour at 37°C

B, 5µL crystal untreated

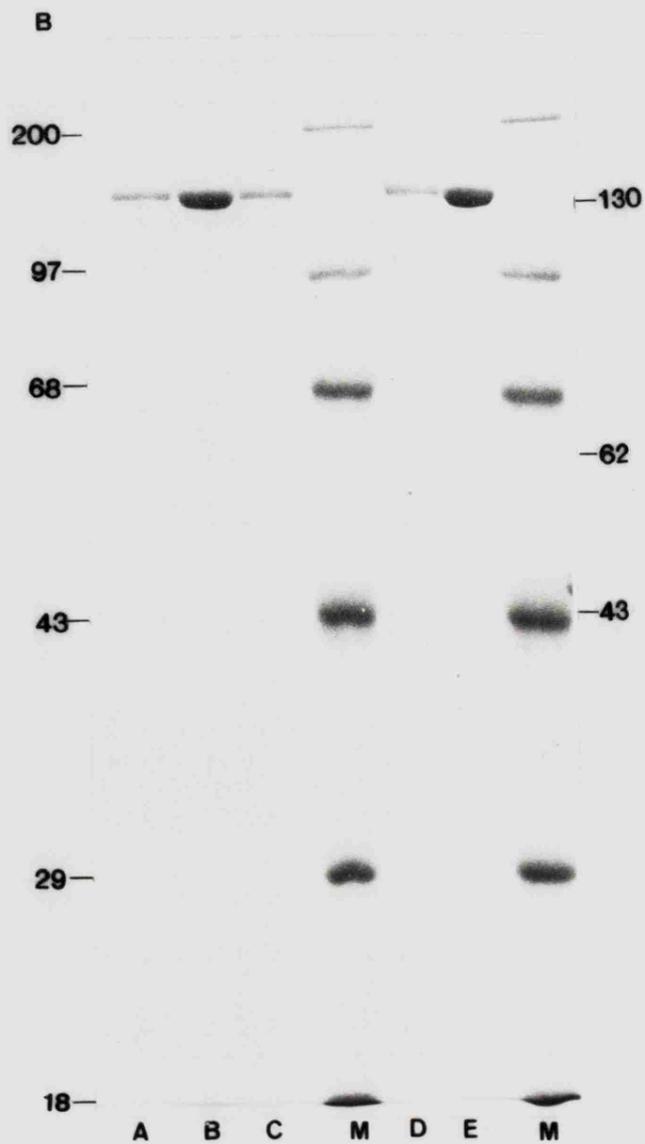
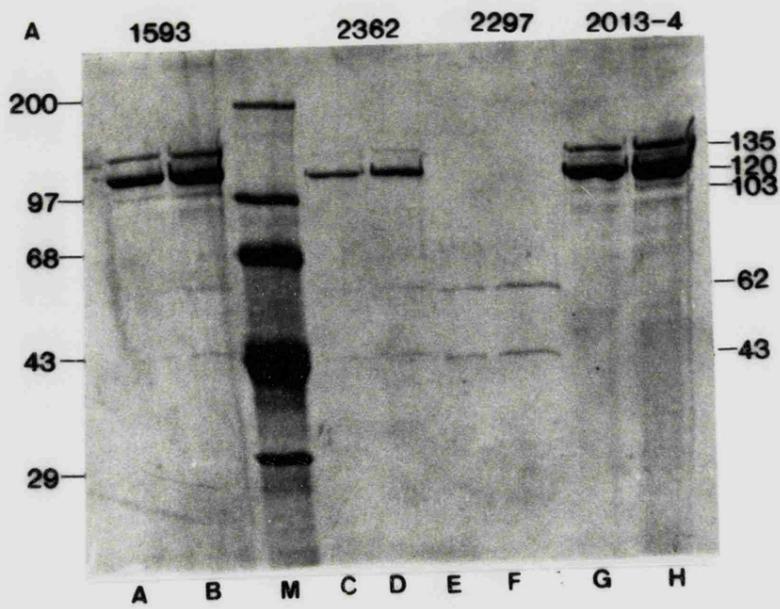
C, 2.5µL crystal suspension treated with 50mM NaOH; 10mM EDTA, pH 12 for 1 hour

D, 2.5µL crystal suspension treated with 50mM NaOH; 10mM EDTA, pH 12 for 2 hours

E, 5µL crystal suspension resuspended in SDS loading buffer, but not boiled

M, Protein standards.

The sizes of protein standards and major proteins in the preparations are indicated in KDa.



protein associated with the crystal preparations of strain 1593 migrated at 125 +/- 5KDa, it will continue to be referred to as the 125KDa protein.

In a further series of experiments to deduce the relationship of the smaller crystal proteins to the 125KDa polypeptide, attempts were made to dissociate the crystal into its constituents by pre-treatment with alkaline sodium carbonate, pH 10.5 (Figure 4.1B, lane A) and 50mM NaOH/ 10mM EDTA at pH 11.7 (Pfannenstiel *et al.*, 1984; Baumann *et al.*, 1985; Figure 4.1B, lanes C and D). However, all the treatments completely failed to disrupt the major constituent of the crystal, the 125KDa protein. Increasingly harsh treatments were then employed. Urea and guanidium chloride at 8 and 6M respectively were found to completely degrade the 125KDa protein, as did the use of 0.1M NaOH, however this treatment did not result in the accumulation of specific substituents (data not shown).

Since it was not possible in this manner unequivocally to demonstrate *in vitro* dissociation of the higher molecular weight crystal proteins into, for example, the 43 and 63KDa forms, I abandoned these methods and concentrated on raising antibodies to the 125KDa protein from strain 1593 in order to analyze its relationship to the 41 and 59KDa proteins detected in minicells.

#### 4.3. Antibodies to the 125KDa crystal protein.

The crystals from *B.sphaericus* 1593 were isolated from NaBr gradients, and recentrifuged until they were microscopically homogeneous, appearing as phase dark shapes, distinct from phase bright spores under a phase contrast microscope. The crystals were then washed several times in distilled water and resuspended in distilled water. Crystal preparations were stored at -20°C.

For preparation of the antigen, the purified crystals were resuspended in SDS loading buffer, heated at 100°C for 3min, and the proteins were separated on a 5% SDS polyacrylamide gel. The 125KDa protein was visualized with Coomassie blue staining. From this stage onwards, all manipulations were

done using sterile solutions and equipment rinsed several times in sterile distilled water. The gel was soaked overnight in 1M Tris-Cl, pH 7.5, and then equilibrated in several changes of 1X phosphate-buffered saline (PBS) over 2 days. The pH was tested, and when it was approximately 7.2, the 125KDa protein band was excised from the gel as described in Chapter 2, Section 2.6.1. The gel slice containing the 125KDa protein was transferred to a sterile eppendorf tube, an equal volume of Complete Freund's Adjuvant was added, and the gel was sonicated until finely homogenized. Approximately 750µg of the 125KDa protein in the gel homogenate was injected subcutaneously into an adult New Zealand White rabbit. For booster doses, the quantity of protein was reduced to 500µg, and the gel fragment containing the protein was sonicated in Incomplete Freund's Adjuvant.

The serum obtained after the second booster dose had an antibody titre of 1:1000 as measured by Western immunoblotting assays against the denatured 125KDa crystal protein. In four booster doses, the titre had risen to 1:10000, indicating that the protein was highly antigenic. Figure 4.2 shows such an immunoblot experiment using the antibodies raised to the 125KDa protein. The results demonstrated a strong reaction of the antibody against total crystal proteins (Figure 4.2, lane A) and against total proteins obtained from sporulated cells of *B.sphaericus* 1593 (Figure 4.2, lane C). In contrast, with the sporulating cells of the non-toxic strain 14577, no 125KDa protein was detected by the antibodies, although there was some non-specific background reaction (Figure 4.2, lane B). These results indicated that the antibody was specifically recognizing a component found in the crystal preparations and present only in crystals and in sporulating cells of *B.sphaericus* 1593.

As also shown in Figure 4.2 (lanes D to G), *E.coli* minicells prepared from strains harbouring the derivatives of the plasmids pAS233, pHAΔEI and pHAΔEV described above (Chapter 2, Section 2.4.1), were transferred to nitrocellulose paper and tested in Western immunoblotting assays against antibodies to the 125KDa protein. No signal was detected in these minicell lanes even though the immunoblot was extensively developed in order to detect any reaction over

Figure 4.2. Western immunoblot of the *B.sphaericus* crystal; sporulated whole cell extracts of *B.sphaericus* 1593 and 14577; and minicells harbouring plasmids conferring toxicity to mosquito larvae, against antibodies to the 125KDa protein.

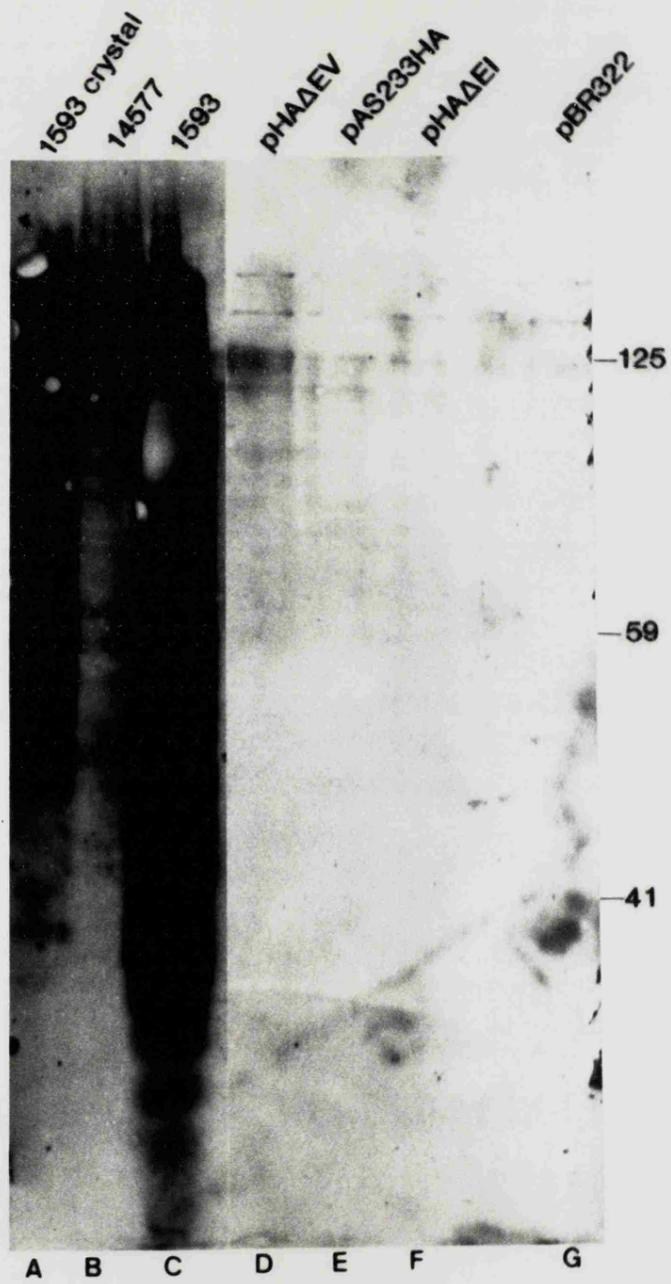
Cells of *B.sphaericus* 1593 and 14577 (Chapter 2, Section 2.1.1) were grown to sporulation in Luria broth. The crystal from strain 1593 was isolated by NaBr density gradient centrifugation (Chapter 2, Section 2.1.5.1).

Minicells containing the plasmids pAS233HA, pHADEI and pHADEV were prepared from *E.coli* DS410" T" cells harbouring the plasmids as described in Chapter 2, Section 2.4.1. A small aliquot of the minicell preparation was separately radiolabelled to identify that the 41 and 59KDa proteins were being expressed (data not shown). 0.5 A<sub>600</sub> Units of unlabelled minicells were heated in SDS loading buffer to 100°C for 3min and the proteins separated on 11% separating, 5% stacking SDS polyacrylamide gels. The proteins were transferred electrophoretically onto nitrocellulose paper, non-specific binding sites blocked with 1% (w/v) casein; 1.5% (w/v) BSA; 10% (v/v) newborn calf serum in 1X PBS at 4°C overnight (Chapter 2, Section 2.6.3). The nitrocellulose paper was then treated sequentially with 1:50 dilution of anti-125KDa antibody, 1:500 dilution of goat anti-rabbit antibody and 1:1000 dilution rabbit peroxidase anti-peroxidase antibody. The immunoblot was developed as described in Chapter 2, Section 2.6.3. The blot was developed extensively to visualize any low level response in the minicell lanes. There is a small amount of spill over of protein from lane C into lane D.

Lanes:

- A, crystal isolated from *B.sphaericus* 1593
- B, 0.5 A<sub>600</sub> Units of *B.sphaericus* 14577 total protein
- C, 0.5 A<sub>600</sub> Units of *B.sphaericus* 1593 total cell protein
- D, 0.5 A<sub>600</sub> Units of *E.coli* minicells containing the plasmid pHADEV encoding the 59KDa toxin protein
- E, 0.5 A<sub>600</sub> Units of *E.coli* minicells containing the plasmid pAS233HA encoding the 41 and 59KDa toxin proteins
- F, 0.5 A<sub>600</sub> Units of *E.coli* minicells containing the plasmid pHADEI encoding the 41KDa toxin protein
- G, 0.5 A<sub>600</sub> Units of *E.coli* minicells containing the vector pBR322.

Protein sizes are shown in KDa. The expected positions of the 125KDa crystal protein and the 41 and 59KDa toxin proteins are indicated.



background.

#### 4.4. Attempts to immunoprecipitate the 41 and 59KDa proteins from minicells.

Although the antibody raised against the 125KDa protein present in crystal preparations from *B.sphaericus* 1593, failed to detect any cross-reacting material by Western immunoblotting corresponding to the 41 or 59KDa proteins expressed in minicells, other more sensitive procedures were then examined in order to determine whether the 41 and 59KDa proteins were in any way related to the 125KDa protein. In these experiments, two alternative approaches were employed in order to increase the probability of detecting possible cross-reactions between the antibodies and the 41 and 59KDa species. One approach involved the use of immunoprecipitation. The second approach was to use an independent source of anti-crystal antibody, in anticipation that antibodies to a different set of crystal or crystal associated antigens might more readily detect any cross reaction with the 41 and 59KDa protein..

In the following experiments, the antibody to the 125KDa crystal protein isolated from SDS polyacrylamide gels described above, is referred to as the "anti-125KDa" antibody. Other antibodies were obtained from Prof. K. Jayaraman (Centre for Biotechnology, Anna University, Madras, India), which had been raised to crystal proteins produced by sporulating cultures of *B.sphaericus* 1593M (Chapter 2, Section 2.1.1). These crystals had been isolated by NaBr density gradient centrifugation prior to injection into rabbits without further purification. This material used to raise antibodies also had the merit of not being denatured by SDS-PAGE or boiling before injection. This antibody is referred to as "anti-crystal" antibody.

#### 4.4.1. Immunoprecipitation of minicell extracts containing the 41 and 59KDa proteins with the "anti-125KDa" antibody.

The 125KDa protein identified in crystal preparations as described by Baumann *et al.* (1985) was reported to contain antigenic determinants of 43 and 63KDa crystal protein substituents, probably corresponding to the 41 and 59KDa proteins seen in *E.coli* minicells containing the plasmid pAS233HA. The anti-125KDa antibody produced in this study was used again in immunoprecipitation experiments, rather than Western immunoblotting, against radiolabelled minicells expressing the 41 and 59KDa proteins (Figure 4.3, lane G). The antibody did not detect either the 41 or the 59KDa protein, even when the X-ray film was overexposed (Figure 4.3, lanes C and D). This result could indicate that, a) the 125KDa protein is completely unrelated to the 41 and 59KDa toxin proteins, b) the SDS-PAGE step had denatured the 125KDa protein, destroying the relevant antigenic determinants, c) the 125KDa protein was a separate, and major, constituent of the crystal, and that the 41 and 59KDa proteins had been eliminated in the gel purification step prior to raising the antibody, d) the 41 and 59KDa proteins were poorly antigenic unless, for example, they were posttranslationally modified by synthesis in *B.sphaericus*.

#### 4.4.2. Immunoprecipitation of minicells containing the 41 and 59KDa proteins with "anti-crystal" antibody.

In an attempt to distinguish some of these possibilities, I used an alternative source of antibody, the "anti-crystal" antibody raised against a native crystal preparation, provided by Prof. K. Jayaraman, in a similar experiment to immunoprecipitate radiolabelled 41 and 59KDa proteins produced in minicells. However, these antibodies also failed to immunoprecipitate either the 41 or 59KDa proteins from minicells (see Figure 4.3, lanes E and F). In contrast, it must be emphasized that in the control, precipitation of  $\beta$ -lactamase protein by anti- $\beta$ -lactamase antibody worked adequately in the experiments, although not as efficiently as anticipated (Figure 4.3, lanes A and B).

Importantly, these results raised the interesting

Figure 4.3. Immunoprecipitation of extracts of minicells containing the plasmid pAS233HA with antibodies to the 125KDa crystal protein and to the non-denatured crystal.

0.1 A<sub>600</sub> Units of radiolabelled minicells containing the plasmid pAS233HA were used for each immunoprecipitation. The minicells were immunoprecipitated with antibody following lysis by boiling in the presence of 1% (w/v) SDS as described in Chapter 2, Section 2.6.5. The minicells in this case were shown to produce clearly enriched amounts of the 41 and 59KDa proteins, detected by radiolabelling.

Lanes:

M, contains protein standards.

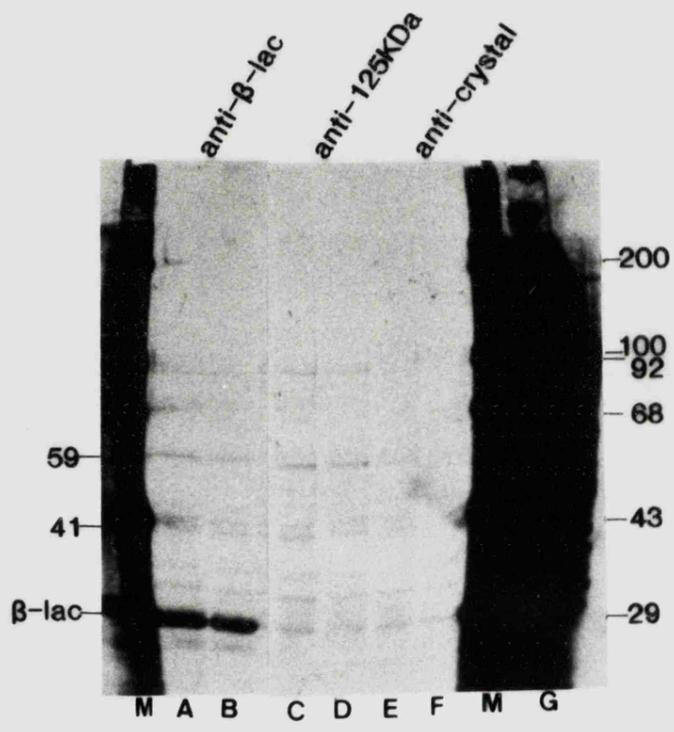
A and B contain 0.1 A<sub>600</sub> Units of minicell proteins incubated with 5 and 10μL respectively of anti-β-lactamase antibody

C and D contain 0.1 A<sub>600</sub> Units of minicell proteins incubated with 5 and 10μL of anti-125KDa antibody

E and F contain 0.1 A<sub>600</sub> Units of minicell proteins incubated with 5 and 10μL of anti-crystal antibody

G contains 0.05 A<sub>600</sub> Units of minicell proteins used in the assay but not immunoprecipitated. This signal shows that the 41 and 59KDa proteins were detected at a lower exposure (not shown).

The immunoprecipitated proteins were separated on an 8.5% separating, 5% stacking minigel. The gel was flurographed, dried and exposed to X-ray film for 10 days. The positions of the protein standards are shown in KDa, and the positions of the 41 and 59KDa proteins are indicated.



possibility that the 41 and 59KDa proteins, although shown to be toxic for larvae, are either absent from either the purified 125KDa or the intact crystal (i.e. do not form a tightly associated oligomeric complex whose size is equivalent to 125KDa), or have been eliminated from the crystal in the NaBr purification step. There is also the distinct possibility that the 41 and 59KDa proteins are associated with the 125KDa protein in the crystal in such small quantities, or are so poorly antigenic that they do not elicit a sufficiently strong antibody response. Since the material obtained as purified crystal from NaBr gradients was shown to be highly toxic for mosquito larvae (Chapter 3, Table 3.1), this indicated that the last possibility might be correct.

#### 4.5. Comparison of the properties of the "anti-125KDa" antibody and the "anti-crystal" antibody.

In view of the results obtained above, it was important to carry out further control experiments in order to confirm the relatedness of the anti-125KDa antibodies and the anti-crystal antibodies. Crystal proteins prepared from *B.sphaericus* strains 1593M, 1593, 2013-4, 2297 and 2362 by the sucrose density gradient centrifugation method (Chapter 2, Section 2.1.5.2), were therefore separated on SDS polyacrylamide gels and transferred to nitrocellulose paper in order to perform an immunoblot. The immunoblot in Figure 4.4 shows the reaction of the various crystal preparations with the two sets of antibodies. Figure 4.4A shows the Coomassie stained profile, whilst Figure 4.4B shows the results of probing with antibodies to the gel purified 125KDa protein from *B.sphaericus* 1593 prepared in this study (Section 4.3). The latter immunoblot shows that the antibodies, as expected, recognized a similar 125KDa protein in all the preparations, except, interestingly, in the crystal preparation from strain 2297, which as seen earlier (Figure 4.1A) contained only 43 and 62KDa species when stained with Coomassie blue. In addition, the antibody also recognized the high molecular weight protein which migrates at approximately 130-135KDa in preparations from strain 1593, 1593M, 2013-4 and 2362.

Figure 4.4C shows the reaction of the same crystal preparations with the anti-crystal antibodies raised against

Figure 4.4 Comparison of the properties of antibodies raised to the denatured 125KDa protein and to the non-denatured crystal.

Crystals from four larvicidal *Bacillus sphaericus* strains 1593, 2013-4, 2297 and 2362 were prepared from sporulated cells by the sucrose density gradient centrifugation method (Chapter 2, Section 2.1.5.2).

5 $\mu$ L of each crystal preparation was heated to 100°C for 3min in SDS loading buffer and the proteins were separated on 8.5% SDS polyacrylamide gels. The proteins were either visualised by staining with Coomassie blue (Panel A), or transferred electrophoretically onto nitrocellulose paper for Western immunoblotting assays. Non-specific binding sites on the paper were blocked overnight with 5% (w/v) Marvel milk in 1X PBS (Chapter 2, Section 2.6.3).

The blots were incubated either with 1:1000 dilution of antibodies to the 125KDa protein (Panel B; Section 4.3) or with 1:1000 dilution of antibodies to non-denatured crystal (Panel C; Section 4.4). The immunoblots were developed following incubation with horseradish peroxidase-conjugated goat anti-rabbit antibodies used at a dilution of 1:5000 (Chapter 2, Section 2.6.3).

Figure 4.4A shows the gel stained with Coomassie blue, Figure 4.4B shows the Western immunoblot with antibodies to the 125KDa protein and Figure 4.4C shows the Western immunoblot with antibodies to the crystal of strain 1593M (obtained from Prof. Jayaraman).

Lanes:

A and B in each figure show the antigens used in the preparation of the crystal antibody from strain 1593M (also a gift from Prof. Jayaraman)

M, protein standards

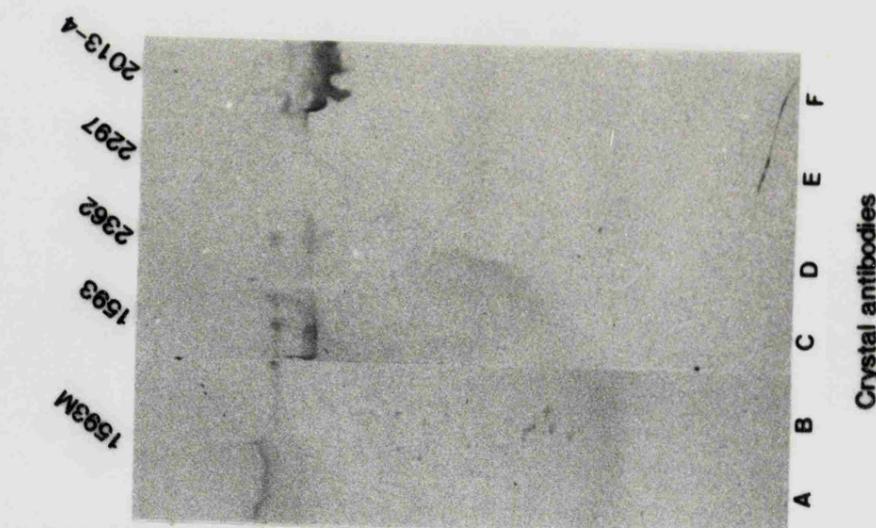
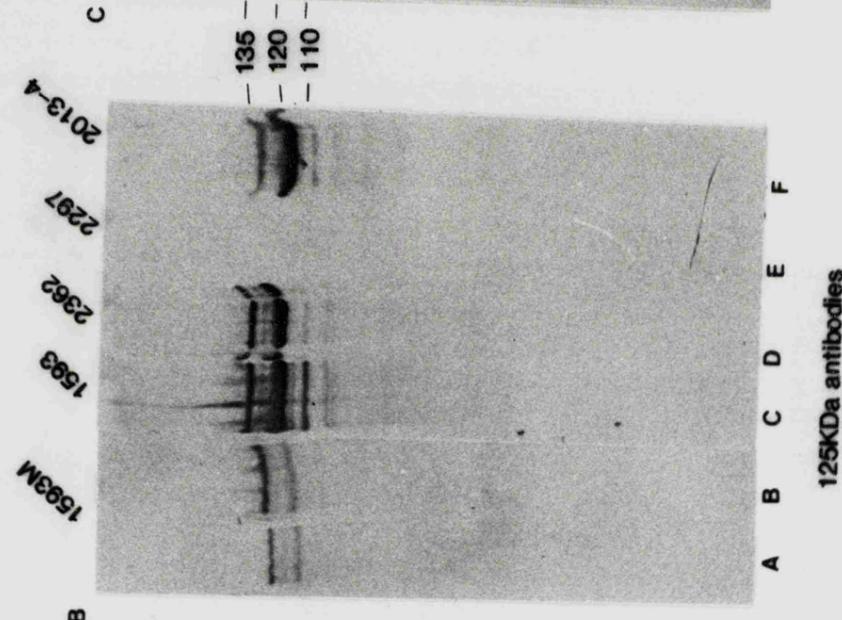
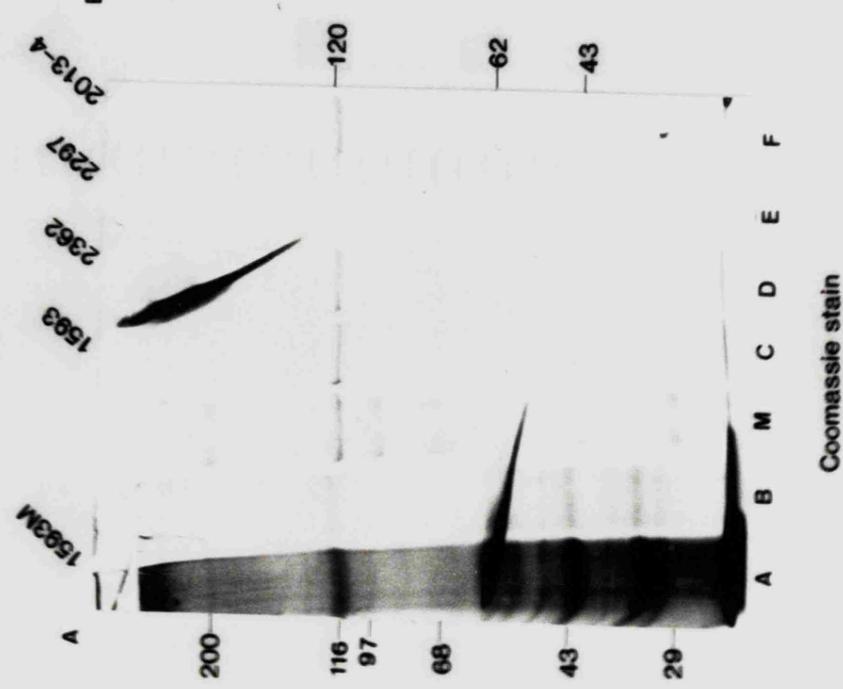
C, crystal proteins isolated from *B.sphaericus* strain 1593

D, crystal proteins isolated from *B.sphaericus* strain 2013-4

E, crystal proteins isolated from *B.sphaericus* strain 2297

F, crystal proteins isolated from *B.sphaericus* strain 2362.

Protein standards and the sizes of major proteins in the preparation are indicated in KDa.



Coomassie stain

125KDa antibodies

Crystal antibodies

200

116

97

68

43

29

120

62

43

135

120

110

1593M

1593

2362

2297

2013-4

A B M C D E F

A B C D E F

A B C D E F

crystals from *B.sphaericus* 1593M, isolated from NaBr gradients, directly into rabbits. The immunoblot showed that the antibodies to the non-denatured crystal also recognized the 130-135KDa and the 125KDa proteins, except for the preparation from the strain 2297. Thus, the two crystal preparations made independently by the same procedure, and either used for immunization as the native complex or a gel purified, denatured 125KDa antigen, elicited antibodies that recognized apparently identical antigens. Nevertheless, neither antibody recognized a 43 and 62KDa species in the crystal preparations.

Following on from earlier observations that the gel purified 125KDa protein was highly antigenic in rabbits (Section 4.3), it is not entirely surprising in retrospect that when the intact, non-denatured crystal complex was used, the 125KDa protein should have induced the production of antibodies, perhaps to the exclusion of any other proteins in, or associated with the crystal. Since it was not profitable to try to adsorb out the 125KDa directed antibodies in the antiserum, firstly because of the very small quantities of antibodies available, and secondly because of lack of assurance that there would be any antibodies directed to other proteins in the preparation, I decided to continue the analysis by attempting to raise antibodies to the toxin proteins themselves. This will be described in subsequent chapters.

In addition, however, in order to determine directly the relationship if any, of the 125KDa protein to the 41 and 59KDa toxins, the 125KDa protein used as antigen in the preparation of antibodies was subjected to N-terminal amino acid sequence analysis described in the next section.

#### 4.6. N-terminal amino acid sequencing of the 125KDa crystal protein.

Following the observations described in earlier sections that the antibodies raised specifically to the 125KDa protein did not cross react with either the minicell products of 41 or the 59KDa, it was important to determine directly whether these proteins were in any way related to the 125KDa protein. To investigate this, purified 125KDa protein was prepared for N-terminal sequencing by Dr. J. N. Keen at the University of Leeds.

For N-terminal sequencing, the protein was isolated from 5% SDS polyacrylamide gels as described in Chapter 2, Section 2.4.8. The protein was electroeluted from the acrylamide gel slices, and the eluate then dialyzed extensively. An aliquot of the protein was removed and the quantity of protein estimated by the Lowry method, and also by estimation against protein standards on an 8.5% SDS polyacrylamide gel. 100µg of the protein was then lyophilized, and used for the analysis.

An appropriate sample of the protein was dried at 56°C for 30min onto a diisothiocyanate-activated membrane disc (Sequelon-DITC™) to covalently attach the protein ready for solid phase sequence analysis (Chapter 2, Section 2.4.9).

The disc-coupled material was then subjected to automated Edman degradation on a Milligen/Biosearch 6600 Prosequencer with on-line HPLC. The amount of protein sequenced was approximately 25pmol. About 30pmol of the protein was found to have been covalently attached, and this sequence, therefore, was "real", rather than low level contaminating material, with the major protein being blocked. Since considerably more protein (150pmol) had been applied to the membrane support, a low coupling efficiency was observed, probably due to steric effects.



Figure 4.5 Comparison of the N-terminal amino acid sequence of the 125KDa protein from *Bacillus sphaericus* 1593 with the predicted amino acid sequence of the S-layer protein from *B.sphaericus* 2362.

The 125KDa protein used for amino acid sequencing was purified by gel electrophoresis of NaBr purified crystal from *B.sphaericus* 1593 (Chapter 2, Section 2.1.5.1). The N-terminal amino acid sequence of the protein was obtained by automated Edman degradation with on-line HPLC. The procedure was carried out at the University of Leeds by Dr. J. Keen.

The N-terminal sequence obtained was compared to the predicted sequence of the 125KDa Surface (S)-layer protein from *B.sphaericus* 2362. The sequence alignment is shown in Figure 4.5. The sequence in bold letters is the amino acid sequence of the N-terminus of the 125KDa protein from *B.sphaericus* 1593 crystal preparations, and the sequence in normal characters is the predicted N-terminal amino acid sequence of the S-layer protein from *B.sphaericus* 2362 (Bowditch et al., 1990). The arrow shows the site of cleavage of the N-terminal signal sequence of the S-layer protein, which removes the signal peptide during translocation to the cell surface.

Amino acid sequence of the N-terminus of the 125KDa crystal associated protein from *B.sphaericus* 1593 compared to the predicted S-layer protein sequence from strain 2362.

1	M	A	K	Q	N	K	G	R	K	F	10
11	F	A	A	S	A	T	A	A	L	V	20
21	A	S	A	I	V	P	V	A	S	A↓	30
31	A	Q	L	N	D	F	N	K	I	S	40
	A	Q	L	N	D	F	N/V	K	I	S?	
41	G	Y	A	K	E	A	V	Q	S	L	50
	X	Y/D	A	A?	E?	X	X	Q			

time in this study, however, the identity of at least proteins involved in toxicity has been demonstrated.

#### 4.7. Presence of the 125KDa protein at various stages of growth of *B.sphaericus* 1593.

In order to analyze the relationship of the 125KDa S-l protein to growth phase, and therefore to its presence in larvicidal crystals, advantage was taken of the availability of the antibodies prepared above to the purified protein (Section 4.3). These antibodies were used to probe whole cells of *B.sphaericus* 1593 harvested at various stages of growth. The cells were lysed by heating to 100°C for 3min in SDS loading buffer and the proteins separated on an 8.5% polyacrylamide gel. The proteins were electrophoretically transferred to nitrocellulose paper. Western immunoblotting was carried out as described in Chapter 2, Section 2.6.3.

The result of the Western immunoblotting of extracts of *B.sphaericus* 1593 at different stages of growth probed with antibodies to the 125KDa protein in Figure 4.6, shows that the 125KDa protein is present at all stages of the growth phase. Synthesis of the protein occurs during the vegetative phase and the protein continues to accumulate as a constant proportion of mass into the stationary phase. The synthesis of this protein was not therefore apparently linked to sporulation or to that expected of a specific crystal protein. Although the protein sequence analysis indicated that the 125KDa protein in the crystal preparation was processed to a smaller form lacking its signal sequence, the two forms were not distinguished on the gels in this analysis.

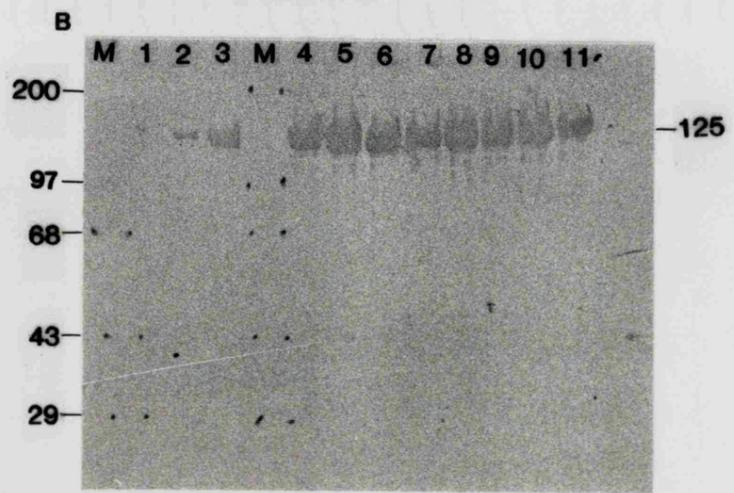
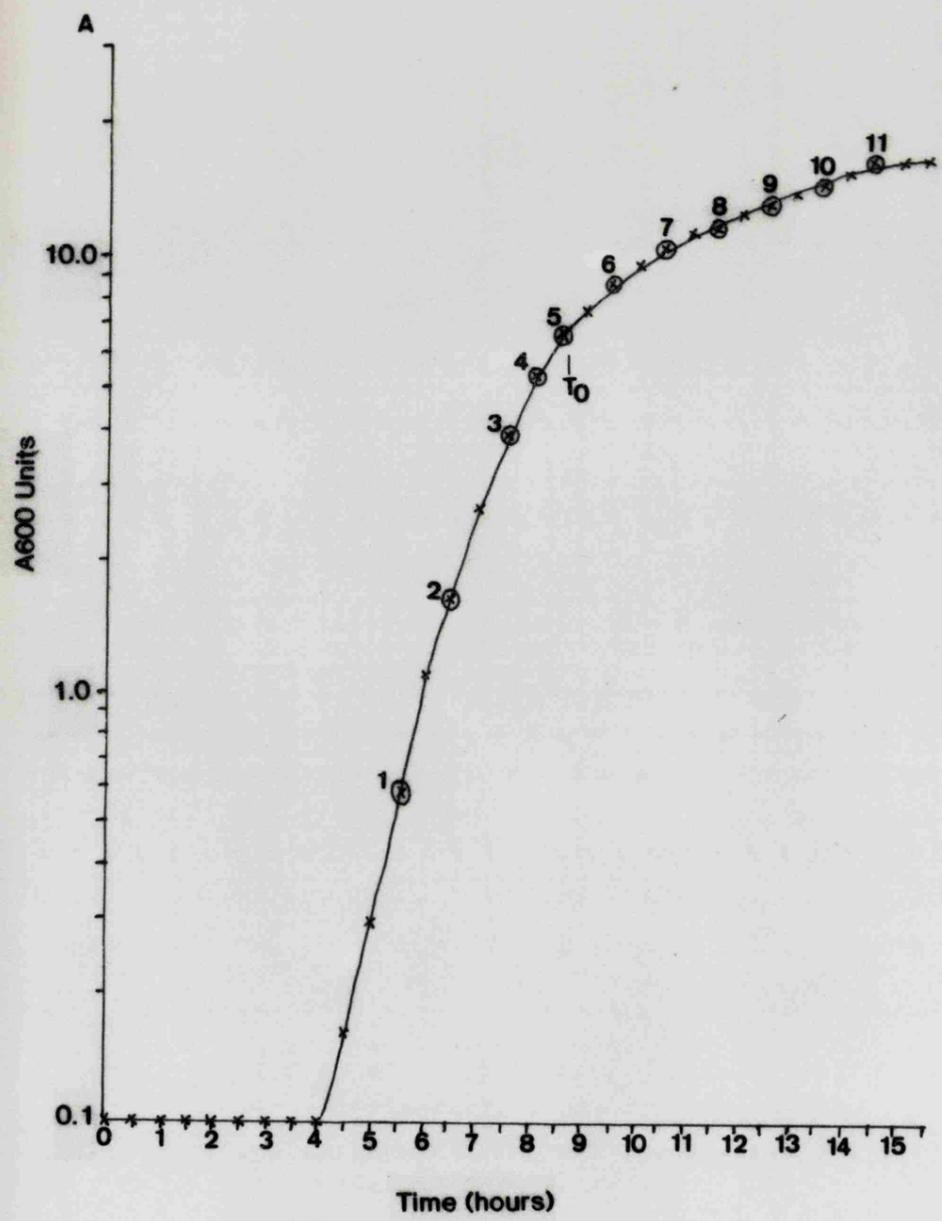
#### 4.8. Summary.

This chapter deals with the identification of the nature of the 125KDa protein present in preparations of the crystals from the *B.sphaericus* strain 1593 and its relationship to the 41 and 59KDa toxins. Antibodies raised to the 125KDa protein indicated that it was not antigenically related to the 41 and 59KDa proteins in contrast to the previous suggestion of Baumann *et al.* (1985).

Figure 4.6 Synthesis of S-layer (125KDa) protein during the growth phase of *Bacillus sphaericus* 1593, investigated using antibodies to the 125KDa protein.

Heat-activated, purified spores (Chapter 2, Section 2.1.6) of *B.sphaericus* 1593 were inoculated into Luria broth to an  $A_{600} = 0.05$ , and grown at 30°C. Samples of cells were taken during different points of growth (Figure 4.6A). Identical 0.05  $A_{600}$  Units of cells for all samples were heated to 100°C for 3min in SDS loading buffer and the proteins separated on an 8.5% separating, 5% stacking SDS polyacrylamide gel. The proteins were transferred electrophoretically onto nitrocellulose paper, and non-specific binding sites on the paper were blocked by incubation overnight in 5% (w/v) Marvel milk in 1X PBS. The Western immunoblot was developed sequentially with a 1:1000 dilution of anti-125KDa antibodies and 1:5000 dilution of horseradish peroxidase conjugated goat anti-rabbit antibodies (Chapter 2, Section 2.6.3).

Lane M shows protein size markers. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 correspond to the sampling points indicated on the growth curve (Figure 4.6A). Protein standards and major proteins are indicated in KDa. The  $T_0$  (onset of sporulation phase) is marked.



The antibodies used in this chapter were obtained from two sources: a gel purified 125KDa protein that was the major constituent of *B.sphaericus* crystals detected by SDS polyacrylamide gel electrophoresis, and from NaBr-purified crystals injected directly into rabbits without further purification. Both sets of antibodies recognized identical antigens in crystal preparations, and neither set recognized the 41 and 59KDa toxin proteins in Western immunoblots or in immunoprecipitation experiments. Subsequent amino acid sequence analysis of purified 125KDa protein showed that it was an S-layer protein, which belongs to a family of such proteins identified on the surface of Gram positive and Gram negative cells (Sleytr and Messner, 1988).

The 125KDa protein was shown to be synthesized during both the vegetative and sporulation phases of growth of the *B.sphaericus* growth phase. Indeed the protein appeared to be synthesized constitutively throughout growth. Constitutive synthesis of other S-layer proteins has previously been observed (Sleytr and Messner, 1988). Surprisingly however, antibodies to the 125KDa protein did not recognize a protein of this size in sporulating cells of the non-toxic *B.sphaericus* strain 14577 (Figure 4.2, lane B). However, it is known that the S-layer proteins of *B.sphaericus* strains vary considerably in amino acid composition, antigenicity and structure (Word et al., 1983; Lewis et al., 1987). Such variation could account for the lack of recognition of the strain 14577 S-layer protein by antibodies to the S-layer protein of strain 1593. A protein migrating at 125KDa was detected in the crystal preparations of strains 1593, 1593M, 2013-4 and 2362, but not in crystal preparations of strain 2297. This discrepancy still raises questions about the nature of larvicidal crystal formation and why apparently identical crystal structures may sometimes be composed primarily of the S-layer protein and sometimes are apparently devoid of this protein.

Following the identification of the 125KDa protein as being unrelated to the 41 and 59KDa toxin proteins, my subsequent work focussed on attempts to overexpress the 41 and 59KDa proteins with the aim of using these expressed proteins

as antigens, to produce antibodies to analyze crystal preparations. This aspect, and the use of such antibody in the case of the 59KDa protein to probe further for the presence of these proteins in the "crystals" and their growth phase regulation of expression, will be described in the following chapters.

## Chapter 5.

### Analysis of the gene encoding the 41KDa toxin protein of *B.sphaericus*, and the attempted high-level expression of this gene.

#### 5.1. Introduction.

The cloned 3.6Kb DNA insert of the plasmid pAS233HA was previously shown to encode information required for *Escherichia coli* strains to display toxic activity for mosquito larvae (Souza et al., 1988). Analysis of plasmid-encoded genes using minicells revealed the presence of two protein products of 41 and 59KDa encoded by the 3.6Kb DNA insert of pAS233HA (Chapter 3, Section 3.3). Furthermore, it was demonstrated that both toxin proteins were required for the toxicity of the recombinant *E.coli* strains for mosquito larvae (see also Chapter 3, Section 3.6).

The nucleotide sequence of a gene encoding a toxin protein from *B.sphaericus* was first determined by Hindley and Berry (1987). This gene was cloned from *B.sphaericus* 1593, by constructing oligonucleotides to the N-terminal amino acid sequence of a 43KDa protein purified from crystals of *B.sphaericus* 2362 by Baumann et al. (1985). The predicted amino acid sequence from the gene cloned by Hindley and Berry (1987) indicated a size of 41.9KDa, very close to the size of the 41KDa detected in this study. In addition, the restriction enzyme sites present on the cloned DNA fragment of Hindley and Berry correlated very closely to those of the 3' end of the 3.6Kb DNA fragment shown to code for a 41KDa protein (Chapter 3, Section 3.3.1; Figure 3.1). This indicated that the 41.9, 43 and 41KDa proteins were the same, and henceforth will be referred to as the 41KDa protein regardless of the source.

My earlier attempts described in Chapter 4 to demonstrate antigenic similarity between the 125KDa crystal protein and the 41 and 59KDa proteins using anti-125KDa antibodies, failed to detect any shared antigenic determinants. My next aim therefore was to attempt in various ways to overexpress the 41 and 59KDa protein encoding genes in order to raise antibodies against these proteins. This would allow, for

example, subsequent attempts to identify the contribution of each protein to the toxicity process. For the DNA manipulations required to overproduce the 41KDa protein, the nucleotide sequence for the gene encoding the 41KDa protein from *B.sphaericus* 1593, then available following the report by Hindley and Berry (1987), was used to design oligonucleotides and to adjust reading frames.

## 5.2. Secretion of the 41KDa protein from *E.coli* using the haemolysin secretion pathway.

The haemolysin secretion system is a well studied export pathway of the Gram negative bacterium *Escherichia coli*. It is unusual in that it is a true secretory pathway, transporting the haemolysin protein from the cytoplasm to the medium independently of the *sec* pathway. Haemolysin secretion requires the presence of two membrane-associated transport proteins, HlyB and HlyD (Mackman *et al.*, 1985), and probably also the TolC protein (Wandersman and Delepelaire, 1990). Secretion also involves the utilization of a non-cleavable signal sequence which is present at the C-terminus of the haemolysin protein. The haemolysin secretion pathway has been shown to be capable of exporting heterologous proteins from *E.coli* when the region of haemolysin containing the signal sequence, a 23KDa peptide, is fused to the C-terminus of the protein to be exported (Mackman *et al.*, 1987; Holland *et al.*, 1990).

Secretion of a protein to the medium simplifies its subsequent purification. In addition, heterologous proteins that are normally rapidly degraded in the cell can be rescued from proteases by transport to the medium. Further, secreted proteins might accumulate to higher concentrations in the medium than would normally be permitted in the cytoplasm in the case of toxic proteins. Membrane active proteins such as the larvicidal toxins might indeed be expected to be toxic. I therefore attempted to secrete a part, or the whole, of the 41KDa protein from *E.coli* using the haemolysin secretion pathway, with the aim of purifying sufficient protein for its use as an antigen.

### 5.2.1. Secretion from *E.coli* of the N-terminal 13KDa of the 41KDa protein fused to the 23KDa signal region of haemolysin.

The rationale for the use of the C-terminal signal region of haemolysin (HlyA) to export the 41KDa protein to the medium has been explained above. The DNA encoding the 23KDa domain of HlyA was already available in the laboratory in all three translational reading frames, on the plasmids pLG609 (-1,-2, -3). The site used to construct the first fusion was at an *Xba*I restriction enzyme site 363bp into the sequence encoding the 41KDa protein. The size of the fusion protein was predicted to be 36KDa. The plasmid construction is shown in Figure 5.1A, and it should be noted that in this construct the 41KDa gene is still transcribed from the original *B.sphaericus* promoter.

The construct described in Figure 5.1A, p41'HlyA, which encodes the N-terminal 13KDa of the 41KDa protein, fused to the 23KDa C-terminal signal region of haemolysin, was transformed into *E.coli* MC4100 carrying pLG575, which expresses the Hly export proteins HlyB and HlyD. Two external controls were used to monitor secretion in this case: the 23KDa signal region of haemolysin placed under the control of the isopropylthio- $\beta$ -D-galactopyranoside (IPTG) inducible *lac* promoter on the plasmid pLG612, and the N-terminus of the chloramphenicol acetyltransferase III (CATIII) gene fused to the 0.8Kb DNA fragment encoding the 23KDa signal region of haemolysin (CATIII'HlyA). The CATIII gene in this construct is under the control of the CATIII promoter, which is switched on in the late exponential phase of growth. The cultures were grown for 5 hours after they had reached an  $A_{600}=0.4$  in order to allow expression of the CATIII'HlyA fusion to occur. No induction was necessary for the 41'HlyA fusion.

After growth, the cells were pelleted in a centrifuge and the proteins in the supernatant were concentrated by precipitation with 10% TCA, and the cell and supernatant samples were separated on 11% SDS polyacrylamide gels. The proteins were transferred onto nitrocellulose paper for Western immunoblot analysis using antibodies directed against the 23KDa peptide of haemolysin. In addition, the samples were analyzed by staining with Coomassie blue. The results

indicated that no protein was visible at the expected position of the 36KDa toxin fusion in either whole cells extracts or the culture supernatant (data not shown). However, the Western immunoblot (Figure 5.1B) detected a faintly staining 36KDa protein in the supernatant of *E.coli* (p41'HlyA) containing the export functions (Figure 5.1B, lane A). The total cell sample corresponding to this supernatant did not contain protein detectable by the anti-23KDa antibody (Figure 5.1B, lane E).

It was noticeable in this experiment that the culture supernatant fraction from cells expressing the toxin fusion also contained an additional protein migrating at 25KDa which is recognized by the anti-23KDa antibodies (Figure 5.1B, lane A). This is presumably a degradation product of the fusion but this was not further investigated. Figure 5.1B also shows the secretion from control constructs of the C-terminal HlyA 23KDa peptide (pLG612; Figure 5.1B, lanes C and D) and the 30KDa CATIII'HlyA fusion protein (pCATIII'HlyA) which in this case also clearly accumulated within the cells (Figure 5.1B, lanes B and F).

All the data in Figure 5.1B refer to cells in which the export proteins HlyB and HlyD were present in the host strain. In the absence of HlyB and HlyD, no HlyA hybrid protein was secreted (data not shown). Nevertheless, the protein was not accumulated in the supernatant to high levels, and it was decided to construct a fusion with the entire 41KDa toxin protein present, and to determine whether, in this case, the efficiency of secretion of the protein to the supernatant would be affected.

#### 5.2.2. Secretion from *E.coli* of the entire 41KDa protein fused to the 23KDa C-terminal signal region of haemolysin.

It was shown above that the N-terminal 13KDa of the 41KDa protein was being secreted to the medium when fused to the 23KDa signal region of haemolysin. In order to establish whether the entire 41KDa protein would be secreted, a unique PvuII restriction enzyme site was created by site-directed mutagenesis at the 3' end of the gene encoding the 41KDa protein. This site for convenience, was located in the fourth

Figure 5.1A Construction of the plasmid p41'HlyA.

The 0.8Kb *SmaI-HincII* fragment containing the 23KDa C-terminal signal region of HlyA was isolated from the plasmid pLG609-2. This fragment was inserted into the *XbaI* site of the plasmid pHAΔEI in order to make a translational fusion to the N-terminal 13KDa of the 41KDa *B.sphaericus* larvicidal protein. The orientation of the 0.8Kb insert in the plasmid p41'HlyA was identified by restriction enzyme digestion analysis with the enzyme *PstI*.

Figure 5.1B Export of the 41'HlyA fusion protein from cells containing the plasmid p41'HlyA in the presence of Hly B, D.

The plasmids p41'HlyA encoding the 41'HlyA fusion; pLG612, encoding the 23KDa signal peptide of HlyA, and pCATIII'HlyA, encoding a fusion of chloramphenicol acetyltransferase fused to the 23KDa signal region of HlyA, were transformed into *E.coli* containing the plasmid pLG575 (HlyB, D). The cells were grown in Luria broth for 5 hours after they had reached an  $A_{600}=0.4$ . Supernatant and cell samples were prepared as described in Chapter 2, Section 2.4.4.  $0.5A_{600}$  Units of cellular protein and  $10 A_{600}$  Units of supernatant proteins were heated to  $100^{\circ}\text{C}$  for 3min in SDS loading buffer and separated on an 8.5% separating, 5% stacking SDS polyacrylamide gel. The proteins were electrophoretically transferred onto nitrocellulose paper and blocked with 5% (w/v) Marvel milk in 1X PBS. The blot was developed with 1:100 antibodies raised to the 23KDa signal peptide of HlyA and 1:10000 goat anti-rabbit antibodies conjugated to horseradish peroxidase enzyme as described in Chapter 2, Section 2.6.3.

Lanes:

- A, supernatant of *E.coli* MC4100 (p41'HlyA), encoding the 41'HlyA fusion
- B, supernatant of *E.coli* JM101 (pCATIII'HlyA), encoding the CATIII'HlyA fusion
- C, supernatant of *E.coli* MC4100 (pLG612), encoding the 23KDa signal peptide of HlyA
- D, supernatant of *E.coli* JM101 (pLG612), encoding the 23KDa signal peptide of HlyA
- E, total cells of *E.coli* MC4100 (p41'HlyA), encoding the 41'HlyA fusion
- F, total cells of *E.coli* JM101 (pCATIII'HlyA), encoding the CATIII'HlyA fusion
- G, total cells of *E.coli* MC4100 (pLG612), encoding the 23KDa signal peptide of HlyA
- H, total cells of *E.coli* JM101 (pLG612), encoding the 23KDa signal peptide of HlyA

All cells contain the export functions HlyB and HlyD, expressed from the plasmid pLG575.

The sizes of proteins are shown in KDa.



codon before the translational stop codon of the gene (for details of the mutagenesis reaction, see Section 5.3 and legend to Figure 5.3). The 0.8Kb *SmaI-HincII* DNA fragment from the plasmid pLG609-2 encoding the 23KDa signal region was fused in the correct translational reading frame at the *PvuII* site of the gene encoding the 41KDa protein (Figure 5.2A). The 41KDa-HlyA fusion in this construct is under the control of the original *B.sphaericus* promoter. The construct was transformed as before into the *E.coli* strain MC4100 containing the export functions present on the plasmid pLG575. Secretion to the medium was monitored after the cells had been grown for 5 hours after they had reached an  $A_{600}=0.4$ . The CATIII'HlyA fusion strain was again used as a positive control.

As before, the toxin fusion protein could not be detected by Western immunoblotting using the anti-23KDa antibodies, either in cells or supernatants of *E.coli* strains lacking the haemolysin export proteins HlyB and HlyD. However, a protein with the expected molecular weight of approximately 63KDa, was clearly detected in Western immunoblots of the supernatant samples of the strains expressing the fusion protein and HlyB and HlyD. This result demonstrated clearly that secretion was HlyB, HlyD dependent (Figure 5.2B, lane H) since no secreted product was detected in the absence of HlyB, D (Figure 5.2B, lane G). Smaller amounts of a 50KDa, 36KDa and a 30KDa protein in the supernatant were detected with the antibodies to the 23KDa peptide (Figure 5.2B, lane H). Interestingly, the full length 63KDa fusion protein, and two of its apparent cleavage products, could be detected in the total cell sample containing the Hly export functions (Figure 5.2, lane D), whereas such proteins could not be detected at all in cells lacking export functions (Figure 5.3B, lane C). It has been observed earlier (B. Kenny and R. D. Haigh, personal communication) that the 23KDa peptide itself is usually rapidly degraded within *E.coli* cells if not exported, and does not therefore accumulate. This may also apply to a heterologous protein to which the 23KDa signal region is tagged. It was therefore surprising that the fusion protein was detected in cells expressing the export proteins HlyB and HlyD. This result suggested that the interaction of the HlyA signal with the translocators in some way protects this particular fusion protein from degradation, and the

Figure 5.2A Construction of the plasmid p41HlyA.

The 0.8Kb *SmaI-HincII* fragment of the plasmid pLG609-2 containing the 23KDa C-terminal signal region of HlyA was isolated and ligated into the *PvuII* site of the plasmid pBRAT-41 in order to make a translational fusion of the 23KDa signal region to the 41KDa protein. pBRAT-41 was earlier constructed by the insertion of the 1.85Kb *EcoRI-HindIII* fragment from M13-41M (Figure 5.2) into pBRAT (pBR322 digested with *EcoRV* and *PvuII* and religated; Chapter 3, Section 3.1). The orientation of the 0.8Kb insert in the plasmid p41HlyA was identified by digestion with the restriction enzyme *PstI*.

Figure 5.3B Export of the 41HlyA fusion protein from cells containing the plasmid p41HlyA.

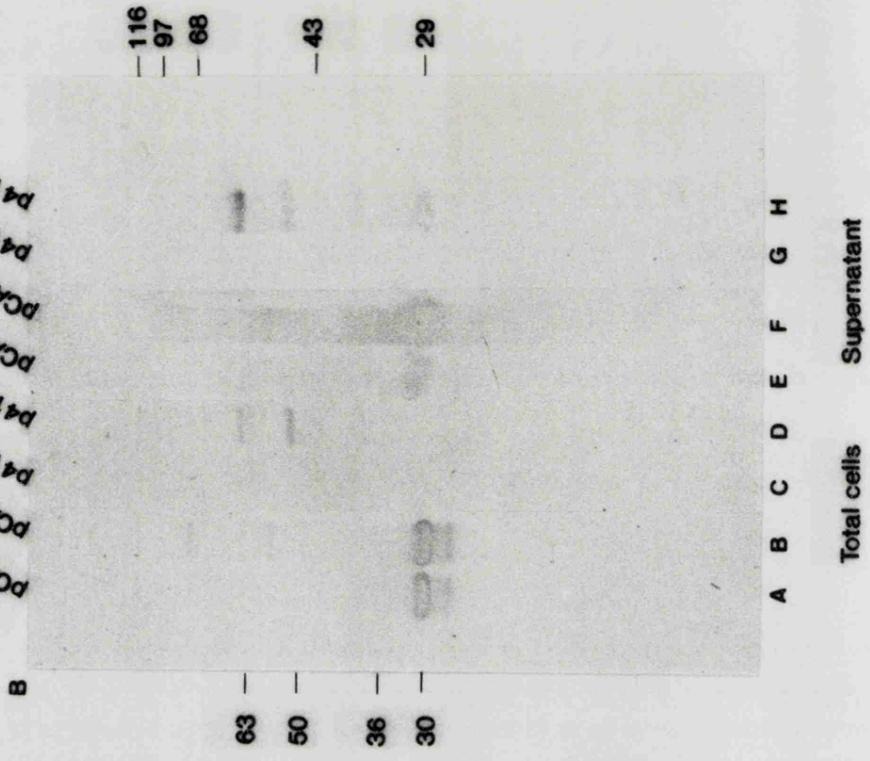
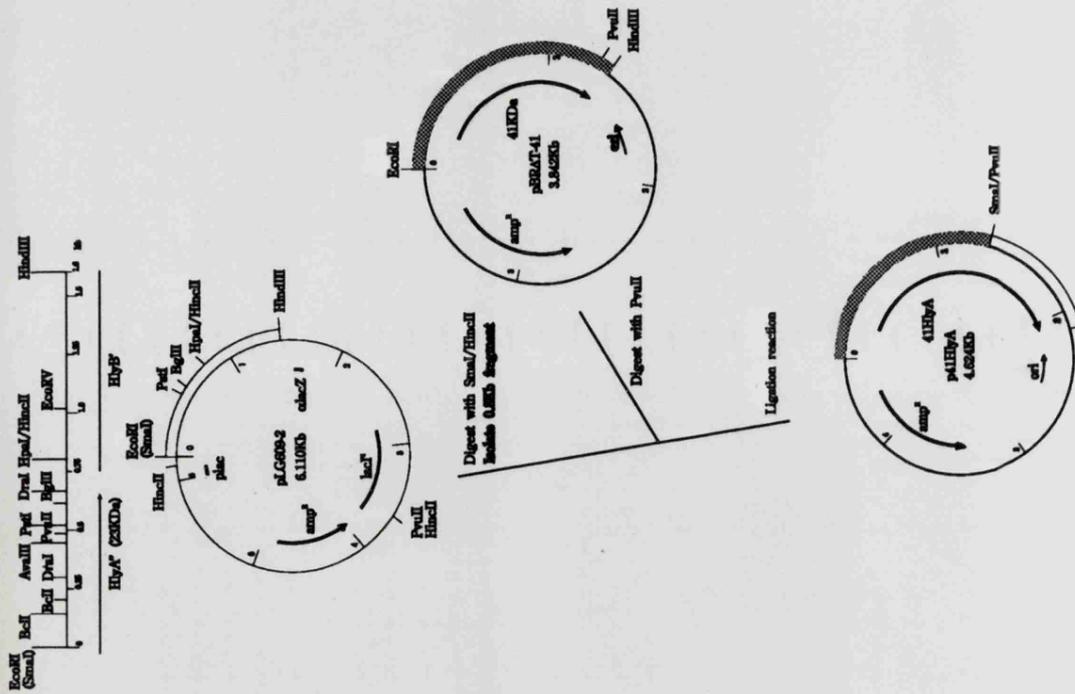
The plasmid p41HlyA, encoding the fusion protein 41HlyA, and pCATIII'HlyA, encoding the chloramphenicol acetyltransferase protein fused to the 23KDa signal region of HlyA, were transformed into *E.coli* cells with and without the plasmid pLG575, encoding the haemolysin export proteins HlyB and HlyD. The cells were grown in Luria broth for 5 Hours after they had reached an  $A_{600}=0.4$ . Supernatant and cell samples were prepared as described in Chapter 2, Section 2.4.4.  $0.05A_{600}$  Units of cells and  $1.0 A_{600}$  Units of supernatant proteins were heated to  $100^{\circ}\text{C}$  for 3min in SDS loading buffer and separated on an 11% separating, 5% stacking minigel. Following separation, the proteins were transferred electrophoretically onto nitrocellulose paper. The nitrocellulose paper was blocked with 5% (w/v) Marvel milk in 1X PBS. The western immunoblot was then developed with 1:100 antibodies raised to the 23KDa signal peptide of HlyA, and 1:1000 goat anti-rabbit antibodies, followed by 1:1000 rabbit peroxidase anti-peroxidase antibodies as described in Chapter 2, Section 2.6.3.

Lanes:

- A, total cells of *E.coli* JM101 (pCATIII'HlyA), without export functions
- B, total cells of *E.coli* JM101 (pCATIII'HlyA), with export functions
- C, total cells of *E.coli* MC4100 (p41HlyA), without export functions
- D, total cells of *E.coli* MC4100 (p41HlyA), with export functions
- E, supernatant of *E.coli* JM101 (pCATIII'HlyA), without export functions
- F, supernatant of *E.coli* JM101 (pCATIII'HlyA), with export functions
- G, supernatant of *E.coli* MC4100 (p41HlyA), without export functions
- H, supernatant of *E.coli* MC4100 (p41HlyA), with export functions

The sizes of proteins and standards are shown in KDa.

A



B

polypeptides detected within the cell are probably undergoing translocation.

In this experiment some apparent "secretion" of the CATIII'HlyA protein to the supernatant of cells lacking the Hly export functions was detected. This is not normally observed, and may indicate some cell lysis in this case.

Although specific secretion of the 41KDa hybrid was clearly obtained in these experiments, the quantity of protein exported to the medium was inadequate for the purpose of raising antibodies. It was therefore decided to attempt high-level expression of the gene encoding the 41KDa protein by replacing its own promoter with a strong *E.coli* promoter.

### 5.3. Attempts to achieve high level expression of the gene encoding the 41KDa protein.

As described above, the levels of secretion of the 41KDa protein obtained when fused to the 23KDa signal region of haemolysin were insufficient for immunization of rabbits. I therefore attempted to achieve a higher level of expression by cloning the gene under the control of a strong *E.coli* promoter. Before this could be done, however, I had to introduce convenient restriction enzyme sites for the manipulations required for such a construction. To do this, the sequence obtained for the gene encoding the 41KDa protein (Hindley and Berry, 1987) was used to design oligonucleotides for site-directed mutagenesis. Using these oligonucleotides, an *SphI* restriction enzyme site was introduced at the ATG start codon and a *PvuII* restriction enzyme site in the fourth codon prior to the termination codon at the 3' end of the gene. The plasmids used in the mutagenesis reaction and the nucleotide and amino acid changes created are shown in Figure 5.3.

Figure 5.3 Construction of the plasmid M13-41 and site-directed mutagenesis for the creation of restriction enzyme sites.

The 1.85Kb fragment containing the gene encoding the 41KDa protein was obtained by digestion of the plasmid pAS233HA with the restriction enzymes *EcoRI* and *HindIII*. This fragment was ligated into the *EcoRI-HindIII* sites in the multiple cloning site of the replicative form of M13mp18 to obtain the plasmid M13-41.

Single stranded DNA was purified from *E.coli* TG1 cells containing the plasmid M13-41 and used in the mutagenesis reaction as described in Chapter 2, Section 2.3.15. The plasmid incorporating the *SphI* and *PvuII* sites was called M13-41M.

Oligonucleotides used in the creation of the restriction enzyme sites 5' and 3' of the gene encoding the 41KDa protein.

The oligonucleotides used for the creation of the *SphI* site at the 5' end and the *PvuII* site at the 3' end of the gene encoding the 41KDa protein are shown below. Also shown are the amino acid sequence changes obtained upon mutagenesis.

5' mutation:

```
5' GGGAGCTAAAAGAC ATG AGA AAT TTG 3'
      G           C
      -----
      Met Arg Asn Leu
```

The nucleotide change from AGA to CGA is silent.

5'GCTAG C3' is the recognition and cleavage sequence for *SphI*.

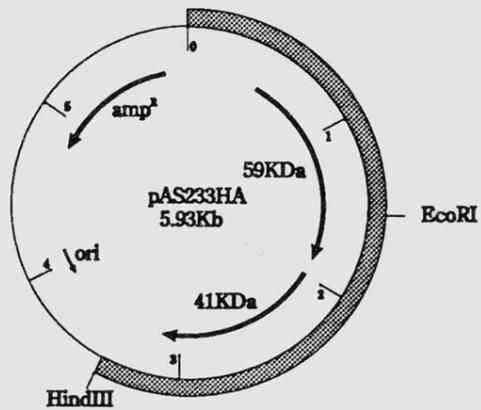
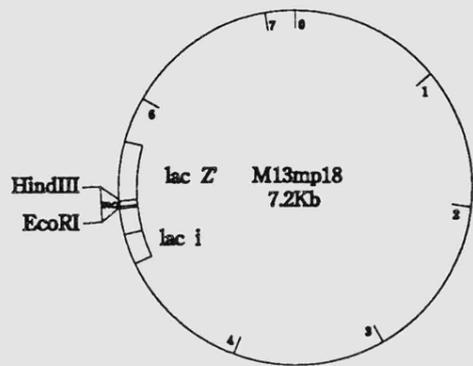
3' mutation:

```
5' AAG ATT ATT ACA GAT GAT CAA AAC TAA 3'
      C
      -----
      Lys Ile Ile Thr Val Asp Gln Asn *
```

The nucleotide change from GAT to GCT changes the amino acid sequence from aspartate to valine.

5'CAT CTG3' is the recognition and cleavage sequence for *PvuII*.

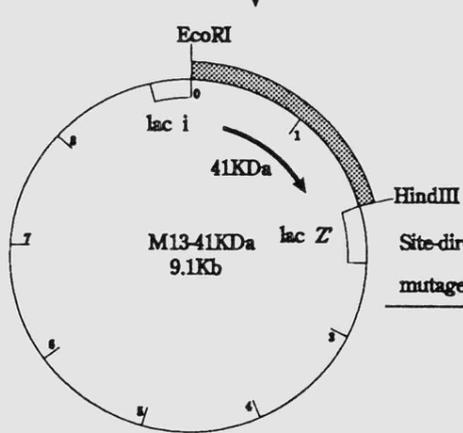
\* denotes the translational stop codon.



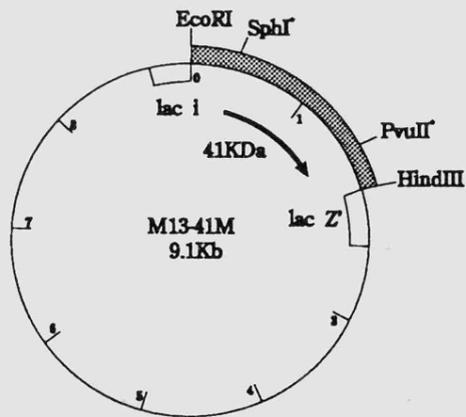
Digest with  
EcoRI/HindIII

Digest with EcoRI/HindIII  
Isolate 1.85Kb fragment

Ligation reaction



Site-directed  
mutagenesis reaction



### 5.3.1. Attempts to clone the gene encoding the 41KDa protein into the *E.coli* expression vector, pET3c.

The vector pET3 is derived from the plasmid pBR322, in the *Bam*HI site of which is located 0.45Kb of sequence containing the  $\Phi$ 10 promoter for the binding of T7 RNA polymerase. This is followed by 13 codons of the *s10* gene of phage T7 (Rosenberg et al., 1987). When manipulations using pET3 are carried out in strains such as *E.coli* MC4100 or HMS174, *E.coli* RNA polymerase, which has no affinity for the T7  $\Phi$ 10 promoter, does not transcribe the *s10* gene. Following the fusion, in the correct translational reading frame, of a gene to the *s10* gene, whose high-level expression is desired, the recombinant vector is transformed into the *E.coli* B strain, BL21-DE3. This strain harbours the excision defective  $\lambda$  lysogen DE3, which contains the T7 polymerase gene downstream of an inducible promoter, *lacUV5*. When expression of the fusion gene is desired, the synthesis of T7 RNA polymerase is induced by the addition of isopropylthio- $\beta$ -D-galactopyranoside (IPTG). T7 RNA polymerase then recognizes and transcribes from the  $\Phi$ 10 promoter on the pET3 vector with very high efficiency and specificity. This process leads to the almost exclusive transcription of the  $\Phi$ 10 promoter controlled gene fusion (Rosenberg et al., 1987).

The gene encoding the 41KDa protein (with the engineered *Sph*I site at the ATG codon, see above) was isolated on a 1.35Kb *Sph*I-*Hinc*II fragment. The 3' *Sph*I overhang was removed with T4 DNA polymerase. This was required in order to clone the gene encoding the 41KDa protein into the *Bam*HI restriction enzyme site in the vector pET3c, making a translational fusion to the *s10* gene on the plasmid. The *Bam*HI site in pET3c had previously been filled in, using the Klenow fragment of DNA polymerase, in order to create compatible blunt-ended sites for the ligation of the 1.35Kb fragment. Due to concern that the enzyme alkaline phosphatase in use at that time in the laboratory had an associated exonuclease activity, the linearized vector was not treated with alkaline phosphatase prior to ligation with the 1.35Kb fragment. Since blunt end ligations are highly inefficient, a large number of transformants had therefore to be screened for the presence of the insert. I decided to do this by colony hybridization

screening under the conditions described in Chapter 2, Section 2.3.12.2; 2.3.13, with the  $^{32}\text{P}$ -labelled 1.35Kb *SphI-HincII* fragment encoding the 41KDa protein as the DNA probe.

Of 440 colonies screened in this manner, 2 colonies showed hybridization to the probe. These colonies were isolated, and the plasmid prepared from them by the minipreparation method (Chapter 2, Section 2.3.2). The plasmids were then digested with a selection of restriction enzymes to check the orientation of the insert in the plasmid. Both the colonies that hybridized contained the insert in the incorrect orientation. Following this, approximately 2000 more colonies were screened in 3 separate experiments, but no colonies containing the insert were obtained. A second vector was therefore chosen, which already contained an *SphI* restriction enzyme site, to simplify the cloning and screening procedure.

### 5.3.2. Cloning of the gene encoding the 41KDa protein into the *E.coli* expression vector, pJLA504.

The vector, pJLA504, is derived from the plasmid pJLf201, which contains the bacteriophage  $\lambda$  promoters  $P_R$  and  $P_L$ , the *CI<sub>857</sub>* gene, and the bacteriophage *fd* transcription terminator. A polylinker containing a multiple cloning site was inserted into the plasmid at a *SalI* restriction enzyme site, downstream of the ribosome binding site of the highly expressed *atpE* gene of *E.coli* (Schauder *et al.*, 1987). Genes cloned in the multiple cloning site under the control of the  $P_L$  and  $P_R$  promoters can then be induced by transfer of the culture to 42°C with consequent inactivation of the *CI<sub>857</sub>* repressor protein.

The gene encoding the 41KDa protein was isolated on the 1.35Kb *SphI-HincII* fragment described above and ligated into the *SphI* and filled-in *EcoRI* site on pJLA504 (Figure 5.4). The *EcoRI* site of the vector pJLA504 was filled-in using the Klenow fragment of DNA polymerase in order to make it compatible with the blunt end generated by the restriction enzyme *HincII*. The ligation reaction mixture was then transformed into *E.coli* MC4100. The ampicillin resistant colonies obtained were analyzed for the presence of the insert in the correct orientation, by minipreparation of the plasmid

and restriction enzyme digestion analysis. Four out of twelve colonies were identified as containing plasmids with the insert in the correct orientation. These four plasmids (pJLA504-41 #6, #7, #8 and #12) were checked by nucleotide sequence analysis over the 5' end of the gene encoding the 41KDa protein, to confirm that the ribosome binding site was positioned correctly. Figure 5.4 shows the plasmid construction map.

The *E.coli* strains containing the four identical recombinant plasmids, together with the control strain containing the vector pJLA504, were grown at 30°C and transferred at an  $A_{600}=0.4$  to 42°C for 90min, for inactivation of the  $CI_{857}$  repressor protein and expression of the 41KDa protein. However, when the cells were lysed by boiling in SDS loading buffer, and the proteins separated on SDS polyacrylamide gels, no highly expressed protein could be detected migrating at 41KDa (Figure 5.5A). This experiment was therefore repeated using an unrelated positive control for induction, a prochymosin-23KDa haemolysin fusion protein with a  $CI_{857}$  controlled promoter. The gel in Figure 5.5B shows that although the positive control protein was induced satisfactorily, there was still no detectable protein being synthesized at 41KDa in the cells containing the plasmid pJLA504-41.

#### 5.4. Detection of the 41KDa protein expressed from the plasmid pJLA504-41 in in vitro transcription-translation assays.

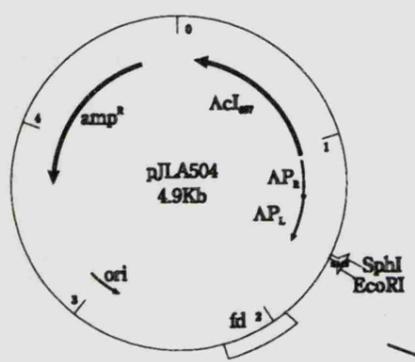
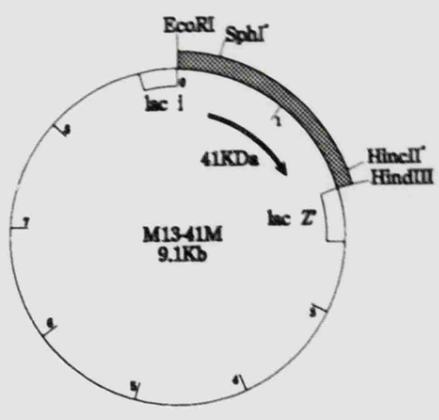
The cloning of the gene encoding the 41KDa protein into an *E.coli* expression vector, pJLA504, did not lead to the detection of high levels of expression of the protein *in vivo*, when cell proteins separated on SDS polyacrylamide gels were stained with Coomassie blue. In order to determine whether the protein was being expressed from pJLA504-41, the recombinant plasmids were purified by CsCl-density gradient centrifugation and subjected to *in vitro* coupled transcription-translation analysis at 30°C and 42°C (Chapter 2, Section 2.4.2).

When the products of a 60min transcription-translation

Figure 5.4 Construction of the plasmid pJLA504-41.

The 1.35Kb fragment containing the gene encoding the 41KDa protein was obtained by digestion of the plasmid M13-41M with the restriction enzymes *SphI* and *HincII*. The vector pJLA504 was digested with the enzyme *EcoRI*, and the 5' overhang created by the digestion was filled in using the Klenow fragment of DNA polymerase I. This produced a blunt end that would be compatible with the *HincII* blunt end. The vector pJLA504 was then digested with *SphI*. The 1.35Kb *SphI-HincII* fragment was then inserted into the *SphI* site and the previously blunt ended *EcoRI* sites of pJLA504, creating the plasmid pJLA504-41. The sequence at the *SphI* site was determined to ascertain that the ribosome binding site was correctly positioned with respect to the ATG start codon of the gene encoding the 41KDa protein. The nucleotide sequence of the region is shown in the inset.

mcs = multiple cloning site  
fd = fd transcription terminator  
RBS = ribosome binding site



Digest with EcoRI  
 Fill in overhang with Klenow  
 Digest with SphI

Digest with SphI/HincII  
 Isolate 1.35Kb fragment

Ligation reaction

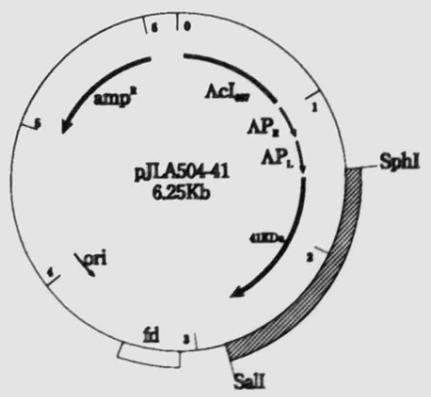


Figure 5.5A Induction of cells containing the plasmid pJLA504-41, which encodes the gene for the 41KDa protein under the control of the  $\lambda$  P<sub>R</sub> and P<sub>L</sub> promoters.

*E. coli* MC4100 cells containing the vector pJLA504, and the plasmids pJLA504-41#8 and pJLA504-41#12, both of which are identical and contain the gene encoding the 41KDa protein, were grown in Luria broth at 30°C from an A<sub>600</sub>=0.05 to an A<sub>600</sub>=0.4. An uninduced sample was taken and the cells were transferred to 42°C to denature the CI<sub>857</sub> protein and allow expression from the  $\lambda$  P<sub>R</sub> and P<sub>L</sub> promoters. After 90min at 42°C, the cells were collected by centrifugation in an MSE microcentrifuge for 10min. 0.05 A<sub>600</sub> Units of cellular protein was heated to 100°C for 3min in SDS loading buffer and the proteins were separated on an 11% separating, 5% stacking SDS polyacrylamide minigel. Following separation, the gel was stained with Coomassie blue to visualize protein.

Lanes:

- A, uninduced *E. coli* MC4100 (pJLA504), negative control
- B, induced *E. coli* MC4100 (pJLA504), negative control
- C, uninduced *E. coli* MC4100 (pJLA504-41#8)
- D, induced *E. coli* MC4100 (pJLA504-41#8)
- E, uninduced *E. coli* MC4100 (pJLA504-41#12)
- F, induced *E. coli* MC4100 (pJLA504-41#12)

Lanes containing uninduced cells are marked "U", and lanes with induced cells are marked "I".

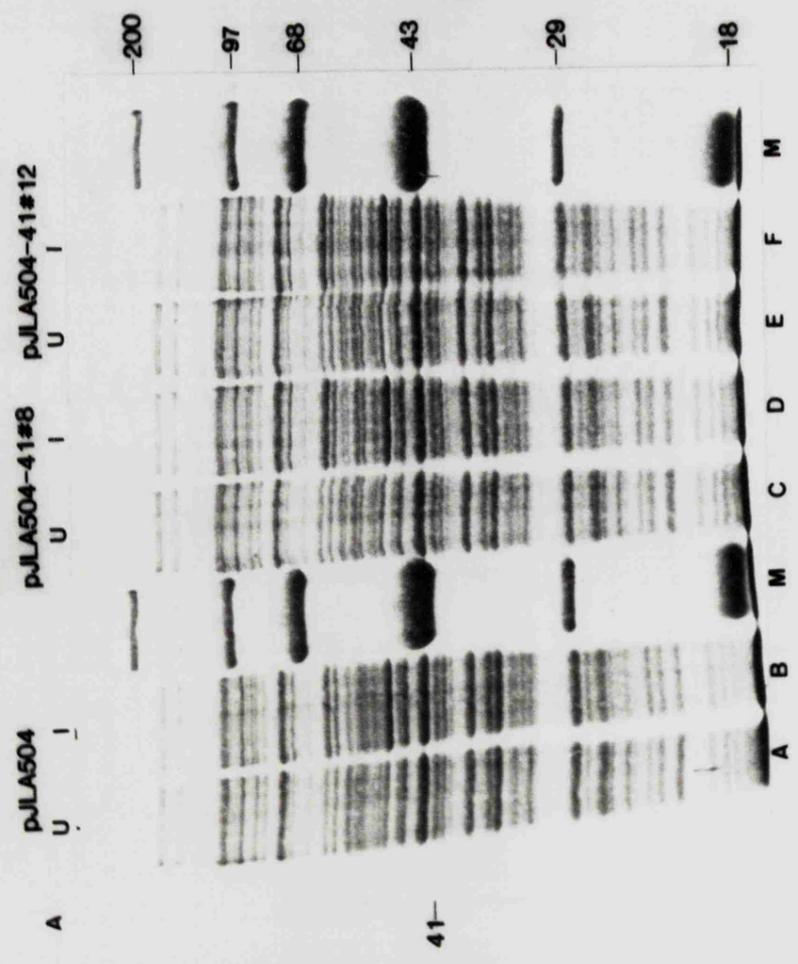
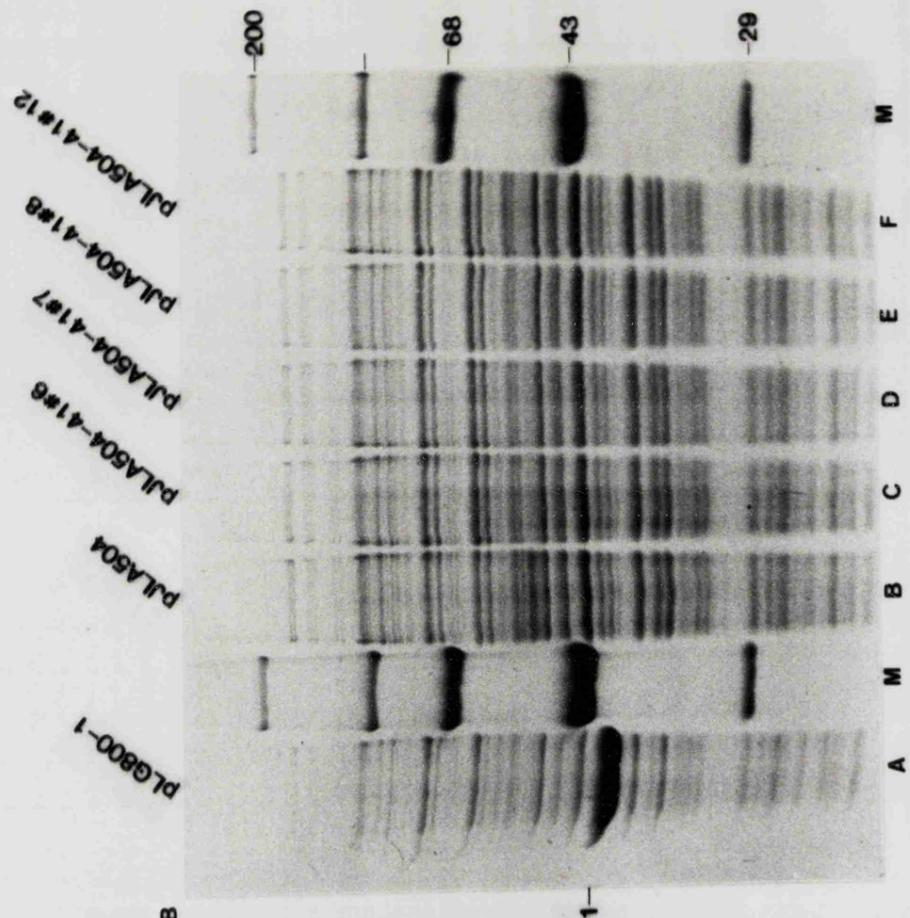
Figure 5.5B Induction of cells containing the plasmid pJLA504-41, encoding the gene for the 41KDa protein, in the presence of a positive control for induction.

*E. coli* MC4100 cells contained the vector pJLA504, or the plasmids pJLA504-41#6, #7, #8 and #12, (all identical), containing the gene encoding the 41KDa protein under  $\lambda$  P<sub>R</sub> and P<sub>L</sub> promoter control. *E. coli* RR1 cells contained the plasmid pLG800-1, encoding a heat-inducible fusion of prochymosin to the 23KDa signal region of HlyA under  $\lambda$  P<sub>R</sub> control. All strains were grown, induced, and the proteins separated exactly as described above (legend to Figure 5.5A). The proteins were visualized by staining the gel with Coomassie blue.

Lanes:

- A, induced *E. coli* RR1 (pLG800-1), expressing the prochymosin-HlyA fusion
- B, induced *E. coli* MC4100 (pJLA504), negative control
- C, induced *E. coli* MC4100 (pJLA504-41#6), containing the gene encoding the 41KDa protein
- D, induced *E. coli* MC4100 (pJLA504-41#7), containing the gene encoding the 41KDa protein
- E, induced *E. coli* MC4100 (pJLA504-41#8), containing the gene encoding the 41KDa protein
- F, induced *E. coli* MC4100 (pJLA504-41#12), containing the gene encoding the 41KDa protein

The sizes of protein standards are shown in KDa. The position expected for the 41KDa protein is indicated.



41-

assay were separated on SDS polyacrylamide gels, fluorographed and the images of radiolabelled proteins obtained on X-ray film, it was clear that a 41KDa protein was indeed being produced from the plasmid pJLA504-41 (Figure 5.6A). This result posed the question of the lack of expression to a high degree *in vivo*. There were several possible reasons for this, including instability of the protein and instability of the plasmid. The cells containing the recombinant plasmid pJLA504-41 nevertheless grew normally upon induction as measured by optical density increase (data not shown).

### 5.5. Analysis of the stability of the 41KDa protein.

In order to investigate the failure of the 41KDa protein to accumulate to detectable levels *in vivo*, the stability of the protein *in vitro* and *in vivo* was assessed.

#### 5.5.1. Analysis of the stability of the 41KDa protein *in vitro*.

The plasmid containing the gene encoding the 41KDa protein, (pJLA504-41), was purified by CsCl density gradient centrifugation and used in a coupled transcription-translation system as described above. The protein was labelled with radioactive <sup>35</sup>S-methionine for 15min, and this was followed by the addition of unlabelled L-methionine as chase. This was followed 5min later by the addition of 10µg/mL rifampicin to stop further transcription from occurring and 10µg/mL chloramphenicol to block further protein synthesis. A control sample was taken prior to the addition of rifampicin and chloramphenicol.

Samples were then taken 10min and 30min following the addition of rifampicin and chloramphenicol to the incubation mixture. Soluble proteins were recovered by centrifugation of the sample for 3min at high speed in an MSE microcentrifuge. The samples were heated to 100°C in SDS loading buffer for 3min before separation of the proteins on an 11% SDS polyacrylamide gel. The gel was then subjected to fluorography. An image of the X-ray film obtained is shown in Figure 5.6B. The gel shows that the 41KDa protein was indeed rapidly degraded, with no protein being present at the end of

Figure 5.6A *In vitro* coupled transcription-translation assays on the plasmid pJLA504-41, containing the gene encoding the 41kDa protein under  $\lambda$  P<sub>R</sub>, P<sub>L</sub> promoter control.

The plasmids used in the assay were purified by the CsCl density gradient centrifugation method (Chapter 2, Section 2.3.3). 1.5 $\mu$ g of plasmid DNA was used in each assay. The S-30 cell extracts used were obtained from the *E.coli* strain Sm<sup>R</sup>. Labelling with <sup>35</sup>S-methionine of proteins encoded by the plasmids was carried out exactly as described in Chapter 2, Section 2.4.2. The reactions were centrifuged for 10min at room temperature in an MSE microcentrifuge to remove ribosomes, and the proteins heated to 100°C for 3min in SDS loading buffer. The proteins were separated on an 8.5% separating, 5% stacking SDS polyacrylamide gel. Following separation, the gel was fluorographed, dried and exposed to X-ray film to obtain an image of the radiolabelled proteins.

Lanes:

- A, assay control without added DNA
- B, assay containing the vector pJLA504, negative control
- C, assay containing the plasmid pJLA504-41#7, containing the gene encoding the 41kDa protein
- D, assay containing the plasmid pJLA504-41#8, containing the gene encoding the 41kDa protein
- E, assay containing the plasmid pJLA504-41#12, containing the gene encoding the 41kDa protein

Figure 5.6B *Pulse-chase of the in vitro transcription-translation assay to measure the stability of the 41kDa protein.*

The proteins encoded by the vector control, pJLA504, and the plasmids containing the gene encoding the 41kDa protein under  $\lambda$  P<sub>R</sub>, P<sub>L</sub> promoter control, pJLA504-41#8 and #12, were labelled for 15min with <sup>35</sup>S-methionine as described in Chapter 2, Section 2.4.2. After the labelling reaction was completed, an excess of cold L-methionine (150 $\mu$ g) was added and incubated for 5min. A 0min sample was taken for control. Rifampicin (10 $\mu$ g/mL) and chloramphenicol (10 $\mu$ g/mL) were then added to interfere with subsequent transcription and protein translation. Samples were taken 10 and 30min after the addition of the antibiotics to follow degradation of the 41kDa protein. The samples were treated as described above (legend to Figure 5.6A) except that they were separated on 11% separating, 5% stacking gels.

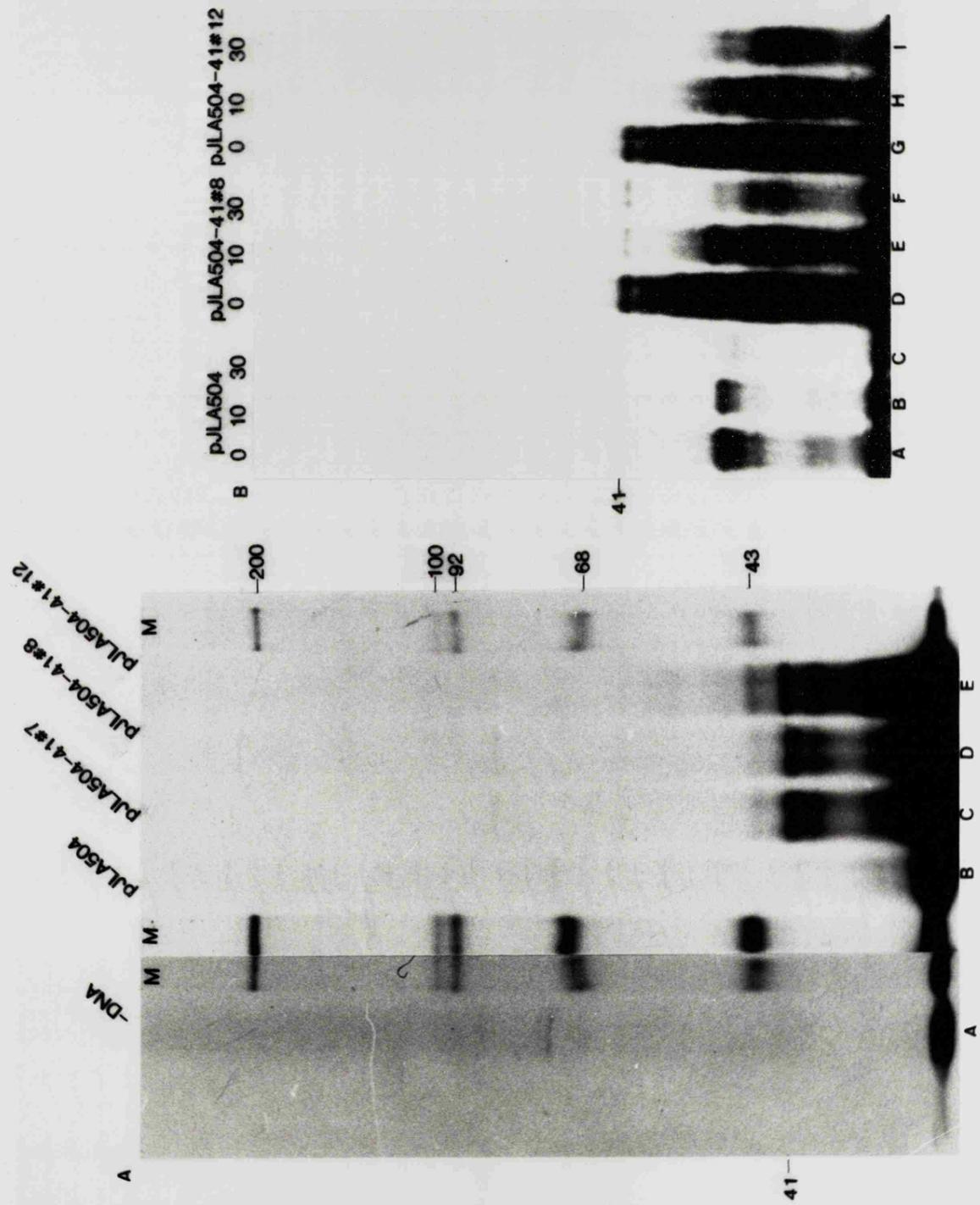
Lanes:

The vector pJLA504 after incubation in the presence of rifampicin and chloramphenicol for A, 0min, B, 10min, C, 30min.

The plasmid pJLA504-41#8 after incubation in the presence of rifampicin and chloramphenicol for D, 0min, E, 10min, F, 30min

The plasmid pJLA504-41#12 after incubation in the presence of rifampicin and chloramphenicol for G, 0min, H, 10min, I, 30min

The sizes of proteins and protein standards are shown in kDa.



a 30min incubation. However, examination of the  $\beta$ -lactamase protein produced by the vector pJLA504 in the same experiment, shows that the  $\beta$ -lactamase protein, which is normally expected to be stable under such conditions, was also turned over at approximately the same rate as the 41KDa protein. Consequently, nothing could be deduced regarding the stability of the 41KDa protein from such an experiment. The addition of phenylmethylsulfonylfluoride (PMSF, a serine protease inhibitor), and the use of Lon protease-deficient extracts prepared from *E.coli* LC137 (*lon*<sup>-</sup>) for the transcription-translation assay, did not affect the rate of degradation of either the 41KDa protein or  $\beta$ -lactamase (data not shown). The reason for the abnormal instability of polypeptides in the *in vitro* system in these experiments was not pursued further and instead an attempt was made to measure stability of the 41KDa protein *in vivo*.

#### 5.5.2. Attempts to determine the half-life of the 41KDa protein *in vivo*.

The pulse-chase analysis performed *in vitro* and described in Section 5.5.1 did not permit any conclusion to be drawn as to the inherent stability of the 41KDa protein. Since *in vivo* conditions are often at variance from those present *in vitro*, I decided to carry out a similar experiment using whole cells.

Cells containing the control vector (pJLA504) and the recombinant plasmid containing the gene encoding the 41KDa protein (pJLA504-41) were grown overnight in minimal medium at 30°C, and then diluted in minimal medium to an  $A_{600}=0.05$ . The cells were grown with vigorous shaking until an  $A_{600}=0.4-0.5$ , following which 20mL of culture from each flask was transferred to fresh, sterile 100mL flasks. The original flasks were retained to measure the  $A_{600}$  values. To each of the 100mL flasks,  $2\mu\text{L}/A_{600}$  Unit of <sup>35</sup>S-methionine was added and the cells were allowed to grow for a further 15min at 30°C. Both sets of flasks were transferred to 42°C and 0, 5, 10 and 15min samples were taken to follow incorporation of radioactive label into the 41KDa protein following heat induction of the cells. L-methionine (10 $\mu\text{L}$  of 30mg/mL solution) was then added as chase to each of the radioactive flasks to follow degradation of the 41KDa protein. Samples

were taken at 0, 5, 10, 15, 20 and 25min following the addition of the chase solution. The  $A_{600}$  reading of the culture was taken at each sampling point. 0.1  $A_{600}$  Unit of each cell sample was then heated to 100°C for 3min in SDS loading buffer, and the radioactive proteins separated by SDS polyacrylamide gel electrophoresis. The gel was then fluorographed as described in Chapter 2, Section 2.4.6. The image of the X-ray film is presented in Figure 5.7. The results obtained failed to detect any visible level of induction of a 41KDa protein either after a short or after a 15min incubation at 42°C. This suggests, although other interpretations are possible, that induction of the synthesis of high levels of the 41KDa protein is being prevented, rather than an overexpressed protein being rapidly degraded.

#### 5.6. Identification of the mRNA transcript of the 41KDa protein encoding gene *in vivo*.

The experiment described in Section 5.5.2 did not lead to the detection of the 41KDa protein *in vivo* in short pulse labelling experiments. I decided therefore to analyze the presence of the mRNA transcript of the gene encoding the 41KDa protein in cells containing the recombinant plasmid pJLA504-41. The cells containing the vector, pJLA504, and the recombinant plasmid, pJLA504-41, were grown at 30°C as described above (Section 5.5.2). Samples were taken from each flask before the flasks were transferred to 42°C for denaturation of the CI<sub>857</sub> protein and consequent induction of transcription of the 41KDa protein encoding gene. Cells of *E.coli* 576, which are wild-type for the *lac* operon, were used as control and induced for the synthesis of  $\beta$ -galactosidase by the addition of 0.4mM IPTG.

Total RNA was prepared from each sample as described in Chapter 2, Section 2.9.1. The RNA samples were then subjected to agarose gel electrophoresis through formamide gels, transferred by capillary action to Hybond-N membranes (Chapter 2, Section 2.9.2.1), and then hybridized to the radiolabelled 1.35Kb *SphI-HincII* DNA fragment containing the gene encoding the 41KDa protein. An image of the X-ray film is shown in Figure 5.8 (Panel C). Controls included radioactive probes directed to an *E.coli* rDNA operon (Figure 5.8, Panel A), and a

Figure 5.7 Pulse chase analysis to determine half-life of the 41KDa protein in vivo.

The *E.coli* MC4100 cells containing the vector, pJLA504, or the recombinant plasmid, pJLA504-41, were grown overnight at 30°C in minimal medium and then inoculated in 100mL minimal medium to an  $A_{600}=0.05$ . The cells were grown to an  $A_{600}=0.4-0.5$ . 20mL from each flask was then transferred to fresh flasks, and 2 $\mu$ L of  $^{35}\text{S}$ -methionine added to the flasks, and grown for a further 15min to allow incorporation of the radioactive label into proteins. The original flasks were used to measure the optical density values.

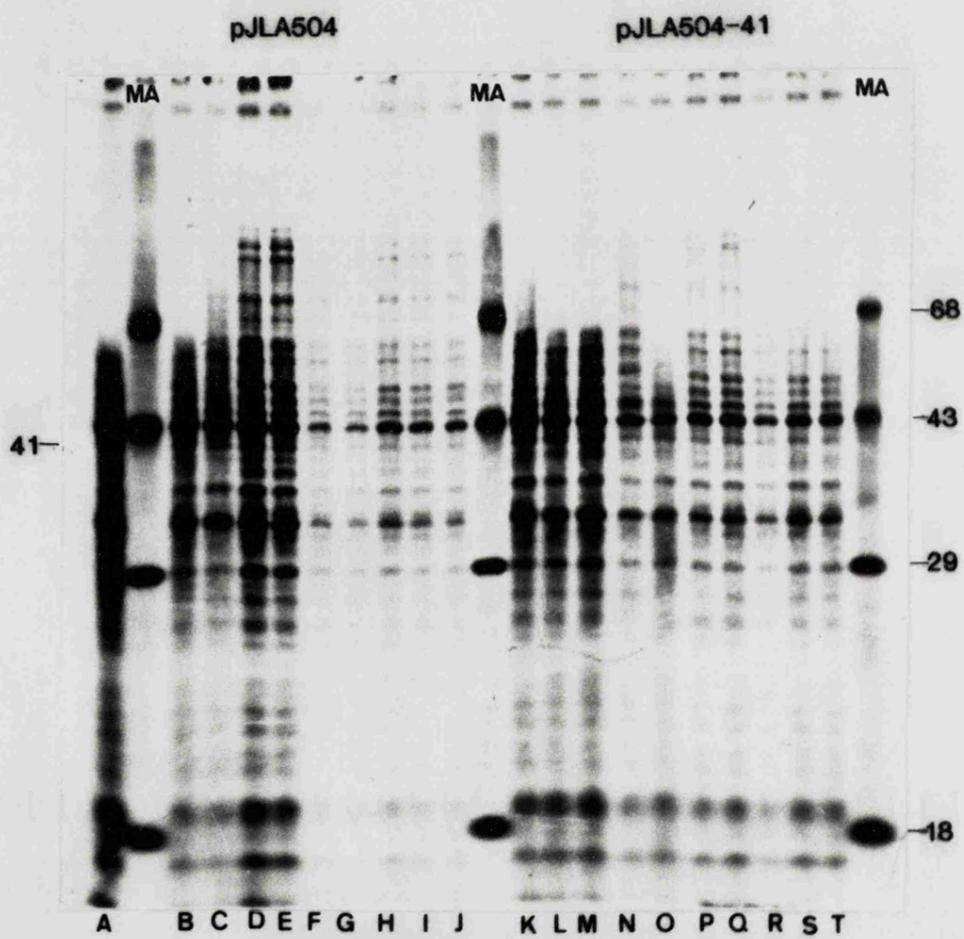
Both sets of flasks were then transferred to 42°C to inactivate the  $\text{CI}_{857}$  repressor protein and induce transcription from the  $\lambda$   $P_R$ ,  $P_L$  promoters. Samples were taken 0, 5, 10, and 15min following transfer to 42°C. Chase (10 $\mu$ L of 30mg/mL L-methionine) was added to the radioactive samples. Optical density and cell samples were taken at 0, 5, 10, 15, 20 and 25min following addition of the chase. 0.1  $A_{600}$  Unit of cells were heated to 100°C for 3min in SDS loading buffer, and separated on an 11% SDS polyacrylamide gel. The gel was fluorographed and exposed to X-ray film to visualize labelled proteins.

Lanes:

A-J : *E.coli* carrying the vector pJLA504; A, 0min after addition of  $^{35}\text{S}$ -methionine at 42°C; B, 5min; C, 10min; D, 15min; E, 0min after addition of chase; F, 5min; G, 10min; H, 15min; I, 20min; J, 25min.

K-T : *E.coli* carrying the recombinant plasmid pJLA504-41; K, 0min after addition of  $^{35}\text{S}$ -methionine at 42°C; L, 5min; M, 10min; N, 15min; O, 0min after the addition of chase; P, 5min; Q, 10min; R, 15min; S, 20min; T, 25min.

The sizes of protein standards (lane MA) and the expected position for the 41KDa protein are shown in KDa.



sequence encoding the *lacZ* gene (Figure 5.8, Panel B).

From the results in Figure 5.8C, it is clear that the RNA species present in the lanes containing the recombinant plasmid, pJLA504-41, hybridizes to the 41KDa gene probe after growth both at 30°C and at 42°C (Figure 5.8C, lanes C and D). Contrary to the expectation, therefore, there was no evidence of a specific heat-inducible transcript. There were, however, two distinct RNA species that hybridized to the probe in each lane, with a faint third species of 4.6Kb in the lane containing the uninduced plasmid. The sizes of the RNA species measured against single-stranded  $\lambda$  (*HindIII*) markers are also shown.

Analysis of this X-ray image therefore showed three RNA species of 7.8Kb, 4.6Kb and 3.5Kb hybridized to the probe for the gene encoding the 41KDa protein. However, none of these RNA species correspond to the size of 1.1Kb that would be expected for the mRNA of the gene encoding the 41KDa protein. This could indicate that the *fd* transcription stop signal present at the 3' end of the 41KDa gene on the vector does not halt transcription. This could have important consequences such as destabilization of the plasmid and difficulty in initiation of translation due to secondary structure of the transcribed mRNA (Ganoza *et al.*, 1987). In order to test this hypothesis, the labelled gene probe encoding the 41KDa protein was stripped from the filter, as described in Chapter 2, Section 2.3.14, and the RNA on the filter then re-hybridized to the linearized and radioactively labelled vector, pJLA504. The results are described in the next section.

#### 5.6.1. The transcription stop signal downstream of the gene encoding the 41KDa protein is not efficient.

The filter used in the experiment described above (Section 5.6) was stripped of the 1.35Kb probe and exposed to X-ray film to ensure complete removal of the probe. The filter was then blocked and subjected to hybridization with the linearized, radiolabelled vector, pJLA504. An image of the filter is presented in Figure 5.8, Panel D. Comparison of the lanes in this panel with those in Figure 5.8, Panel C reveals that the RNA species at the higher molecular weights of 4.6

Figure 5.8 Analysis of mRNA transcripts from the plasmids pJLA504 and pJLA504-41.

*E.coli* MC4100 strains containing the plasmids pJLA504 and pJLA504-41 and *E.coli* W576 (wild type for the *lac* operon) were grown in Luria broth at 30°C and 37°C, respectively, from  $A_{600}=0.05$  to  $A_{600}=0.4$ . Samples of the cultures for preparation of RNA were taken, and then *E.coli* MC4100 containing pJLA504 and pJLA504-41 were induced by transfer to 42°C, and *E.coli* W576 was induced by the addition of 0.4mM IPTG. The cultures were induced for 90min, and samples removed for RNA preparation.

Total RNA was prepared from the cells as described in Chapter 2, Section 2.9.1. 4.8µg of the RNA samples were transferred following electrophoresis through three identical formamide agarose gels to Hybond-N as described in Chapter 2, Section 2.9.2.1). The RNA was bound to the membrane by exposure to long wavelength ultraviolet radiation for 4min. Single-stranded  $\lambda$  (*Hind*III) DNA was used as size markers.

The RNA on the filters was subjected to hybridization with radioactively labelled RNA probes:

Panel A: 2.5Kb *S*alI fragment from  $\lambda$ rif<sup>d</sup>18 containing the genes encoding an *E.coli* rRNA operon

Panel B: 6.3Kb *S*maI fragment from pLKC480 containing the *lacZ* and *lacY* genes

Panel C: 1.35Kb *S*phI-*H*incII fragment containing the gene encoding the 41KDa protein

Panel D shows the filter in Panel C stripped of radioactive probe as described in Chapter 2, Section 2.3.14, and subjected to hybridization with the radioactively labelled plasmid pJLA504, previously digested with *E*coRI. The filter was exposed before the second hybridization step to ensure complete removal of the probe.

Lanes:

A, Total RNA prepared from uninduced (U) cells of *E.coli* MC4100 (pJLA504), negative control

B, Total RNA prepared from induced (I) cells of *E.coli* MC4100 (pJLA504), negative control

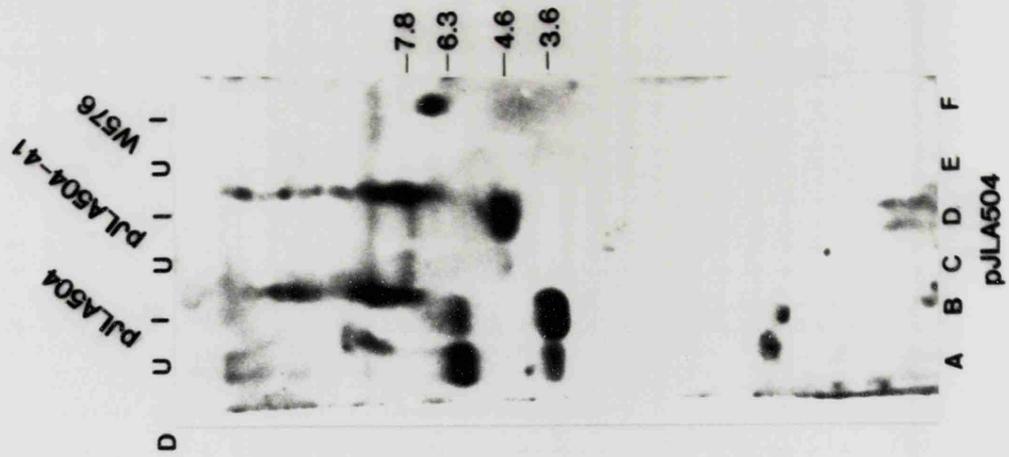
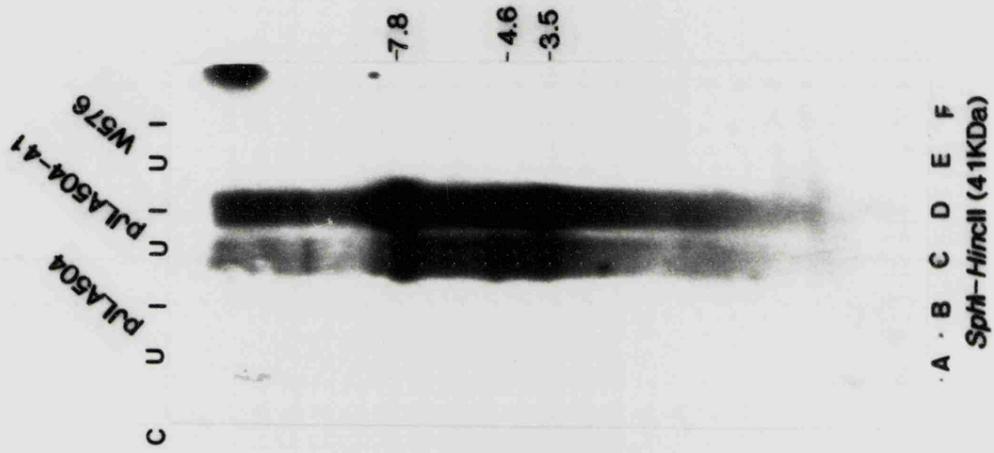
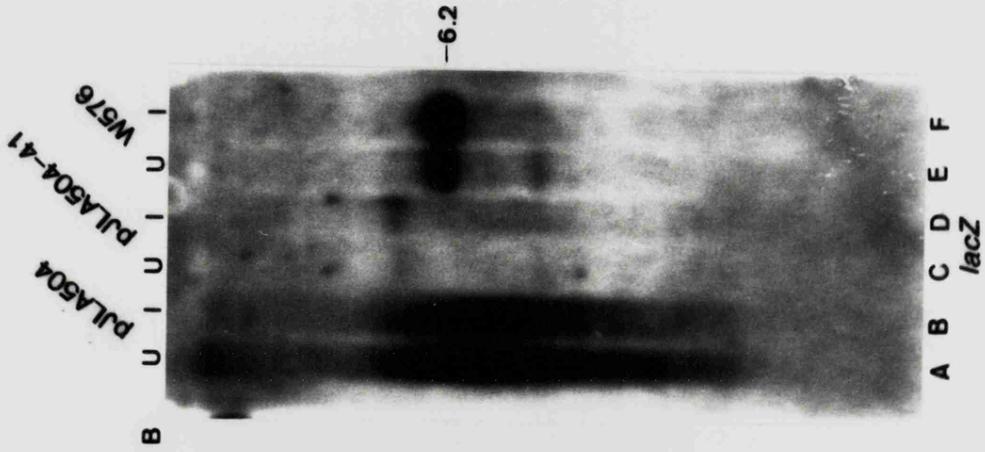
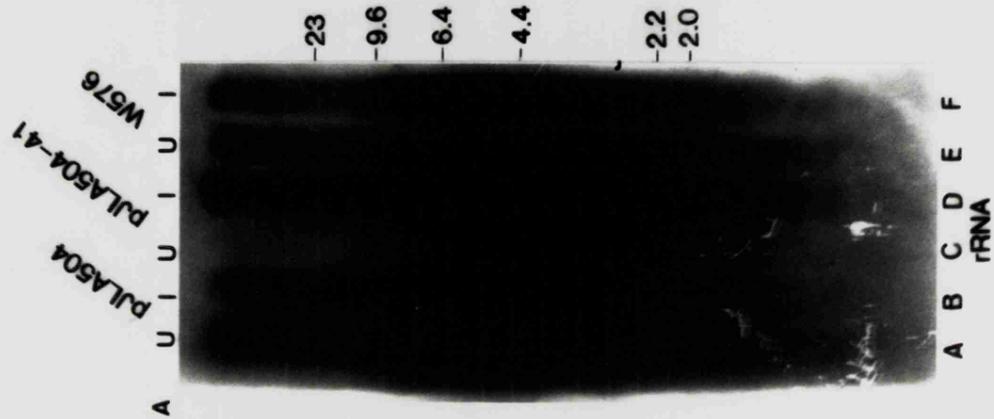
C, Total RNA prepared from uninduced (U) cells of *E.coli* MC4100 (pJLA504-41), containing the gene encoding the 41KDa protein

D, Total RNA prepared from induced cells (I) of *E.coli* MC4100 (pJLA504-41), containing the gene encoding the 41KDa protein

E, Total RNA prepared from uninduced (U) cells of *E.coli* W576, wild type for the *lac* operon

F, Total RNA prepared from induced (I) cells of *E.coli* W576, wild type for the *lac* operon

The sizes of the RNA hybridizing to the probes was calculated from single-stranded  $\lambda$  (*Hind*III) markers, and are indicated in Kb.



and 7.8Kb correspond to the vector pJLA504, and that only the RNA species at 3.5Kb in lanes C and D of Figure 5.8C, correspond to the mRNA encoding the 41KDa protein and terminating at the *fd* terminator (see Figure 5.4). This shows that the hypothesis presented in Section 5.6 above, that the *fd* transcription stop signal is inefficient and allows substantial unterminated transcription of the entire plasmid from the  $\lambda$  P<sub>L</sub> and P<sub>R</sub> promoters, is correct. Inefficient translation from these mRNAs could be an explanation for the lack of accumulation of the 41KDa protein in cells containing the recombinant plasmid. Puzzlingly, the size of the RNA species hybridizing to the probe for the 41KDa encoding gene itself in fact did not correspond to the size of 1.1Kb predicted for the minimal mRNA transcript of the gene. The markers used were  $\lambda$  DNA digested with the restriction enzyme *HindIII* and converted to single strands by alkaline treatment rather than single-stranded RNA markers. I suggest that in these experiments, the single-stranded  $\lambda$  DNA markers ran at a significantly lower molecular weight than the corresponding size of RNA markers would have done.

### 5.7. Summary.

Constructs were prepared which allowed the successful secretion of a 41KDa-HlyA fusion protein, but the level of secretion obtained was insufficient for the purpose of rabbit immunization for antibody production. Attempts were next made to obtain high-level expression of the gene encoding the 41KDa protein. Initial attempts to clone the gene into the vector pET3c, under the control of a T7 RNA polymerase-recognized  $\Phi$ 10 promoter, led to the detection of two colonies containing the insert but in the incorrect orientation. Subsequent attempts did not lead to the detection of any colonies containing the insert. A  $\lambda$  P<sub>L</sub>, P<sub>R</sub> based vector was then chosen that already contained an *SphI* site to facilitate subcloning of the gene.

The plasmid pJLA504-41, containing the gene encoding the 41KDa protein, was sequenced to ensure correct positioning of the ribosome binding sequence and the ATG start codon. The colonies containing the recombinant did not express to detectably high levels the protein *in vivo*. Nevertheless, a protein of the expected size was expressed from the same

recombinants *in vitro* at 30°C and 42°C confirming the presence of the correct construct.

Total RNA was prepared from uninduced and heat-induced cells containing the recombinant plasmid, and this RNA was analyzed for transcription of the gene encoding the 41KDa protein. There were three major RNA species that hybridized to the probe, none of which corresponded to the position of 1.1Kb that would be expected for mRNA encoding a 41KDa protein. The DNA probe was then stripped from the filter, which was reprobbed with radiolabelled linearised vector pJLA504. In this case, the probe hybridized to two of the three higher molecular weight RNA species that had initially hybridized to the *SphI-HincII* probe (7.8 and 4.6Kb), but not to the lower molecular weight mRNA species of 3.5Kb. This mRNA species should therefore encompass the coding region for the 41KDa gene. This data indicated some form of aberrant transcription of the gene, perhaps leading to poor levels of translation. Perhaps more seriously, there was no detectable increase in 41KDa specific transcripts upon heat induction of the Lambda promoters. These data in turn suggested some aberrant transcription of the gene encoding the 41KDa protein at low temperature, when transcription should have been blocked. For all these reasons, and for lack of time, further attempts to enhance the level of expression of the 41KDa protein were not continued.

## Chapter 6.

### DNA sequencing and high-level expression of the gene encoding the 59KDa toxin protein of *B.sphaericus*.

#### 6.1. Introduction.

The data presented in Chapter 3 showed that the 3.6Kb DNA insert of the plasmid pAS233HA encodes two proteins of 41 and 59KDa. Since antibodies raised against the putative "crystal" protein of 125KDa failed to recognize either the 41 or the 59KDa proteins, my aim was to produce sufficient amounts of the 41 and 59KDa proteins to use as antigen in rabbits for the purpose of raising antibodies for further studies of the relation of these proteins to the crystal.

Chapter 5 dealt with attempts to overexpress the gene encoding the 41KDa protein, following site-directed mutagenesis to create restriction enzyme sites that could be used to place the gene under the control of a strong *E.coli* promoter. The sequence for the gene from *B.sphaericus* 1593, encoding the 41KDa protein was reported by Hindley and Berry (1987), in the early stages of this study. There was also evidence that a number of groups had cloned a similar segment of DNA from larvicidal strains of *B.sphaericus* (Souza *et al.*, 1988; Baumann *et al.*, 1987), although there was no other report to substantiate the nature or sizes of the toxin proteins produced by *B.sphaericus* 1593. Chapter 3 in this thesis described the identification of a 59KDa protein also encoded by the 3.6Kb insert of the plasmid pAS233HA, which was required, together with the 41KDa protein, for toxicity of recombinant *E.coli* to mosquito larvae (Chapter 3, Section 3.6). As indicated above, the similarity of the restriction enzyme sites on the cloned DNA fragments encoding the 43 and 63KDa proteins (Baumann *et al.*, 1987), with that of the 3.6Kb *HindIII* fragment encoding the 41 and 59KDa proteins, indicated that the 41 and 43KDa proteins are the same, and that the 63KDa protein described by Baumann *et al.* (1987) is identical to the 59KDa protein product of pAS233HA. The nucleotide sequence of the gene encoding the 59KDa protein had not been reported earlier, and we therefore decided to sequence this gene from *B.sphaericus* 1593M in order to approach overexpression of the protein in a systematic manner. In an

alternative approach for overexpression and purification, I shall describe attempts to secrete the 59KDa protein using the haemolysin secretion pathway of *E.coli*. As with the 41KDa toxin whis was to be achieved by fusing the 23KDa signal region of haemolysin to the 59KDa protein to make a translational fusion.

## 6.2. Secretion from *E.coli* of a portion of the 59KDa protein using the haemolysin secretion system.

Concomitantly with the secretion experiments described above (Chapter 5, Section 5.2.1) to secrete a portion of the 41KDa protein, I attempted to secrete a part of the 59KDa protein in a similar manner. The site chosen for the fusion of the 59KDa protein to the haemolysin signal region was a unique *StuI* restriction enzyme site, approximately two-thirds of the way into the gene encoding the 59KDa protein. This gene in the plasmid pHAΔEV is under the control of the original *B.sphaericus* promoter. The 23KDa C-terminal signal region was isolated on a 0.8Kb *SmaI-HincII* in-frame fragment from the vector pLG609-1, and ligated to the plasmid pHAΔEV linearized with the restriction enzyme *StuI*. The construction of the plasmid p59'HlyA is shown in Figure 6.1A.

Ampicillin resistant colonies obtained following transformation of the ligation mixture into *E.coli* MC4100 were screened for the presence of insert in the correct orientation by minipreparation of the plasmids and restriction enzyme digestion analysis. *E.coli* MC4100 cells containing the plasmid p59'HlyA were transformed with a second plasmid, pLG575, which contains the two membrane-associated transport proteins HlyB and HlyD. Cells with and without the Hly export functions were then grown in Luria broth for 5 hours after they had reached an  $A_{600}=0.4$ . The reason for growing the cells into late exponential phase in this way was that the positive control used for secretion was a 30KDa fusion of chloramphenicol acetyltransferase III (CATIII) gene to the 23KDa signal region of haemolysin. The CATIII'HlyA fusion gene was under the control of the CATIII promoter that is expressed in the late exponential growth phase. In order to compare secretion levels between the CATIII'HlyA protein and the 59'HlyA fusion protein, all the cultures were grown into

Figure 6.1A Construction of the plasmid p59'HlyA.

The 0.8Kb *SmaI-HincII* fragment of the plasmid pLG609-1 encoding the 23KDa C-terminal signal region of HlyA was isolated and ligated into the *PvuII* site of the plasmid pHAAEV in order to make a translational fusion of the 23KDa signal to two-thirds of the gene encoding the 59KDa protein. The size of the fusion protein was 66KDa. The orientation of the 0.8Kb insert in the plasmid was identified by digestion with the restriction enzyme *PstI*.

Figure 6.1B Export of the 59'HlyA fusion product from cells containing the plasmid p59'HlyA.

The plasmid p59'HlyA, containing the fusion protein 59'HlyA, and pCATIII'HlyA, encoding part of the CATIII protein fused to the 23KDa C-terminal signal region of HlyA, were transformed into *E.coli* cells with and without the plasmid pLG575, encoding the haemolysin export proteins HlyB and HlyD. The cells were grown in Luria broth for 5 hours after they had reached an  $A_{600}=0.4$ . Supernatant and cell samples were prepared as described in Chapter 2, Section 2.4.4. 0.05  $A_{600}$  Units of cells and 1.0  $A_{600}$  Units of supernatant proteins were heated to 100°C for 3min in SDS loading buffer and the proteins separated on an 8.5% separating, 5% stacking SDS polyacrylamide minigel. The proteins were then electrophoretically transferred onto nitrocellulose paper. Non-specific binding sites on the paper were blocked by incubation overnight with 5% (w/v) Marvel milk in 1X PBS. The Western immunoblot was developed sequentially with a 1:100 dilution of antibodies raised to the 23KDa signal peptide of HlyA, 1:1000 dilution of goat anti-rabbit antibodies and 1:1000 dilution of rabbit peroxidase anti-peroxidase antibodies (Chapter 2, Section 2.6.3)

Lanes:

- A, total cells of *E.coli* JM101 (pCATIII'HlyA) without export functions
- B, total cells of *E.coli* JM101 (pCATIII'HlyA) with export functions
- C, total cells of *E.coli* MC4100 (p59'HlyA) without export functions
- D, total cells of *E.coli* MC4100 (p59'HlyA) with export functions
- E, supernatants of *E.coli* JM101 (pCATIII'HlyA) without export functions
- F, supernatants of *E.coli* JM101 (pCATIII'HlyA) with export functions
- G, supernatants of *E.coli* MC4100 (p59'HlyA) without export functions
- H, supernatants of *E.coli* MC4100 (p59'HlyA) with export functions

The sizes of protein standards and major proteins are indicated in KDa.



the late exponential phase of growth before samples were taken.

The supernatant and cell fractions prepared from each culture were separated on an SDS polyacrylamide gel. When the proteins in the gel corresponding to supernatant fractions were stained with Coomassie blue, no exported protein corresponding to the 59'HlyA fusion over the background was detected (data not shown). However, transfer of the proteins to nitrocellulose paper and Western immunoblot analysis carried out using polyclonal antibodies to the 23KDa peptide, led to the detection of an exported protein migrating at 63KDa (Figure 6.1B, lane H), from supernatants of cells containing export functions. This protein corresponds to the size expected for approximately 40KDa of the 59KDa protein fused to the 23KDa signal sequence. No such protein was visible in the supernatants of cells lacking the export functions (Figure 6.1b, lane G), demonstrating the specificity of the transport process. In contrast to the presence inside cells containing the HlyB and HlyD proteins of the 41KDa protein fused to the HlyA signal, no such protected protein was visible inside cells in the case of the 59KDa protein fused to the HlyA signal. However, as in the case of the 41KDa protein fused to the signal of HlyA, the quantity of heterologous protein exported to the medium was insufficient to purify and use as antigen. Therefore approaches to overexpress the protein were attempted.

### 6.3. DNA sequence analysis of the gene encoding the 59KDa protein.

The DNA sequence of the gene encoding the 59KDa protein had not yet been reported at the time of these experiments. Since the sequence of the gene was essential to approach high-level expression of its product in a directed manner, I decided to obtain the sequence of the gene.

A 1.65Kb *EcoRI-HindIII* fragment of DNA was shown to contain a portion of the 59KDa protein by deletion analysis of the plasmid pAS233HA in *E.coli* minicells (Chapter 3, Section 3.3.1). This fragment was sonicated and size-selected on agarose gels using the "Death-wish" method of DNA fragment

recovery (Chapter 2, Section 2.3.7.3) for fragments between 0.2 to 0.5Kb. The DNA termini were repaired using the Klenow fragment of DNA polymerase I and subcloned into the alkaline phosphatase-treated *Sma*I restriction enzyme site of M13mp18. Recombinant M13 phage plaques were identified by the inability of recombinant phage to convert the colourigenic substrate X-gal into a blue pigment. Wild-type phage produce blue plaques.

M13 phage was prepared by the method described in Chapter 2, Section 2.5.1. The single-stranded DNA was then isolated and sequenced by the chain termination method (Sanger *et al.*, 1977) using commercially available M13 sequencing primers. The sequences obtained were entered into UWGGC DNA sequence handling computer programmes (Chapter 2, Section 2.10). Gaps that were left in the sequence were filled by sequencing using oligonucleotides designed for the purpose and synthesized in Leicester in the Department of Biochemistry by Mr. J. Keyte. The remainder of the sequence was obtained by subcloning the entire gene encoding the 59KDa protein on a 1.73Kb *Kpn*I-*Eco*RV DNA fragment into M13mp18, and using specifically designed oligonucleotides to read across the gaps in the sequence. The sequencing strategy is presented in Figure 6.2. The nucleotide sequence of the gene encoding the 59KDa protein is presented in Figure 6.3. It should be pointed out that only one strand of the DNA was sequenced. This was because the sequence of the corresponding gene from the *B.sphaericus* strain 2362 was published (Baumann *et al.*, 1988) while the sequencing of this gene from the strain 1593M was underway. Importantly, the sequences were identical except for 3 nucleotides at positions 1890, 1891 and 1892 3' to the coding sequence, which read TAT for the sequence from *B.sphaericus* 1593M (Figure 6.3), and ATA for the sequence from strain 2362 (Baumann *et al.*, 1988).

The major features of the gene encoding the 59KDa toxin are shown in Figure 6.3. The promoter of the 59KDa protein could be identified at positions 508-513 and 452-457 by comparison to known *Bacillus* promoters. A putative ribosome binding site was identified in the corresponding *B.sphaericus* 2362 sequence by Baumann *et al.* (1988). This sequence reading 5'AAGGAGATGA3' had a 9 out of 10 nucleotide match to the 3'

Figure 6.2 Strategy for the nucleotide sequencing of the gene encoding the 59KDa protein.

The gene encoding the 59KDa protein was sequenced by a combination of overlapping strategies. Initially, the fragment of DNA deduced to contain the gene was sonicated and fragments of 200-500bp in length were isolated from an agarose gel. These fragments were treated with the Klenow fragment of DNA polymerase I to repair the sheared ends. The fragments were then subcloned in to M13mp18 and subjected to dideoxynucleotide sequencing (Sanger *et al.*, 1977). The sequences were then entered into a computer programme to identify overlaps and assembled into longer sequences. Oligonucleotide sequencing primers were then synthesized to sequence the regions of the gaps. These sequences were then assembled using computer programmes.



**Figure 6.3 Nucleotide sequence of the gene encoding the 59KDa protein in *Bacillus sphaericus* 1593M.**

The nucleotide sequence of the gene encoding the 59KDa protein is shown in the figure. Numbering starts at the second "A" in the *Hind*III restriction enzyme site at the 5' of the gene. The DNA encoding this gene was initially cloned by Souza (1987) from the *B.sphaericus* strain 1593M (Chapter 1, Section 1.4.2.1).

The strategy used to sequence the gene is shown in Figure 6.2. In the sequence, non-coding regions are single spaced. Regions containing the open reading frame of the 59KDa protein are divided into codons with the predicted amino acid shown below. The presumed -35 and -10 promoter sequences, and the ribosome binding site (RBS) are underlined. The ATG start site of the gene encoding the 41KDa protein downstream of the sequence for the 59KDa protein is indicated. The 3 nucleotide change at nt 1890, 1891 and 1892 (TAT to ATA), seen in the sequence determined by Arapinis *et al.* (1988) for the same isolate is also underlined. The putative terminators of the gene encoding the 59KDa protein are indicated in the coding sequence for the 41KDa protein. Sequences encoding putative membrane binding domains are italicized.

Sequence 2093 onwards was obtained from Hindley and Berry (1987).

Nucleotide sequence of the gene encoding the 59KDa protein from *Bacillus sphaericus* 1593M.

1 AGCTTGTCAA CATGTGAAGA TTAAAGGTAA CTTTCAGTTT CTTTCTGTGT  
 51 AACAAATACAC GAAGTAATAT ATGTATTTAT ATAGAAATTA ATCAAAAAAA  
 101 GACCTAGTGA ACTGCACCCT GTC AAGTAGA TAGTGGAAAT AATAAAAATG  
 151 TATTAATCGG CTTGTGCTCT TAATTCTAAG AAGGTGATGA AAAACTTGCA  
 201 CTCTAGGGCG TACCAAGCAA TCAAAGGAGA AACGTTGTAT CTCCGGATTA  
 251 ATTCGAACTA CAACGTCTAT TG TAGATTAA ACCGTAAGAG ATTATATAAT  
 301 TATTATAACA ATA CTTTAAT TAATTCAATT GAAATTAAC AAACAAC TTT  
 351 TTCGGTATAT ACTATCTAAC ATATCGGTTT CAGTCCCATC GTTCTAAAGG  
 -35  
 401 TACCTTCTTT TTGGTTAACG TTATTTAATG AACTTTT TAG GTTTTAAATA  
 -10 RBS  
 451 ATATAATGAG AAGTATTTTT TATCAATGAT AAGGAGATGA AGAAAGC  
 498 ATG TGC GAT TCA AAA GAC AAT TCT GGC GTT TCA GAA AAA TGC  
 Met Cys Asp Ser Lys Asp Asn Ser Gly Val Ser Glu Lys Cys  
 540 GGA AAG AAA TTT ACT AAT TAC CCG CTA AAT ACT ACT CCT ACA  
 Gly Lys Lys Phe Thr Asn Tyr Pro Leu Asn Thr Thr Pro Thr  
 582 AGC CTA AAT TAT AAC CTT CCA GAA ATA TCA AAA AAA TTT TAT  
 Ser Leu Asn Tyr Asn Leu Pro Glu Ile Ser Lys Lys Phe Tyr  
 624 AAC CTT AAG AAT AAA TAT TCA CGG AAT GGT TAT GGT TTA TCA  
 Asn Leu Lys Asn Lys Tyr Ser Arg Asn Gly Tyr Gly Leu Ser  
 666 AAA ACC GAA TTT CCT TCA AGT ATC GAA AAT TGC CCA TCT AAC  
 Lys Thr Glu Phe Pro Ser Ser Ile Glu Asn Cys Pro Ser Asn  
 708 GAA TAT TCA ATA ATG TAT GAT AAT AAA GAT CCT CGA TTC TTG  
 Glu Tyr Ser Ile Met Tyr Asp Asn Lys Asp Pro Arg Phe Leu  
 750 ATT CGG TTT TTA TTA GAT GAT GGT AGA TAT ATT ATT GCA GAT  
 Ile Arg Phe Leu Leu Asp Asp Gly Arg Tyr Ile Ile Ala Asp  
 792 AGA GAC GAT GGA GAA GTT TTT GAT GAA GCA CCT ACT TAT TTG  
 Arg Asp Asp Gly Glu Val Phe Asp Glu Ala Pro Thr Tyr Leu  
 834 GAT AAT AAC AAT CAC CCT ATC ATA AGT AGA CAT TAT ACC GGA  
 Asp Asn Asn Asn His Pro Ile Ile Ser Arg His Tyr Thr Gly  
 876 GAA GAG AGA CAA AAG TTT GAG CAG GTA GGT AGT GGA GAT TAT  
 Glu Glu Arg Gln Lys Phe Glu Gln Val Gly Ser Gly Asp Tyr  
 918 ATT ACG GGA GAG CAA TTT TTT CAA TTC TAT ACA CAA AAC AAA  
 Ile Thr Gly Glu Gln Phe Phe Gln Phe Tyr Thr Gln Asn Lys  
 960 ACA CGT GTA TTG TCA AAT TGT AGG GCG CTT GAC AGT AGG ACA  
 Thr Arg Val Leu Ser Asn Cys Arg Ala Leu Asp Ser Arg Thr  
 1002 ATA TTA CTA TCT ACT GCA AAA ATC TTC CCA ATT TAC CCT CCA  
 Ile Leu Leu Ser Thr Ala Lys Ile Phe Pro Ile Tyr Pro Pro  
 1044 GCT TCT GAA ACT CAA CTA ACA GCT TTC GTT AAT AGT TCA TTT  
 Ala Ser Glu Thr Gln Leu Thr Ala Phe Val Asn Ser Ser Phe

1086 TAT GCT GCG GCA ATT CCT CAA TTA CCC CAA ACA TCC TTA CTT  
 Tyr Ala Ala Ala Ile Pro Gln Leu Pro Gln Thr Ser Leu Leu  
 1128 GAG AAT ATT CCT GAG CCT ACT AGT CTC GAT GAT TCT GGA GTA  
 Glu Asn Ile Pro Glu Pro Thr Ser Leu Asp Asp Ser Gly Val  
 1170 TTA CCA AAA GAT GCA GTA AGA GCA GTT AAA GGA AGT GCG CTA  
 Leu Pro Lys Asp Ala Val Arg Ala Val Lys Gly Ser Ala Leu  
 1212 TTA CCT TGT ATA ATA GTA CAT GAT CCT AAT TTA AAC AAT TCC  
 Leu Pro Cys Ile Ile Val His Asp Pro Asn Leu Asn Asn Ser  
 1254 GAT AAA ATG AAA TTT AAT ACC TAC TAT CTT TTA GAA TAT AAA  
 Asp Lys Met Lys Phe Asn Thr Tyr Tyr Leu Leu Glu Tyr Lys  
 1296 GAA TAC TGG CAT CAA TTA TGG TCA CAA ATT ATA CCT GCT CAT  
 Glu Tyr Trp His Gln Leu Trp Ser Gln Ile Ile Pro Ala His  
 1338 CAA ACT GTA AAA ATA CAG GAA CGA ACA GGA ATA TCT GAA GTT  
 Gln Thr Val Lys Ile Gln Glu Arg Thr Gly Ile Ser Glu Val  
 1380 GTA CAA AAT AGC ATG ATT GAA GAT TTA AAT ATG TAT ATT GGA  
 Val Gln Asn Ser Met Ile Glu Asp Leu Asn Met Tyr Ile Gly  
 1422 GCA GAT TTT GGC ATG CTT TTT TAT TTT AGA TCT AGT GGA TTT  
 Ala Asp Phe Gly Met Leu Phe Tyr Phe Arg Ser Ser Gly Phe  
 1464 AAG GAA CAA ATA ACA AGG GGG CTA AAT AGG CCT TTA TCC CAA  
 Lys Glu Gln Ile Thr Arg Gly Leu Asn Arg Pro Leu Ser Gln  
 1506 ACG ACC ACT CAG TTA GGA GAA AGA GTA GAA GAA ATG GAG TAT  
 Thr Thr Thr Gln Leu Gly Glu Arg Val Glu Glu Met Glu Tyr  
 1548 TAT AAT TCT AAT GAT TTG GAT GTT AGA TAT GTG AAA TAC GCA  
 Tyr Asn Ser Asn Asp Leu Asp Val Arg Tyr Val Lys Tyr Ala  
 1590 TTG GCT AGA GAA TTC ACA CTA AAA CGC GTT AAT GGT GAA ATT  
 Leu Ala Arg Glu Phe Thr Leu Lys Arg Val Asn Gly Glu Ile  
 1632 GTA AAA AAT TGG GTT GCT GTA GAT TAT CGA TTG GCA GGT ATA  
 Val Lys Asn Trp Val Ala Val Asp Tyr Arg Leu Ala Gly Ile  
 1674 CAA TCG TAT CCT AAT GCA CCT ATA ACT AAT CCA CTT ACG CTA  
 Gln Ser Tyr Pro Asn Ala Pro Ile Thr Asn Pro Leu Thr Leu  
 1716 ACA AAA CAT ACA ATT ATT CGA TGT GAA AAT AGT TAC GAT GGA  
 Thr Lys His Thr Ile Ile Arg Cys Glu Asn Ser Tyr Asp Gly  
 1758 CAC ATA TTT AAA ACA CCT TTA ATC TTT AAA AAT GGT GAA GTT  
 His Ile Phe Lys Thr Pro Leu Ile Phe Lys Asn Gly Glu Val  
 1800 ATT GTA AAA ACG AAT GAA GAA TTA ATA CCT AAA ATT AAC CAG  
 Ile Val Lys Thr Asn Glu Glu Leu Ile Pro Lys Ile Asn Gln

1842 TGA

\*

1845 TACTTTAACT TCAAATATTC ATTACCATGT TATTTAAAAT AGTAGTATG  
 1895 ATGAAATAAA TAGTATATAT TAAGACAACA ACTTAATTTT GACACATAA  
 1945 GAATAATTTT TAAATGTATA AATAGTATTT AGAGTGTTAT TGCAATATA  
 1995 TTTTTTGAAA GGGAGCTAAA AGAC

2019 ATG AGA AAT TTG GAT TTT ATT GAT TCT TTT ATA  
 Met Arg Asn Leu Asp Phe Ile Asp Ser Phe Ile

2052 CCC ACA GAA GGA AAG TAC ATT CGC GTT ATG GAT  
 Pro Thr Glu Gly Lys Tyr Ile Arg Val Met Asp

2085 TTT TAT AAT AGC GAG TCA GAA ATC TGT AGC AGA  
 Phe Tyr Asn Ser Glu Ser Glu Ile Cys Ser Arg  
 --- ---

2138 GAA AAT AAT CAA TAT TTT ATT TTT TTT CCT  
 Glu Asn Asn Gln Tyr Phe Ile Phe Phe Pro  
 -- --- - -- --- --

end of the *B.subtilis* 16S rRNA (Baumann et al., 1988). The sequence of the *B.sphaericus* 16S rRNA is not yet known. At 25nt upstream of the ribosome binding sequence was a putative promoter sequence. The -10 sequence read 5'TATAAT3', which is the canonical -10 sequence for the vegetative  $\sigma_{55}$  promoter of *B.subtilis*, and 18nt upstream of the -10 sequence was a putative -35 sequence. This -35 sequence, reading 5'ATGAAC3', resembles the canonical sequence, 5'TTGACA3' for the  $\sigma_{55}$  vegetative promoter of *B.subtilis* in 3 out of the 6 positions. The strong *rrnO* P2 promoters of *B.subtilis* contain the promoter sequences -35, 5'TTGACC3' and -10, 5'TACTAT3' recognized by the  $\sigma_{55}$  subunit of RNA polymerase (Ogasawara et al., 1983). This is a 4 out of 6nt match to the -35 sequence observed for the 59KDa gene. Baumann et al. (1988), in their analysis of this region failed to observe a promoter consensus, and stated merely that the sequence is AT-rich, as would be normal in the case of *B.sphaericus* (AT content 65% mol). Nevertheless, this sequence does appear to be a possible transcription start site because of the 100% identity of the -10 sequence to the canonical sequence and strong sequence similarity to other *B.subtilis* promoters. However, the presence of other upstream promoters cannot be ruled out. Indeed, the toxin gene for *B.thuringiensis* subspecies *kurstaki* has been shown to have two promoter sequences which are transcribed at different stages of growth (Schnepf et al., 1987).

The location of the termination codon for the gene encoding the 59KDa protein and the predicted amino acid sequence are shown in Figure 6.3. The predicted amino acid sequence of the "59KDa" protein adds up to an estimated size of 51.4KDa, at some variance from the observed size of 59KDa from the *E.coli* minicell analysis of the plasmid pAS233HA. In order to avoid confusion in this thesis, this protein will continue to be referred to as the 59KDa protein.

The DNA and the predicted amino acid sequences for the 59KDa protein were compared to those in the GenBank and NBRF databases. No significant matches were obtained. However, as first noted by Baumann et al. (1988), when the predicted amino acid sequence of the 59KDa protein was compared in a dotplot against the predicted amino acid sequence of the 41KDa toxin

protein of *B.sphaericus* sequenced from the strain 1593, significant regions of sequence similarity were identified (Figure 6.4A). Baumann et al. (1988) have defined the regions of identity in great detail and have observed that there are 4 regions of maximum amino acid sequence conservation between these proteins, two of which correspond to hydrophobic regions in the protein and two which correspond to regions of hydrophilic amino acid sequences (Figure 6.4B). In addition, the last 163 amino acids of the two proteins could be aligned without any gaps. Figure 6.4A shows the dotplot output with a window of 30 and a stringency of 15 for comparison of the two proteins. No similar DNA sequence similarity was seen in a DNA dotplot.

The codon usage table for the sequence of the gene encoding the 59KDa protein shows a strong preference for A or T at the wobble position. The codon bias reflects the high AT composition of the *B.sphaericus* genome, with the exception of the codons for the amino acid cysteine: TGT 50% (predicted 80.6%), TGC 50% (predicted 19.4%). Other codons show more or less comparable values to the predicted usage values.

The sequence of the gene encoding the 59KDa protein from the same isolate *B.sphaericus* 1593M was also published before sequencing in this laboratory was completed (Arapinis et al., 1988) and that sequence was reported to correspond entirely to that of the strain 2362 determined by Baumann et al. (1988). The three nucleotide change at position 1890-1892 from ATA to TAT was observed by me, but not by Arapinis et al. (1988). This TAT substitution for ATA at these positions was also found by Hindley and Berry (1987) in their nucleotide sequence of the region 5' to the sequence of the gene encoding the 41KDa protein from the *B.sphaericus* strain 1593.

The sequence of the gene encoding the 59KDa protein obtained above was subsequently used to design oligonucleotides in order to create site-specific mutations of the gene. These mutations incorporated restriction enzyme sites at the 5' and 3' ends of the gene encoding the 59KDa protein that could be used to subclone the gene under the control of strong promoters, in order to achieve high-level expression.

Figure 6.4A Comparison between the predicted amino acid sequences of the 59 and 41KDa proteins in a dotplot.

The predicted amino acid sequences of the 59KDa protein (this work) and the 41KDa protein (Hindley and Berry, 1987), were entered into a COMPARE programme with a window of 30 amino acids and a stringency of 15 on the University of Wisconsin Genetics Computer Group (UWGCG) sequence handling programmes run on the University of Leicester VAX/VMScluster computer. The output was plotted using the DOTPLOT programme.

Figure 6.4B Hydropathy index of the 41 and 59KDa proteins.

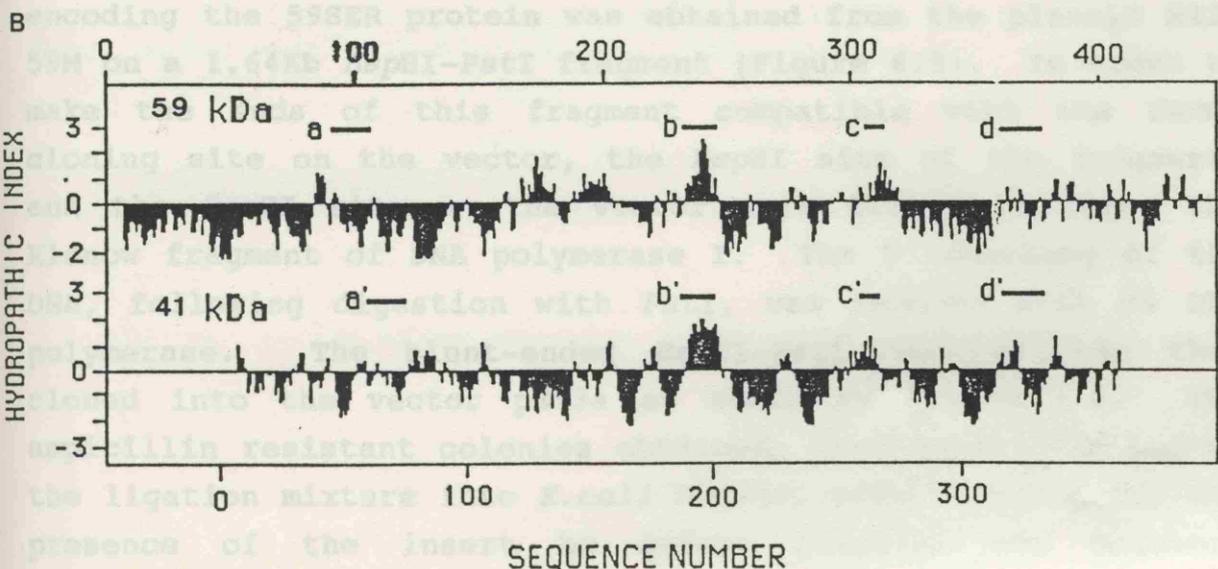
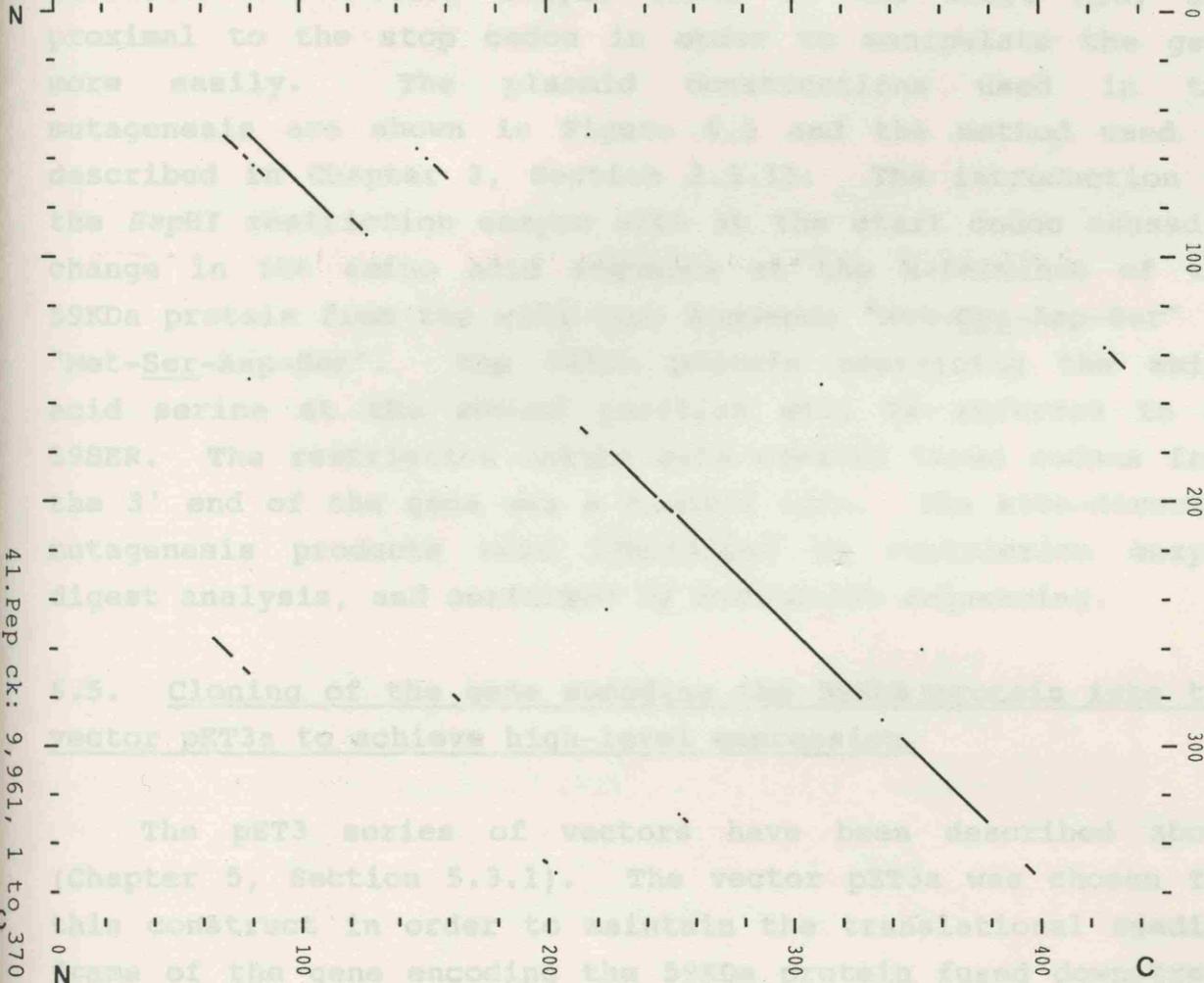
The figure shows the hydropathy index and a comparison of the hydrophobic and hydrophilic regions of the predicted amino acid 41 and 59KDa proteins. The plots were aligned by the identical sequences in b and b'.

Figure reproduced from Baumann *et al.* (1988).

A DOTPLOT of: 41.pnt Density: 511.36

COMPARE Window: 30 Stringency: 15.0 Points: 237

59.Pep ck: 4,957, 1 to 448



#### 6.4. Mutagenesis and overexpression of the gene encoding the 59KDa protein.

The DNA sequence of the gene encoding the 59KDa protein determined above was used to design oligonucleotides to introduce restriction enzyme sites at the start ATG, and proximal to the stop codon in order to manipulate the gene more easily. The plasmid constructions used in the mutagenesis are shown in Figure 6.5 and the method used is described in Chapter 2, Section 2.3.15. The introduction of the *Bsp*HI restriction enzyme site at the start codon caused a change in the amino acid sequence at the N-terminus of the 59KDa protein from the wild-type sequence "Met-Cys-Asp-Ser" to "Met-Ser-Asp-Ser". The 59KDa protein containing the amino acid serine at the second position will be referred to as 59SER. The restriction enzyme site created three codons from the 3' end of the gene was a *Hind*III site. The site-directed mutagenesis products were identified by restriction enzyme digest analysis, and confirmed by nucleotide sequencing.

#### 6.5. Cloning of the gene encoding the 59KDa protein into the vector pET3a to achieve high-level expression.

The pET3 series of vectors have been described above (Chapter 5, Section 5.3.1). The vector pET3a was chosen for this construct in order to maintain the translational reading frame of the gene encoding the 59KDa protein fused downstream of the *s10* gene of the phage T7. The mutagenized gene encoding the 59SER protein was obtained from the plasmid M13-59M on a 1.64Kb *Bsp*HI-*Pst*I fragment (Figure 6.5). In order to make the ends of this fragment compatible with the *Bam*HI cloning site on the vector, the *Bsp*HI site of the fragment, and the *Bam*HI site on the vector were filled in using the Klenow fragment of DNA polymerase I. The 5' overhang of the DNA, following digestion with *Pst*I, was removed with T4 DNA polymerase. The blunt-ended *Bsp*HI-*Pst*I fragment was then cloned into the vector pET3a as shown in Figure 6.6. The ampicillin resistant colonies obtained, upon transformation of the ligation mixture into *E.coli* MC4100, were screened for the presence of the insert by colony transfer and Southern hybridization analysis (Chapter 2, Section 2.3.12.2 and 2.13),

Figure 6.5 Construction of the plasmid M13-59 and site-directed mutagenesis for the creation of restriction enzyme sites.

The 1.73Kb fragment containing the gene encoding the 59KDa protein was isolated by digestion of the plasmid pAS233HA with the restriction enzymes *KpnI* and *EcoRV*. The fragment was ligated into the *KpnI-HincII* restriction enzyme sites in the multiple cloning site of M13mp18 to obtain the plasmid M13-59.

Single-stranded DNA was purified from the supernatants of *E.coli* TG1 cells harbouring M13-59 and used in the mutagenesis protocol as described in Chapter 2, Section 2.3.15. The plasmid containing the *BspHI* and *HindIII* restriction enzyme sites was called M13-59M.

Oligonucleotides used for the creation of the restriction enzyme sites at the 5' and 3' of the gene encoding the 59KDa protein.

Shown below are the oligonucleotides used in the mutagenesis reaction to create a *BspHI* restriction enzyme site at the 5' end and the *HindIII* site at the 3' end of the gene encoding the 59KDa protein. Also shown below are the amino acid changes caused by the nucleotide substitutions.

5' end:

```
5' GAAAGC ATG TGC GAT TCA AAA 3'
      T      A
      -----
      Met Ser Asp Ser Lys
```

The nucleotide change from TGC to AGC changes the amino acid sequence at position 2 from a cysteine to a serine.

5'T CATGA3' is the recognition and cleavage sequence for the enzyme *BspHI*.

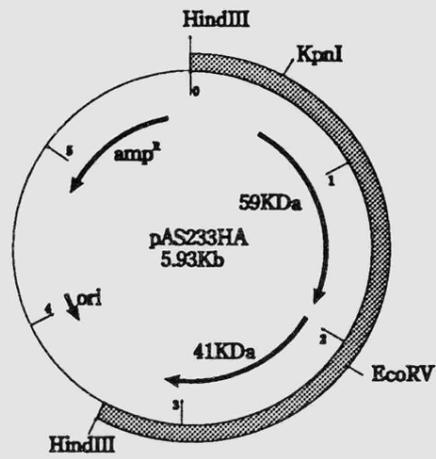
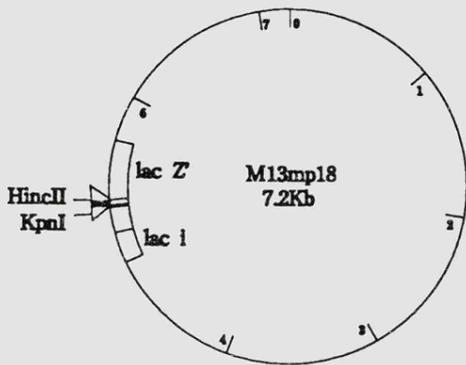
3' end:

```
5' ATA CCT AAA ATT AAC CAG TGA 3'
      G C
      -----
      Ile Pro Lys Leu Asn Gln *
```

The nucleotide sequence change from AAA to AAG is silent, whereas the change from ATT to CTT changes the amino acid sequence from an isoleucine to a leucine.

"\*" indicates the translation stop codon.

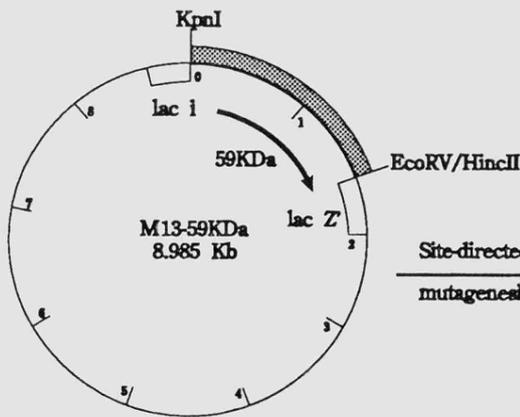
5'A AGCTT3' is the recognition and cleavage sequence for the restriction enzyme *HindIII*.



Digest with  
KpnI/HincII

Digest with KpnI/EcoRV  
Isolate 1.73Kb fragment

Ligation reaction



Site-directed  
mutagenesis

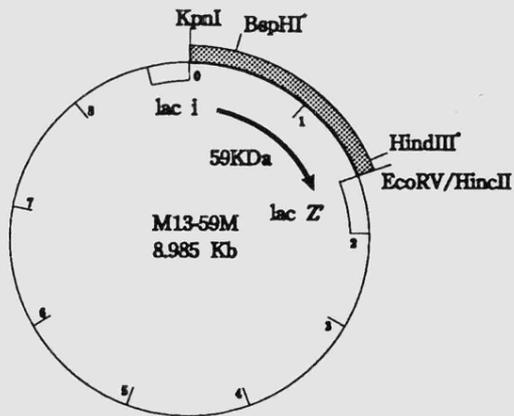


Figure 6.6 Construction of the plasmid pET3a-59SER.

M13-59M (Figure 6.5) was derived by site-directed mutagenesis of the sequences at the 5' and 3' of the gene encoding the 59KDa protein to the recognition sites for the restriction enzymes *BspHI* and *HindIII*, respectively.

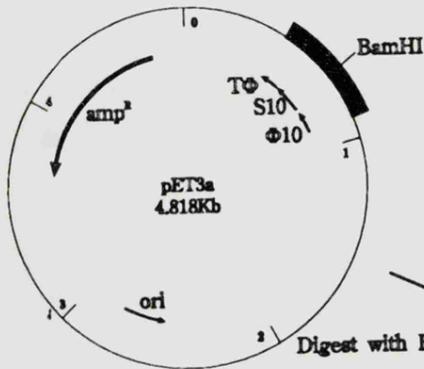
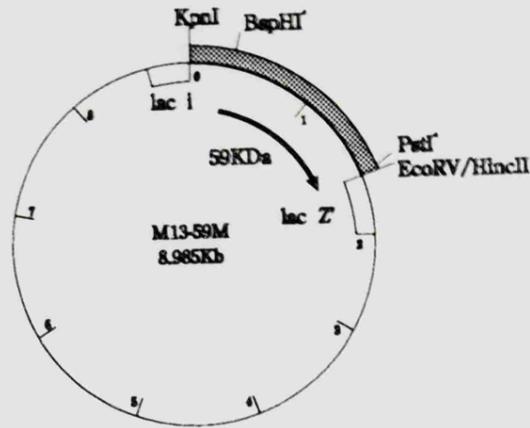
M13-59M was digested with the restriction enzyme *BspHI*. The overhang generated was filled in with the Klenow fragment of DNA polymerase I. The plasmid was then digested with the enzyme *PstI*. This digestion generates a 1.64Kb fragment containing the gene encoding the 59KDa protein. This protein contains the amino acid serine at the second position instead of the wild-type cysteine. The 5' overhang generated by the enzyme *PstI* was removed with T4 DNA polymerase. The 1.64Kb fragment was now blunt-ended on both sides.

The plasmid pET3a was digested with the restriction enzyme *BspHI*. The *BamHI* overhang was filled in with the Klenow fragment of DNA polymerase I. The 1.64Kb fragment from M13-59M was ligated into the filled-in *BamHI* site pET3a. This would lead to a translational fusion between the gene encoding the S10 protein of the phage T7, which is under T7 polymerase control. The plasmid pET3a-59SER was identified by colony transfer followed by Southern hybridization analysis using the 1.64Kb fragment as the DNA probe.

The inset shows the nucleotide sequence over the region of the fusion of the gene encoding the 59KDa protein to the *s10* gene. The fusion site is overlined.

TΦ = transcription stop signal

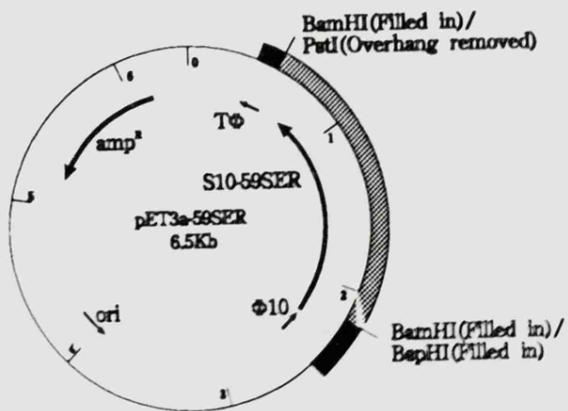
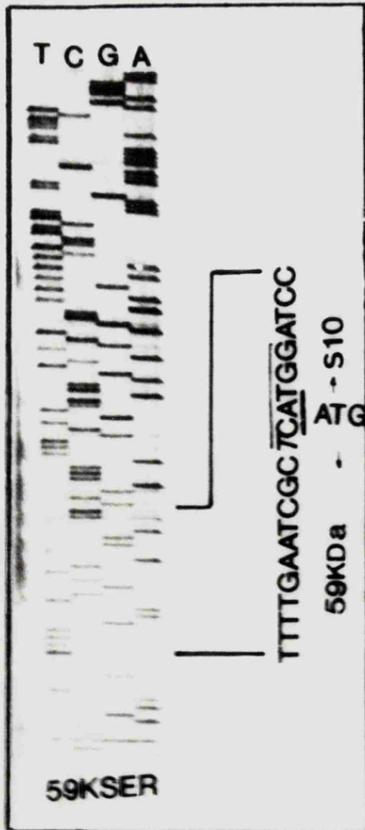
Φ10 = promoter recognized by T7 RNA polymerase



Digest with BamHI  
Fill in overhang with Klenow

Digest with BspHI  
Fill in overhang with Klenow  
Digest with PstI  
Remove overhang with T4 DNA polymerase  
Isolate 1.643Kb fragment

Ligation reaction



using the *BspHI-PstI* fragment described above as the DNA probe. Two colonies out of approximately 40 hybridized to the probe. Plasmid DNA was then prepared from these colonies by the minipreparation method (Chapter 2, Section 2.3.2). The plasmids were analyzed for the presence of the insert in the correct orientation by restriction enzyme digestion. One plasmid was obtained with the correct restriction enzyme pattern. This plasmid, called pET3a-59SER, was then sequenced to determine the reading frame at the fusion site of the gene encoding the 59KDa protein to the *s10* gene. The S10-59SER protein in this case contains 13 amino acids of the S10 protein at the N-terminus.

*E.coli* MC4100 does not contain the gene encoding the T7 RNA polymerase protein required for transcription from the  $\Phi$ 10 promoter upstream of the S10-59SER translational gene fusion. Therefore, the plasmid, pET3a-59SER was transformed into the *E.coli* strain BL21-DE3, which contains an isopropylthio- $\beta$ -D-galactopyranoside (IPTG) inducible T7 RNA polymerase gene, in order to determine if the S10-59SER protein was overexpressed by the construct.

The vector, pET3a, was also transformed into *E.coli* BL21-DE3 to serve as a negative control. Both the strains were grown overnight at 37°C in Luria broth and then diluted to an  $A_{600}=0.05$  in Luria broth and grown to an  $A_{600}=0.4$  at 37°C. The cells were then treated with 0.4mM IPTG to induce the production of T7 RNA polymerase from the *lacUV5* promoter on the chromosome of the strain BL21-DE3. Following induction, the cultures were grown for 5 hours to allow expression from the  $\Phi$ 10 promoter to occur. The cells were then harvested by centrifugation for 10min at room temperature in an MSE microcentrifuge at 13Krpm, heated to 100°C for 3min in SDS loading buffer, and the proteins separated by SDS polyacrylamide gel electrophoresis. Figure 6.7 shows the gel stained with Coomassie blue. Other strains were included in this experiment to determine which strain supported maximum production of the S10-59SER fusion protein. The strains, BL21-DE3 (pLysS) and BL21-DE3 (pLysE) contain the gene encoding the T7 lysozyme protein, which normally cleaves the T7 RNA polymerase protein. These strains are used to minimize premature expression from the  $\Phi$ 10 promoter by T7 RNA

Figure 6.7A Induction of the expression of proteins encoded by the plasmids pET3a and pET3a-59SER.

The plasmids pET3a-59SER and the control pET3a were transformed into the *E.coli* strains BL21 and HMS174, which do not contain the gene encoding T7 RNA polymerase and the strains BL21-DE3, BL21-DE3 (pLysS) and BL21-DE3 (pLysE), which contain the IPTG-inducible T7 RNA polymerase gene. The cells were transformed by the rapid CaCl<sub>2</sub> method (Chapter 2, Section 2.2.3.1). The plasmids pLysS and pLysE contain the gene encoding the T7 lysozyme protein, which cleaves T7 RNA polymerase.

The strains containing the plasmids pET3a and pET3a-59SER were inoculated into Luria broth to an A<sub>600</sub>=0.05. The cells were grown to an A<sub>600</sub>=0.4 at 37°C. The cells were then induced with 0.4mM IPTG. Induction was continued for 5 hours. The cells were harvested and 0.1 A<sub>600</sub> Units of cells were heated to 100°C for 3min in SDS loading buffer, and the proteins were separated on an 11% separating, 5% stacking SDS polyacrylamide gel. The proteins in the gel were stained with Coomassie blue.

Lanes:

- A, induced total cells of *E.coli* HMS174 (pET3a-59SER)
- B, uninduced total cells of *E.coli* HMS174 (pET3a-59SER)
- C, induced total cells of HMS174 (pET3a)
- D, uninduced total cells of *E.coli* HMS174 (pET3a)
- E, induced total cells of *E.coli* BL21-DE3 (pLysE; pET3a-59SER)
- F, uninduced total cells of *E.coli* BL21-DE3 (pLysE; pET3a-59SER)
- G, induced total cells of *E.coli* BL21-DE3 (pLysE; pET3a)
- H, uninduced total cells of *E.coli* BL21-DE3 (pLysE; pET3a)
- I, induced total cells of *E.coli* BL21-DE3 (pLysS; pET3a-59SER)
- J, uninduced total cells of *E.coli* BL21-DE3 (pLysS; pET3a-59SER)
- K, induced total cells of *E.coli* BL21-DE3 (pLysS, pET3a)
- L, uninduced total cells of *E.coli* BL21-DE3 (pLysS; pET3a)
- M, induced total cells of *E.coli* BL21-DE3 (pET3a-59SER)
- N, uninduced total cells of *E.coli* BL21-DE3 (pET3a-59SER)
- O, induced total cells of *E.coli* BL21-DE3 (pET3a)
- P, uninduced total cells of *E.coli* BL21-DE3 (pET3a)
- Q, induced total cells of *E.coli* BL21 (pET3a-59SER)
- R, uninduced total cells of *E.coli* BL21 (pET3a-59SER)
- S, induced total cells of *E.coli* BL21 (pET3a)
- T, uninduced total cells of *E.coli* BL21 (pET3a)

Protein standards (lane MA) and the position of the 59KDa protein are shown in KDa.

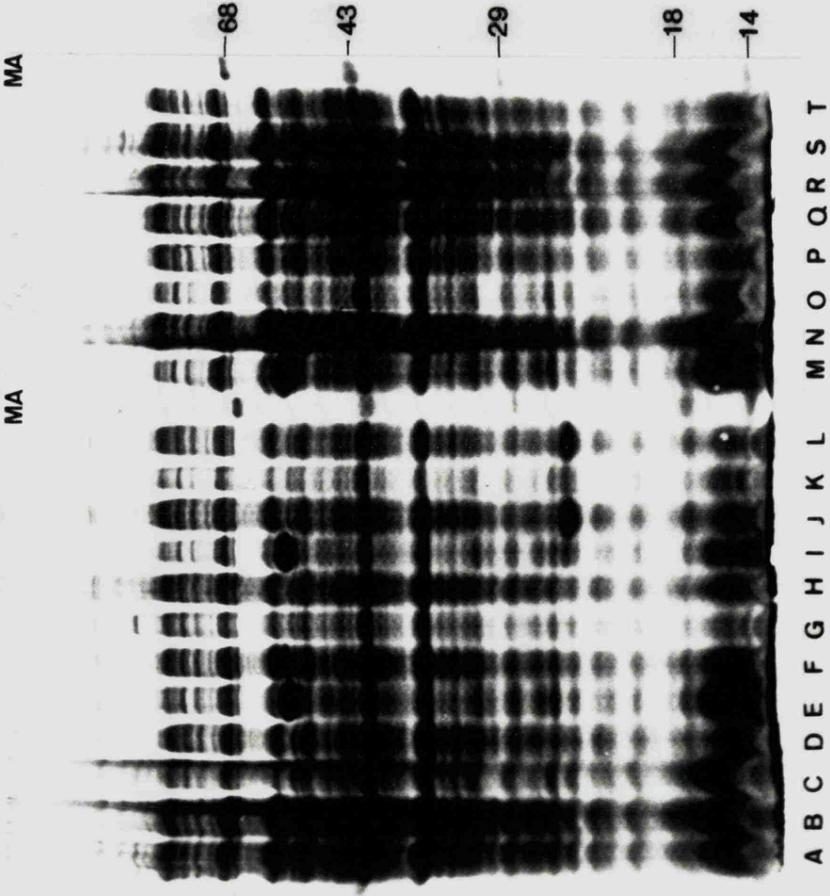
Figure 6.7B Growth curve of *E.coli* BL21-DE3 cells containing pET3a-59SER or pET3a induced by the addition of IPTG.

Cells of *E.coli* BL21-DE3 containing the negative control vector pET3a, and the plasmid containing the gene encoding the 59KDa protein, pET3a-59SER, were inoculated into Luria broth to an A<sub>600</sub>=0.05. The cells were grown at 37°C at 250rpm to an A<sub>600</sub>=0.4. The cells were induced by the addition of 0.4mM IPTG and grown for 5 hours. A<sub>600</sub> readings were taken at half-hour intervals. The arrow shows point of induction.

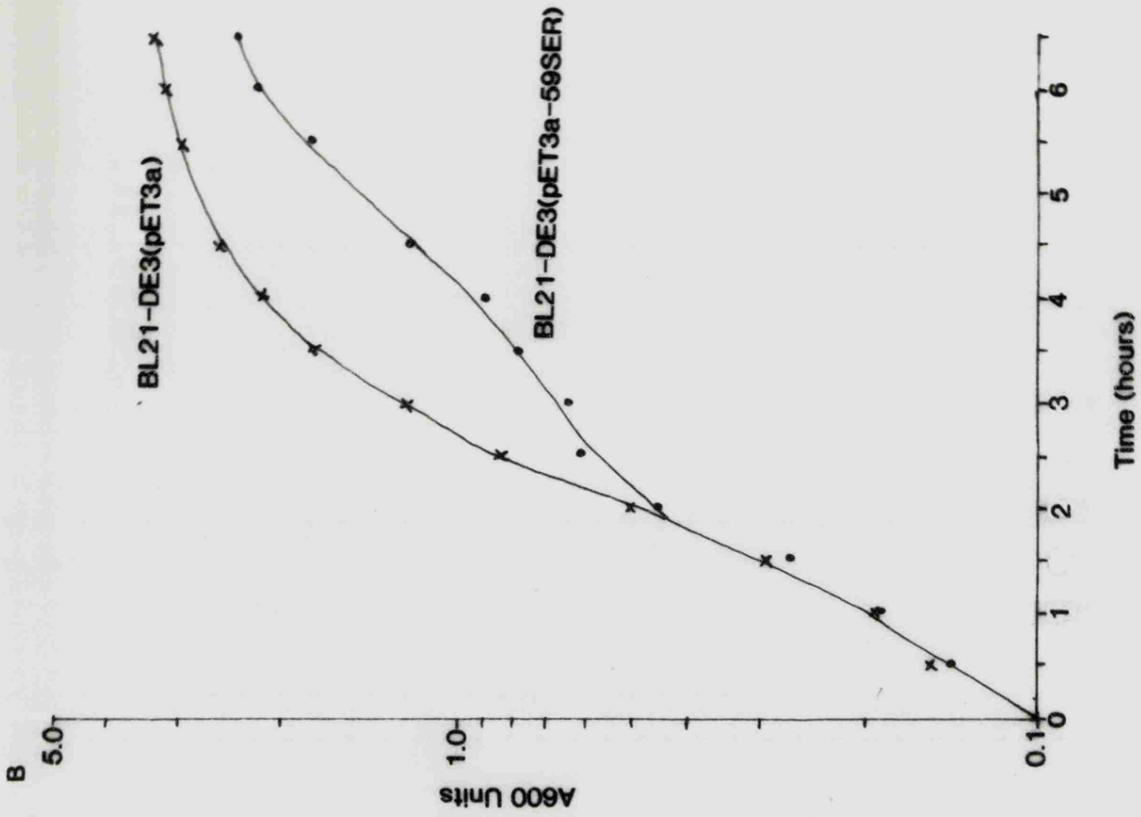
x = BL21-DE3

o = BL21-DE3 (pET3a-59SER)

A



59



polymerase, which is under the control of the leaky *lacUV5* promoter (Studier *et al.*, 1990). The other strain used as a control was *E.coli* BL21 without the lysogen harbouring the T7 RNA polymerase gene.

It can be seen from this gel (Figure 6.7, lanes E, I and M) that induction of the T7 RNA polymerase in the *E.coli* BL21-DE3 cell clearly leads to the expression of a protein with the expected mobility of the S10-59SER protein. In fact, the protein accumulates as inclusion bodies within the cell (data not shown). In complete contrast, *E.coli* BL21 lacking the Lambda lysogen containing the T7 RNA polymerase gene did not express the protein (Figure 6.7, Lanes Q and R). The strains containing the T7 lysozyme genes did not significantly affect the amount of protein accumulated after induction. No protein accumulation was apparent before the cells were induced with IPTG.

The growth curve of the *E.coli* BL21-DE3 strain containing the plasmid pET3a-59SER, encoding the S10-59SER fusion protein, clearly reveals growth inhibition when IPTG is added, compared to the strain containing the vector pET3a (Figure 6.7B). However, no evidence of lysis of the culture is seen, indicating that the high-level synthesis of the S10-59SER fusion protein is not particularly toxic to the cells.

#### 6.5.1. Secretion of the S10-59SER fusion protein from *E.coli* BL21-DE3 via the haemolysin secretion system.

Following the high-level expression of the gene encoding the S10-59SER fusion protein on the vector pET3a, we were interested to determine whether the secretion of the protein by the haemolysin system would be increased compared to when the protein was under *B.sphaericus* promoter control. For this construction, a derivative of pET3a (pET3a $\Delta$ H) was prepared by digestion of pET3a with *HindIII*, the ends were filled in and religated to destroy the site. The gene encoding the 59KDa protein was reinserted into this vector on a *SalI-EcoRV* fragment from pET3a-59SER. This plasmid was called pET3a-59, to distinguish it from pET3a-59SER.

The 23KDa C-terminal signal region from the vector

pLG609-3 was obtained with the correct translational reading frame and fused to the S10-59SER fusion gene in pET3a-59 at the filled-in *Hind*III site. This site is three codons from the C-terminus of the gene encoding the 59KDa protein. The plasmid construction is shown in Figure 6.8A. This construct, pET3a-59-HlyA, was transformed into the *E.coli* strain BL21-DE3 together with the plasmid pLG575 containing the essential haemolysin export proteins, HlyB and HlyD.

This strain, together with one lacking the export functions was grown overnight in Luria broth and then inoculated into Luria broth to an  $A_{600}=0.05$  at 37°C. The cells were grown to an  $A_{600}=0.4-0.5$ , and then induced by the addition of 0.4mM IPTG. After growth for a further 90min, the cell and supernatant samples were obtained, and the proteins in the supernatant were concentrated by TCA precipitation (Chapter 2, Section 2.4.4). The proteins in the cell and supernatant samples were separated on 8.5% SDS polyacrylamide gels, and then stained with Coomassie blue to visualise total protein (data not shown), or transferred to nitrocellulose paper and subjected to Western immunoblot analysis using antibodies raised to the 23KDa haemolysin signal peptide.

Western immunoblot analysis (Figure 6.8B) revealed that the S10-59SER protein fused to the 23KDa signal region (designated 59HlyA) was exported from the cell (Figure 6.8B, lane H). However, there was also considerable degradation of the protein within the cell (Figure 6.8B, lanes C and D). Moreover, apparently truncated proteins at 63, 43 and 31KDa were detected by the 23KDa antibody in the supernatant samples from the strain secreting the fusion protein (Figure 6.8B, lane H). The size expected for the 59HlyA fusion protein was 82KDa, and a very faint band can be seen in the total cells at this position (Figure 6.8B, lanes C and D), but not in the supernatant fractions (Figure 6.8B, lane H). The amounts of 59HlyA protein in the medium compared with the amount of secreted CATIII'HlyA fusion expressed from the weaker promoter indicated that the haemolysin export system is not an efficient pathway for export of the toxin fusion protein under these conditions. This may be because the 59HlyA fusion protein is slowly and inefficiently translocated and therefore rapidly saturates the translocation proteins HlyB and HlyD;

Figure 6.8A Construction of the plasmid pET3a-59HlyA, encoding the fusion of the 59KDa protein under the T7 promoter control to the 23KDa C-terminal signal region of HlyA.

The 0.8Kb *SmaI-HincII* fragment containing the 23KDa C-terminal signal region was isolated from the plasmid pLG609-3. The plasmid pET3a-59SER was digested with the restriction enzyme *HindIII* and the overhangs were filled in with the Klenow fragment of DNA polymerase I. The 0.8Kb fragment was inserted into the filled-in *HindIII* site in order to make a translational fusion of the 23KDa signal peptide to the S10-59SER fusion protein under the control of the  $\Phi$ 10 promoter. The orientation of the 0.8Kb fragment was identified by digestion with the enzyme *PstI* (see text, Section 6.5.2 for further details).

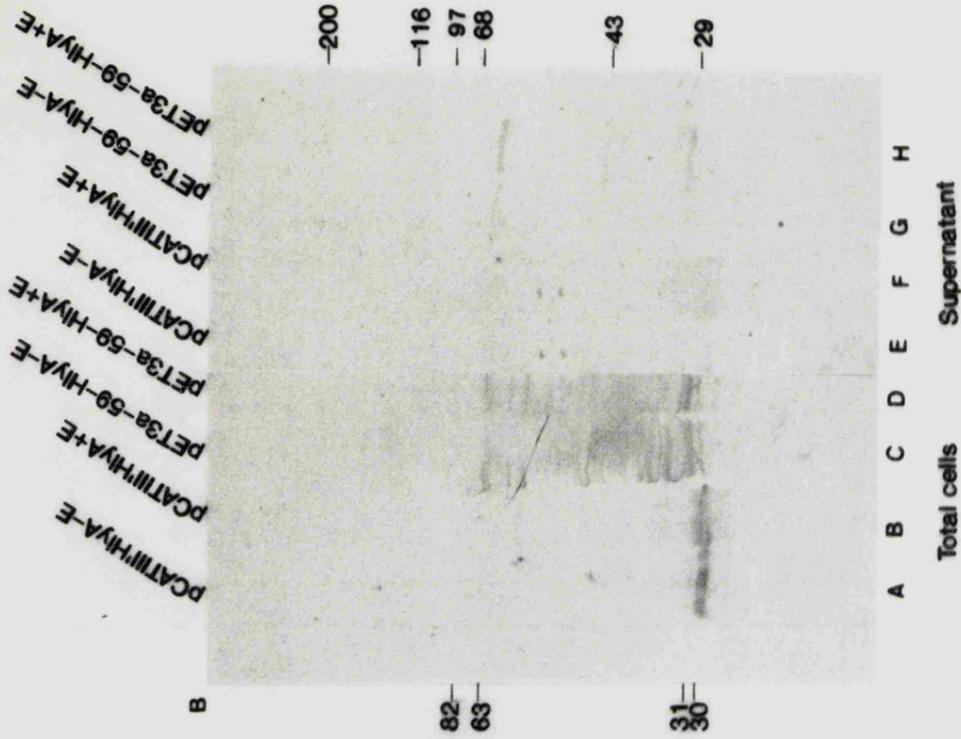
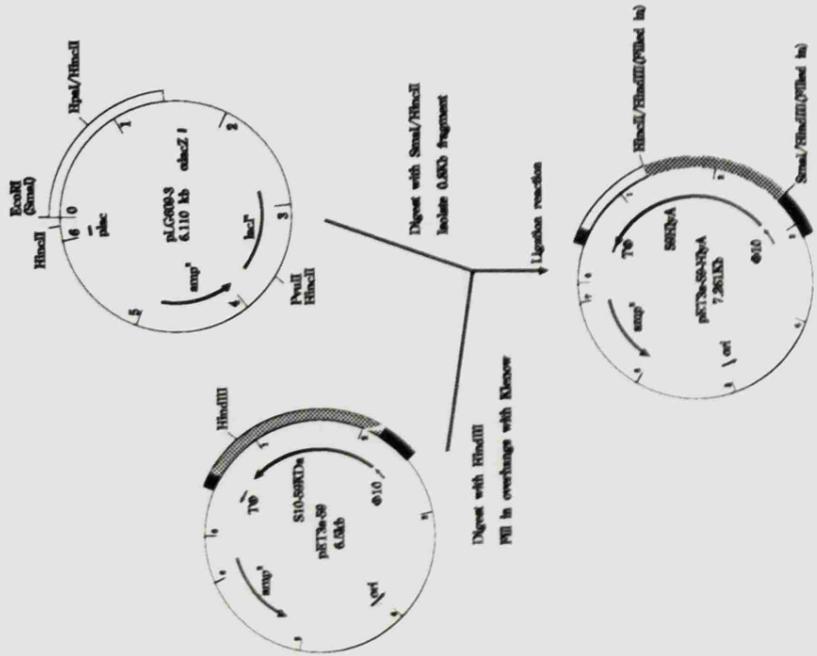
Figure 6.8B Export of the 59HlyA protein from *E.coli* BL21-DE3 cells containing the plasmid pET3a-59-HlyA.

The plasmids pET3a-59-HlyA encoding the 59HlyA fusion protein and CATIII'HlyA, encoding a translational fusion of a part of the chloramphenicol acetyltransferase gene to the 23KDa signal peptide of HlyA, were transformed into *E.coli* cells with and without the plasmid pLG575, encoding the export proteins HlyB and HlyD. The cells were grown in Luria broth at 37°C to an  $A_{600}=0.4$ . The *E.coli* BL21-DE3 cells were induced by the addition of 0.4mM IPTG. Both sets of cells were grown for a further 5 hours. Supernatant and cell proteins were prepared as described in Chapter 2, Section 2.4.4. 0.5  $A_{600}$  Units of cell proteins and 1.0  $A_{600}$  Units were heated to 100°C for 3min in SDS loading buffer, and separated on an 8.5% separating, 5% stacking SDS polyacrylamide gel. The proteins were transferred onto nitrocellulose paper for Western immunoblot analysis. Non-specific binding sites on the membrane were blocked by incubation overnight in 5% (w/v) Marvel milk in 1X PBS. The blot was developed by sequential incubation with a 1:100 dilution of antibodies raised to the 23KDa signal peptide, 1:1000 dilution of goat anti-rabbit antibodies and 1:1000 rabbit peroxidase anti-peroxidase antibodies (Chapter 2, Section 2.6.3).

Lanes:

- A, total cells of *E.coli* JM101 (pCATIII'HlyA), without export functions
- B, total cells of *E.coli* JM101 (pCATIII'HlyA; pLG575), with export functions
- C, total cells of *E.coli* BL21-DE3 (pET3a-59-HlyA), without export functions
- D, total cells of *E.coli* BL21-DE3 (pET3a-59-HlyA; pLG575), with export functions
- E, supernatants of *E.coli* JM101 (pCATIII'HlyA), without export functions
- F, supernatants of *E.coli* JM101 (pCATIII'HlyA; pLG575), with export functions
- G, supernatants of *E.coli* BL21-DE3 (pET3a-59-HlyA), without export functions
- H, supernatants of *E.coli* BL21-DE3 (pET3a-59-HlyA; pLG575), with export functions

The sizes of protein standards and major proteins are shown in KDa.



82—  
63—  
31—  
30—

because the protein is rapidly sequestered into insoluble inclusion bodies or perhaps due to intracellular instability of the fusion protein even in the absence of the HlyB and HlyD export proteins. This observation is comparable to that seen when the 41KDa protein was fused to the 23KDa signal region of haemolysin.

#### 6.6. Toxicity analysis of cells producing high levels of the S10-59SER fusion protein by *in vivo* bioassays with mosquito larvae.

Following the successful high level expression of the 59KDa protein as a fusion to 13 amino acids of the S10 protein from the plasmid pET3a-59SER, we were interested to determine whether this fusion protein still expressed toxicity to mosquito larvae *in vivo* in the presence of the 41KDa protein.

*E.coli* BL21-DE3 cells harbouring the plasmid pET3a-59SER expressing the S10-59SER fusion protein were grown and induced with 0.4mM IPTG as described above (Section 6.5). Cells containing the vector pET3a alone were used as a control. The cells were resuspended in 10mL of distilled water containing 10 larvae of the *Culex quinquefasciatus* mosquito for the bioassay. Since it had been established earlier that the 59KDa protein by itself was not toxic but required the presence of the 41KDa protein for larvicidal activity, the assays were carried out in the presence of various amounts of the 41 and S10-59SER proteins. The source of the 41KDa protein was *E.coli* MC4100 cells containing the plasmid pJLA504-41 induced at 42°C (Chapter 5, Section 5.4).

Table 6.1 presents the results obtained. The *E.coli* BL21-DE3 or MC4100 cells containing the vectors pET3a, pJLA504 and pBR322, or *E.coli* MC4100 lacking any plasmid did not cause death of the larvae in the 24 hours over which the assay was monitored. Furthermore, induced cells expressing the S10-59SER fusion protein (pET3a-59SER) or the 41KDa protein (pJLA504-41) separately did not lead to mortality. However, when cells expressing the S10-59SER fusion protein (pET3a-59SER) were mixed together in equal quantities with induced cells expressing the 41KDa protein (pJLA504-41), mortality of the larvae was observed within 12 hours of the start of the

Table 6.1 In vivo toxicity assays of E.coli strains containing plasmids encoding the 41 and 59KDa proteins.

The toxicity assays were carried out using second to third instar larvae of *Culex quinquefasciatus* collected from the wild. The larvae were washed in tap water, resuspended in tap water and starved for 6 hours. 10 larvae were then resuspended in 10mL of tap water in a sterile scintillation vial for the assay.

The *E.coli* BL21-DE3 cells containing the plasmid pET3a-59SER were grown in Luria broth at 37°C to an  $A_{600}=0.4$ . They were then induced by the addition of 0.4mM IPTG. The cells were grown for a further 3 hours before harvesting. The cells were resuspended in distilled water. The *E.coli* MC4100 cells containing the plasmid pJLA504-41 encoding the 41KDa protein under the control of the  $\lambda$  P<sub>L</sub> and P<sub>R</sub> promoters were also grown in Luria broth at 30°C to an  $A_{600}=0.4$ . The cells were induced by transfer to 42°C for 3 hours, harvested and resuspended in distilled water. *E.coli* MC4100 cells containing the plasmids pHAΔEI and pHAΔEV were grown for 3 hours after reaching an  $A_{600}=0.4$ .

Different concentrations of the cells were added to the vials containing the larvae ( $A_{600} = 1.4 = 10^9$  cells/mL; Miller, 1976). A total of 20 larvae were used at each concentration. Controls included larvae starved for the period of the assay and larvae to which an equivalent amount of distilled water as the experiments was added. No mortality was seen in the controls over the duration of the assay.

Table 6.1.

Toxicity of *E.coli* cells containing plasmids encoding the 59KDa and 41KDa proteins.

<u>Plasmids(protein)</u>	<u>No. of cells/mL</u>	<u>% survival</u>	
		<u>24 hours</u>	<u>36 hours</u>
pET3a <sup>a</sup> (none)	1.4 x 10 <sup>8</sup>	95%	95%
pJLA504 (none)	1.4 x 10 <sup>8</sup>	100%	100%
pET3a-59SER <sup>a</sup> (59SER)	0.355 x 10 <sup>7</sup>	100%	100%
pJLA504-41 (41)	1.4 x 10 <sup>8</sup>	95%	90%
pJLA504-41 (41)	0.71 x 10 <sup>7</sup>	100%	100%
pJLA504-41 (41)	0.355 x 10 <sup>7</sup>	100%	100%
pHAΔEI (41)	1.4 x 10 <sup>8</sup>	100%	100%
pHAΔEI (41)	0.71 x 10 <sup>7</sup>	100%	94.4%
pHAΔEI (41)	0.355 x 10 <sup>7</sup>	100%	100%
pHAΔEV (59)	1.4 x 10 <sup>8</sup>	100%	100%
pHAΔEV (59)	0.71 x 10 <sup>7</sup>	100%	100%
pHAΔEV (59)	0.355 x 10 <sup>7</sup>	100%	100%
pHAΔEI/pHAΔEV* (41/59)	2.8 x 10 <sup>8</sup>	76.2%	38%
pHAΔEI/pHAΔEV* (41/59)	1.4 x 10 <sup>8</sup>	85%	45%
pHAΔEI/pHAΔEV* (41/59)	0.71 x 10 <sup>7</sup>	100%	52.4%
pHAΔEI/pHAΔEV* (41/59)	0.355 x 10 <sup>7</sup>	45%	35%
pET3a-59SER <sup>a</sup> / pJLA504-41* (59SER/41)	1.4 x 10 <sup>8</sup>	11.1%	5.5%
pET3a-59SER <sup>a</sup> / pJLA504-41* (59SER/41)	0.71 x 10 <sup>8</sup>	10%	0%
pET3a-59SER <sup>a</sup> / pJLA504-41* (59SER/41)	0.355 x 10 <sup>7</sup>	5%	0%
pJLA504-41/pHAΔEV* (41/59)	1.4 x 10 <sup>8</sup>	81%	38.1%
pJLA504-41/pHAΔEV* (41/59)	0.71 x 10 <sup>8</sup>	57.8%	26.3%
pJLA504-41/pHAΔEV* (41/59)	0.355 x 10 <sup>8</sup>	55%	25%

a: plasmids present in *E.coli* strain BL21-DE3; the *E.coli* strain used for all other plasmids was MC4100.

\*: the number of cells shown from each strain were added.

bioassay. Importantly, these results clearly demonstrated that the plasmid pJLA504-41 did indeed express a functional 41KDa toxin, confirming the *in vitro* transcription-translation data. Even more importantly, the results demonstrated that the 59KDa toxin was still active, despite the absence of the N-terminal cysteine and the presence of 13 additional residues at the N-terminus.

It can also be seen by comparison of the larvicidal activities for a mixture of pJLA504-41/pHAA $\Delta$ EV and PHAAEI/pHAA $\Delta$ EV (Table 6.1), that the plasmids pHAAEI and pJLA504-41 produce the 41KDa protein at approximately the same level despite the presence of the  $\lambda$  promoter in the latter construct. The plasmid pHAAEI contains the gene encoding the 41KDa protein under the control of its *B.sphaericus* promoter. This observation confirms the low level expression of the 41KDa protein in cells containing the plasmid pJLA504-41, and suggests that expression of this protein is tightly regulated, perhaps at the level of translation. The 59KDa fusion construct under T7 promoter control expressed higher amounts of the 59KDa fusion, than when the 59KDa protein was expressed from the *B.sphaericus* promoter in *E.coli*. The former construct also conferred higher levels of toxicity on *E.coli* when used in bioassays.

A very interesting observation that arose from this assay was that, as indicated above, the replacement of cysteine in the second position of the protein with serine did not detectably affect the biological activity of the protein. This could mean either, that the cysteine in this position is not critical in the secondary structure of the protein, or that in any event, this portion of the molecule is removed by larval proteases during the activation of the protein. Other indications that the N-terminal region is not critical for function arises from the fact that the presence of the 13 amino acids of the *s10* gene at the N-terminus of the 59KDa protein did not apparently affect toxicity of the 59KDa protein in bioassays. I have recently carried out site directed mutagenesis in order to convert the serine at the position 15 in the S10-59SER construct to cysteine. This construct, S10-59CYS, has not yet been tested for larvicidal activity in bioassays, but is expressed at the same high level

as S10-59SER from the  $\Phi$ 10 promoter in pET3a.

#### 6.7. Raising antibodies to the S10-59SER protein.

The S10-59SER fusion protein was produced in large quantities upon induction of the plasmid pET3a-59SER in the *E.coli* strain BL21-DE3. In order to use this protein as antigen, the cellular proteins from an induced culture were separated on an 8.5% SDS polyacrylamide gel. Proteins in the gel were visualized by staining with Coomassie blue. The gel was soaked in 1M Tris, pH 7.5 and 1XPBS over two days to neutralize the acid, and the overexpressed protein was then excised from the gel with a scalpel and sonicated in Complete Freund's Adjuvant as described above (Chapter 4, Section 4.3, and Chapter 2, Section 2.6.1). Approximately 100 $\mu$ g of this protein was used to inject an adult New Zealand White rabbit subcutaneously, followed at 4 week intervals with 75 $\mu$ g booster doses in Incomplete Freund's Adjuvant. After 4 booster injections, an antibody reaction could be detected in Western immunoblot assays at a titre of 1:50. After 7 booster doses, the titre had risen to 1:500. Thus, the protein was not very antigenic compared with the 125KDa crystal protein, which induced the production of antibodies with a titre of 1:10000 on Western immunoblots after four booster doses (Chapter 4, Section 4.3). This result provides evidence for earlier speculation (Chapter 4, Section 4.5) that any 59KDa protein present in the *B.sphaericus* crystal preparations may not be sufficiently antigenic to elicit the production of antibodies.

#### 6.8. Presence of the 59KDa protein in purified crystals of *B.sphaericus*.

In order to determine whether the 59KDa protein was present in the preparations of *B.sphaericus* crystals from the toxic strains 1593, 2297, 2013-4 and 2362, the proteins present in each crystal preparation were separated on 8.5% SDS polyacrylamide gels and transferred electrophoretically onto nitrocellulose paper. The blots were probed with antibodies raised to the 59KDa protein and antibodies raised to the 125KDa crystal-associated protein (Chapter 2, Section 2.6.3). The results obtained are shown in Figure 6.9, where it is clear that the 59KDa protein can be identified using

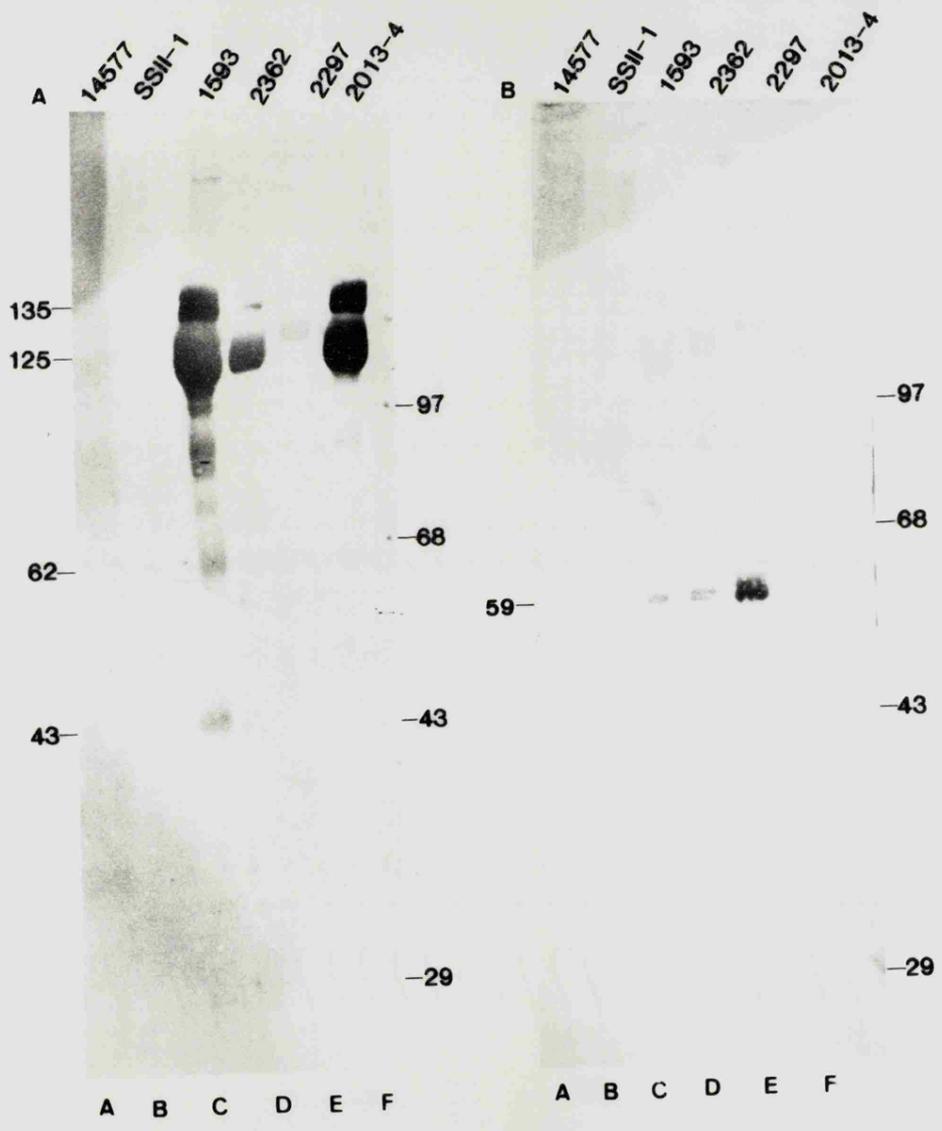
Figure 6.9 Presence of the 59KDa protein in the crystal of *Bacillus sphaericus* strains 1593, 2013-4, 2297 and 2362 investigated using anti-59SER and anti-125KDa antibodies.

Crystals from *B.sphaericus* 1593, 2362, 2297 and 2013-4 were prepared by sucrose density gradient centrifugation (Chapter 2, Section 2.1.5.2). The crystal proteins were separated on 8.5% separating, 5% stacking SDS polyacrylamide gels and transferred onto nitrocellulose paper. Non-specific binding sites on the paper were blocked by incubation in 5% (w/v) Marvel milk in 1X PBS. The Western immunoblot was developed by incubation in either 1:1000 dilution of abtu-125KDa antibodies or 1:100 dilution of anti-59KDaSER antibodies, followed by serial incubations in 1:1000 dilution of goat anti-rabbit antibodies and 1:1000 rabbit peroxidase anti-peroxidase antibodies (Chapter 2, Section 2.6.3).

Lanes:

- A, Whole cells of *B.sphaericus* 14577
- B, Whole cells of *B.sphaericus* SSII-1
- C, Crystal from *B.sphaericus* 1593
- D, Crystal from *B.sphaericus* 2362
- E, Crystal from *B.sphaericus* 2297
- F, Crystal from *B.sphaericus* 2013-4

Protein standards and major proteins are indicated in KDa.



125KDa antibodies

S10-59SER antibodies

antibodies in the crystals of *B.sphaericus* strains 1593, 2362 and 2297. However, no 59KDa protein was detected in the crystals from the strain 2013-4. This strain was earlier shown not to contain the 43 and 62KDa proteins, detectable by Coomassie staining of the crystal proteins (Chapter 4, Section 4.5). In contrast, strain 2297, which contains no detectable 125KDa protein recognizable by the antibodies, gave a strong signal with the anti-59SER antibodies. The results therefore confirmed quite unequivocally that the antibody prepared against the purified 125KDa protein did not recognize any shared epitopes with the 59KDa antibody. Finally, neither antibody recognized proteins in the whole cell profile of sporulated cells of *B.sphaericus* 14577 and SSII-1, which do not produce crystals.

#### 6.9. Summary.

The gene encoding the 59KDa protein was localized to the 5' region of the 3.6Kb insert by deletion analysis of the plasmid pAS233HA examined in minicells. In order to achieve high-level expression of the protein in a systematic manner, we decided first to obtain the sequence of the gene encoding the 59KDa protein. During the sequencing, the sequence of the corresponding gene from the *B.sphaericus* 2362 was reported (Baumann et al., 1988). Shortly afterwards, the sequence of the 59KDa gene from the same isolate used in this study, *B.sphaericus* 1593M, was also reported (Arapinis et al., 1988). The three sequences were in fact identical in the coding sequence for the 59KDa protein, but differed at 3 nucleotides 3' to the coding sequence. The sequence obtained for the gene encoding the 59KDa protein is shown in Figure 6.3.

The sequence obtained was used to design oligonucleotides in order to mutagenize the 3' and 5' ends of the gene in order to incorporate restriction enzyme sites for subsequent genetic manipulation of the gene. The mutagenized gene, containing a serine in the second position rather than the wild type cysteine, was then cloned downstream of the strong  $\Phi 10$  promoter as a fusion to 13 codons of the *s10* gene of phage T7. One transformant was obtained with the insert in the correct orientation, and the recombinant plasmid expressed the protein at high levels when transformed into the strain *E.coli* BL21-

DE3. This protein was used as an antigen for the immunization of rabbits, and antibody was successfully raised.

The 59KDa protein was specifically exported, albeit at low level, to the medium, when a truncated portion of the protein under the control of the *B.sphaericus* promoter was fused to the 23KDa haemolysin signal region. When the S10-59SER fusion protein under the control of the  $\Phi$ 10 promoter was fused to the 23KDa signal region of haemolysin, specific secretion of the protein was again obtained, but at approximately the same low level. This was accompanied by cleavage of the fusion protein inside the cell.

When induced *E.coli* BL21-DE3 cells containing the plasmid pET3a-59SER, which encodes the S10-59SER fusion protein, were mixed with induced *E.coli* MC4100 cells containing the plasmid pJLA504-41, encoding the 41KDa protein, toxicity to mosquito larvae in bioassays was demonstrated. Toxicity was not seen when cells containing either the 59KDa or the 41KDa protein alone were used in bioassays.

Antibodies to the 59KDa protein were used to probe whole cells of the non-toxic *B.sphaericus* strain 14577 and the poorly toxic strain SSII-1, and crystals isolated from the toxic *B.sphaericus* strains 1593, 2362, 2297 and 2013-4. A protein migrating at 59KDa was recognized by the antibodies in the strains 1593, 2362 and 2297. No proteins were recognized in the other strains. Remarkably, as also observed earlier, strain 2297 appears capable of forming crystals containing relatively high levels of the 59KDa protein, in the complete absence of detectable 125KDa protein at either the stainable or the antibody-detectable level.

## Chapter 7.

### Preliminary studies on the regulation of expression of the genes encoding the 41 and 59KDa toxin proteins during the growth phase in *E.coli*, *B.subtilis* and *B.sphaericus*.

#### 7.1. Introduction.

It has been shown in this study that both the 41 and 59KDa proteins are required for toxicity to mosquito larvae. It has been reported that vegetative cells of *B.sphaericus* were non-toxic to mosquito larvae, whilst cells undergoing sporulation were toxic (Myers and Yousten, 1981; Jamuna et al., 1982). This indicates that the 41 and 59KDa proteins may be synthesized or activated in a growth phase-dependent manner. However, DNA sequencing of the gene encoding the 41KDa protein (Hindley and Berry, 1987) indicated that the promoter of this gene resembled a vegetative promoter of *B.subtilis*. This promoter would be expected to be recognized by the  $\sigma_{55}$  subunit of *Bacillus* RNA polymerase. In addition, DNA sequencing of the gene encoding the 59KDa protein (Chapter 6, Section 6.3), showed that the apparent promoter region for this gene also contained the -10 recognition sequence of the  $\sigma_{55}$  subunit of *Bacillus* RNA polymerase, and a region with a 3 out of 6bp match to the the canonical -35 recognition sequence of the  $\sigma_{55}$  subunit of RNA polymerase. Although the identity of these promoters has not been confirmed by DNA footprinting assays, the nucleotide sequences do indicate that a vegetative  $\sigma$ -factor could control transcription of these genes.

It was of interest therefore to investigate the regulation of the genes encoding the 41 and 59KDa proteins in *Bacillus* and also in the cloning host *E.coli*, to determine if these genes were expressed in a growth phase-dependent manner. To study this, I first used the product of the *lacZ* gene of *E.coli*,  $\beta$ -galactosidase (LacZ), as a reporter enzyme. I made fusions of the *lacZ* gene to the 41 and 59KDa protein encoding genes, which were under the control of their own *B.sphaericus* promoters. The expression of the gene encoding the 59KDa protein could also be studied in *B.sphaericus* using antibodies that had previously been raised to the S10-59SER fusion protein (Chapter 6, Section 6.7). The lack of antibodies to the 41KDa protein made such an approach impossible for this

protein. In addition, synthesis of the transcripts of the genes encoding the 41 and 59KDa proteins in *B.sphaericus* could be studied, using the cloned DNA fragments encoding the respective genes as radioactively labelled probes.

## 7.2. Construction of fusions of the *E.coli lacZ* gene to the genes encoding the 41 and 59KDa proteins.

The *lacZ* gene used in these constructs was present, together with the lactose permease (*lacY*) gene, in the promoter probe plasmid series pLKC480 (Tiedemann and Smith, 1987). This plasmid was derived from a series of plasmids incorporating the *lacZ*, *lacY* genes (Minton, 1984) and contains the ampicillin resistance gene of pBR322 and the kanamycin resistance gene from the plasmid pNEO (Pharmacia Inc.). The promoterless *lacZ* gene, the *lacY* gene and the gene for kanamycin resistance are present on a 6.3Kb *SmaI* restriction enzyme fragment, and can be subcloned as a "cassette". Upstream of the *lacZ* gene is a multiple cloning site (see Figure 7.1 for plasmid details).

### 7.2.1. Fusing the 41KDa protein encoding gene to *lacZ*.

The promoter and the 5' region of the gene encoding the 41KDa protein were isolated on a 1.57Kb *Sau3A* restriction enzyme fragment and ligated into the multiple cloning site upstream of *lacZ* at the *BamHI* site of the vector pLKC481 (Figure 7.1). This fusion should maintain the correct translational reading frame. Dephosphorylation of the vector with alkaline phosphatase was not carried out due to concerns about exonuclease contamination of the enzyme. The ligation mixture was transformed into *E.coli* MC4100, which lacks the *lac* operon and thus remains colourless on McConkey agar plates. The transformants were plated out on McConkey agar containing ampicillin and kanamycin at 100µg/mL and 50µg/mL respectively, and grown overnight at 37°C. Colonies containing the required fusion should be red due to fermentation of lactose and the consequent red colour of the phenol red indicator in acidic medium. Such red colonies were picked and plasmid DNA isolated from them by the minipreparation method (Chapter 2, Section 2.3.2). The orientation of the insert in the recombinant plasmid was then

Figure 7.1 Construction of the plasmid p41-LacZ.

The plasmid pAS233HA was digested with the restriction enzyme *Sau3A* and the 1.57Kb fragment containing the 5' sequence encoding the 41KDa protein together with the promoter of the gene was isolated. This fragment was ligated into the *Bam*HI site of the promoter probe plasmid pLKC481 in order to make a translational fusion of the gene encoding the 41KDa protein to the *lacZ* gene encoded by the plasmid. The ligation reaction was transformed into the *E.coli* strain MC4100. Transformants were screened for ampicillin and kanamycin resistance and Lac<sup>+</sup> phenotype. The identity of the plasmids from such transformants was confirmed by restriction enzyme digestion analysis.

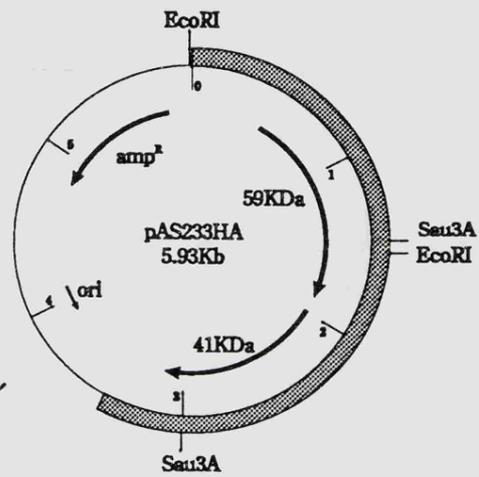
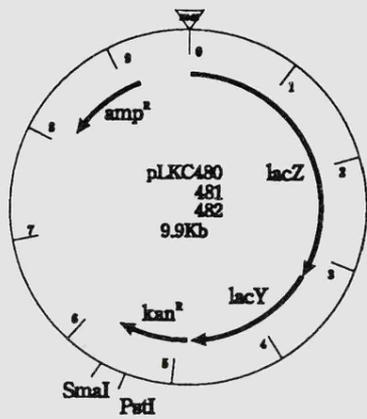
The multiple cloning site (*mcs*) contained the following sequences, with the *Bam*HI site underlined.

pLKC480: GA ATT CCC GGG GAT CCG TCG ACC TGC AGC CAA GCT TGC

pLKC481: GAA TTC CCG GGG ATC CGT CGA CCT GCA GCC AAG CTT GC

-----

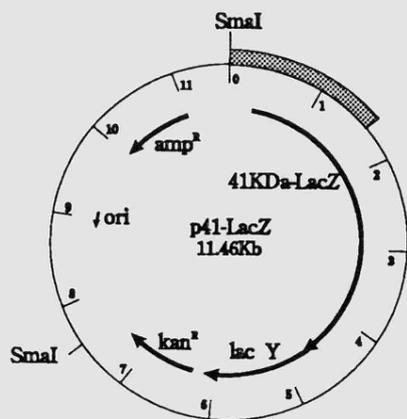
pLKC482: G AAT TCC CGG GGA TCC GTC GAC CTG CAG CCA AGC TTG



Digest pLKC481  
with BamHI

Digest with Sau3A  
Isolate 1.567Kb fragment

Ligation reaction



determined by restriction enzyme digestion analysis. All the red colonies contained the plasmid with the insert in the required orientation. One colony was chosen for further work, and the plasmid was named p41-LacZ.

#### 7.2.2. Inserting the 41KDa-LacZ fusion into the shuttle vector, pHV33.

The plasmid, p41-LacZ, was constructed by inserting a 1.57Kb *Sau3A* fragment into pLKC481, which contains a Gram negative replication origin and thus cannot replicate in a Gram positive host. Since the promoter upstream of the gene fusion was originally derived from a Gram positive host, it may not be functionally normal in a Gram negative strain. Therefore, I decided to move the 41KDa-LacZ fusion into the shuttle vector pHV33, which can replicate both in Gram negative and Gram positive backgrounds.

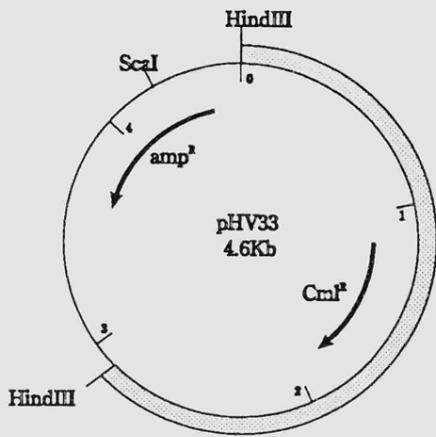
The 41KDa-LacZ fusion was isolated on a 7.5Kb *SmaI* DNA fragment from the plasmid p41-LacZ (Section 7.2.1) and ligated into the *ScaI* restriction enzyme site in the gene encoding  $\beta$ -lactamase on pHV33. The plasmid construction is shown in Figure 7.2. *E.coli* MC4100 transformants were selected on McConkey agar plates containing chloramphenicol (5 $\mu$ g/mL). These transformants were then screened for ampicillin sensitivity. A plasmid containing the gene fusion in the *ScaI* site of pHV33 was identified by restriction enzyme digestion analysis of the plasmid DNA. This plasmid conferred chloramphenicol resistance and a Lac<sup>+</sup> phenotype on the cells and was named pHV41-LacZ.

#### 7.2.3. Fusing the gene encoding the 59KDa protein to lacZ.

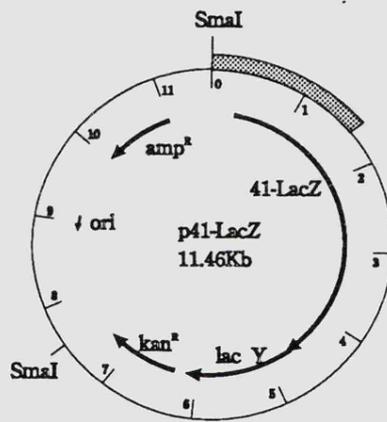
The 5' portion containing the promoter and a portion of the gene encoding the 59KDa protein was isolated on a 1.63Kb *EcoRI* DNA fragment and ligated into the *EcoRI* site in the multiple cloning site of the vector, pLKC481. This fusion should place *lacZ* downstream of the gene encoding the 59KDa protein in the correct translational reading frame. The ligation mixture was transformed into *E.coli* MC4100, and transformants that were Lac<sup>+</sup> and resistant to ampicillin and kanamycin (100 $\mu$ g/mL and 50 $\mu$ g/mL respectively), were isolated on

Figure 7.2 Construction of the plasmid pHV41-LacZ.

The 7.5Kb fragment obtained by *Sma*I digestion of the plasmid p41-LacZ contains the gene encoding the 41KDa protein under the control of its *Bacillus sphaericus* promoter fused to the *lacZ* gene of *E.coli*. This fragment was ligated into the *Sca*I site of the shuttle vector pHV33. The ligation reaction was transformed into *E.coli* MC4100 and transformants were screened for chloramphenicol resistance, ampicillin sensitivity and a Lac<sup>+</sup> phenotype.

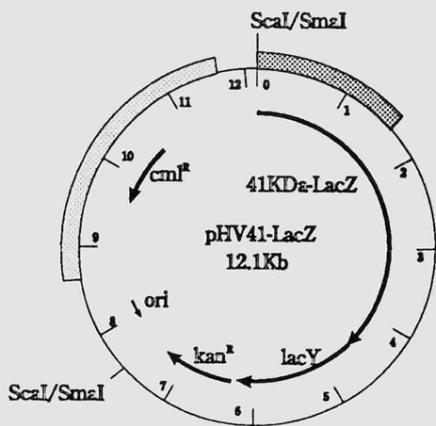


Digest with Scal



Digest with SmaI  
Isolate 7.5Kb fragment

Ligation reaction



McConkey agar plates. Plasmid identity was determined by isolation of plasmid DNA by the minipreparation method (Chapter 2, Section 2.3.2), and restriction enzyme digestion analysis. A plasmid with the insert in the correct orientation was named p59-LacZ (Figure 7.3).

#### 7.2.4. Inserting the 59KDa-LacZ fusion into the shuttle vector, pHV33.

As explained above, the plasmid, pLKC481, is unable to replicate in a Gram positive background due to the absence of a Gram positive origin of replication. In order to analyze expression of the gene fusion in such backgrounds, a Gram positive replicon was required.

A 2.9Kb fragment produced by *Hind*III restriction enzyme digestion of the plasmid pHV33 contains a gene encoding chloramphenicol resistance in addition to containing a Gram positive replicon. This fragment was isolated and ligated into the unique *Sca*I site in the gene encoding  $\beta$ -lactamase of the plasmid p59-LacZ (Figure 7.4). Since the plasmids pLKC481 and pHV33 contain the same ColE1 replicon for Gram negative backgrounds, the copy number of the two constructs in Gram negative bacteria is the same (Old and Primrose, 1985). This plasmid was called pHV59-LacZ, following confirmation of its identity and orientation of the insert by plasmid preparation from transformants that had Lac<sup>+</sup>, chloramphenicol resistant and ampicillin sensitive phenotype.

#### 7.2.5. Transformation of *B.subtilis* BR151 with the plasmids pHV41-LacZ and pHV59-LacZ.

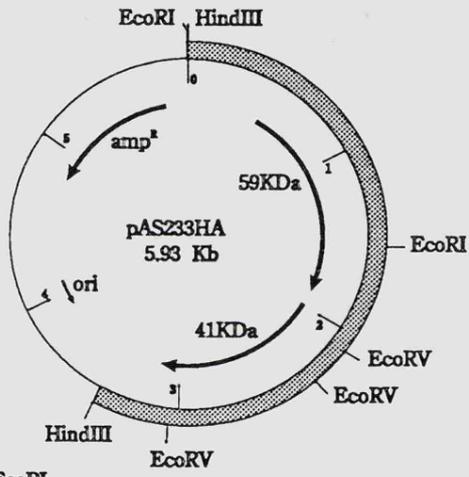
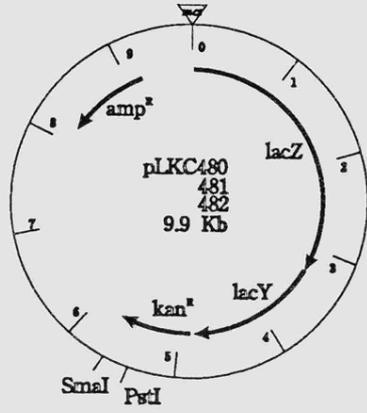
Attempts to transform *B.sphaericus* strains 1593 and 14577 by the competence transformation method (Hardy, 1985; McDonald and Burke, 1984) or by electroporation (Bone and Ellar, 1989) were not successful. Selection was complicated by the observation that *B.sphaericus* 1593 was already resistant to chloramphenicol up to a concentration of 20 $\mu$ g/mL. In addition, *B.sphaericus* 1593 is reported to contain the enzyme *R-Bsp*, having the recognition sequence GGCC (Koncz *et al.*, 1978), an isoschizomer for the enzyme *Hae*III. The presence of these restriction sites on the plasmids pHV41-LacZ (12.1Kb) and

Figure 7.3 Construction of the plasmid p59-LacZ.

The plasmid pAS233HA was digested with the restriction endonuclease *EcoRI*. The 1.63Kb fragment obtained from the digestion contained the control sequences and the 5' coding sequence of the 59KDa toxin protein. This fragment was ligated into the *EcoRI* site of the plasmid pLKC481 in order to create a translational fusion of the gene encoding the 59KDa protein to the *lacZ* gene encoded by the promoter probe plasmid pLKC481. The ligation reaction was transformed into *E.coli* MC4100 and transformants were screened for ampicillin and kanamycin resistance and a Lac<sup>+</sup> phenotype. Plasmid identity was confirmed by restriction enzyme digestion analysis.

The multiple cloning site (*mcs*) contained the following sequences, with the *EcoRI* site underlined.

pLKC480: GA ATT CCC GGG GAT CCG TCG ACC TGC AGC CAA GCT TGC  
pLKC481: GAA TTC CCG GGG ATC CGT CGA CCT GCA GCC AAG CTT GC  
-----  
pLKC482: G AAT TCC CGG GGA TCC GTC GAC CTG CAG CCA AGC TTG



Digest pLKC481  
with EcoRI

Digest with EcoRI  
Isolate 1.629Kb fragment

Ligation reaction

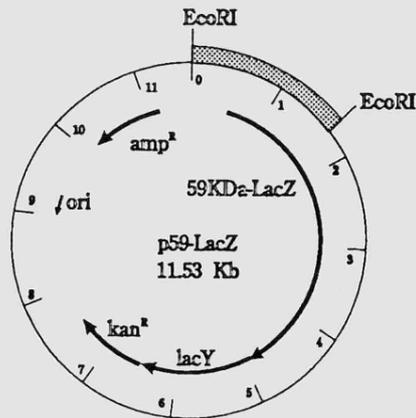
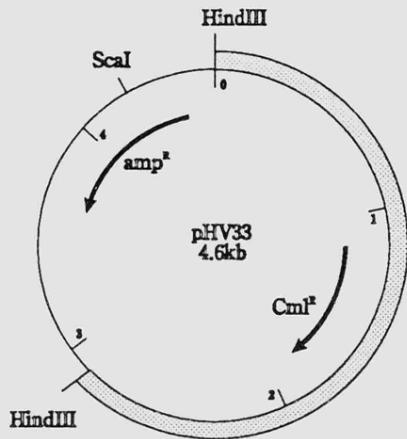


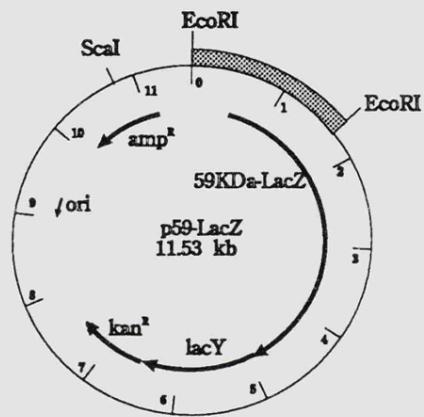
Figure 7.4 Construction of the plasmid pHV59-LacZ.

The plasmid pHV33 was digested with the restriction enzyme *Hind*III. The 2.9Kb fragment released by the digestion was isolated. This 2.9Kb fragment contains the gene encoding a chloramphenicol acetyltransferase III gene of Gram positive origin, and a Gram positive replication origin. The overhangs generated by the *Hind*III digestion were filled in with the Klenow fragment of DNA polymerase.

The plasmid p59-LacZ was digested with the restriction enzyme *Sca*I. The 2.9Kb *Hind*III fragment with the blunt ends was ligated into the *Sca*I site of the plasmid p59-LacZ. The ligation reaction was transformed into *E.coli* MC4100. Transformants were screened for chloramphenicol resistance, ampicillin sensitivity and a Lac<sup>+</sup> phenotype.



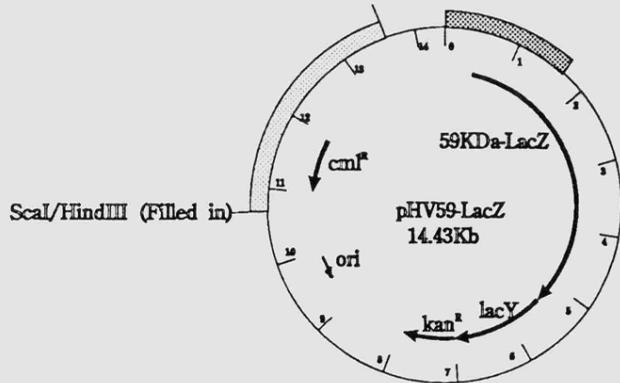
Digest with HindIII  
 Fill in overhangs with Klenow  
 Isolate 2.9Kb fragment



Digest with Scal

Ligation reaction

Scal/HindIII (Filled in)



Scal/HindIII (Filled in)

pHV59-LacZ (14.43Kb) could have contributed to the inability to obtain the expected transformants. Therefore, I decided to use another *Bacillus* strain for transformation, assuming that regulatory elements in related Bacilli may be highly homologous. For this purpose the strain *B.subtilis* BR151 was chosen. Attempts to transform the *B.subtilis* strain by electroporation were not successful, however, and transformation was subsequently carried out by the competence method of Hardy (1985).

*Bacillus* transformants were screened for Lac<sup>+</sup>, and the chloramphenicol resistance (5µg/mL) phenotype. The frequency of transformation of *B.subtilis* BR151 obtained for pHV33 was  $2.3 \times 10^4$  transformants/µg DNA, and a frequency of  $2.8 \times 10^4$ /µg DNA was obtained for pHV41-LacZ. The frequency of transformation of pHV59-LacZ obtained was lower at  $6 \times 10^1$  transformants/µg DNA.

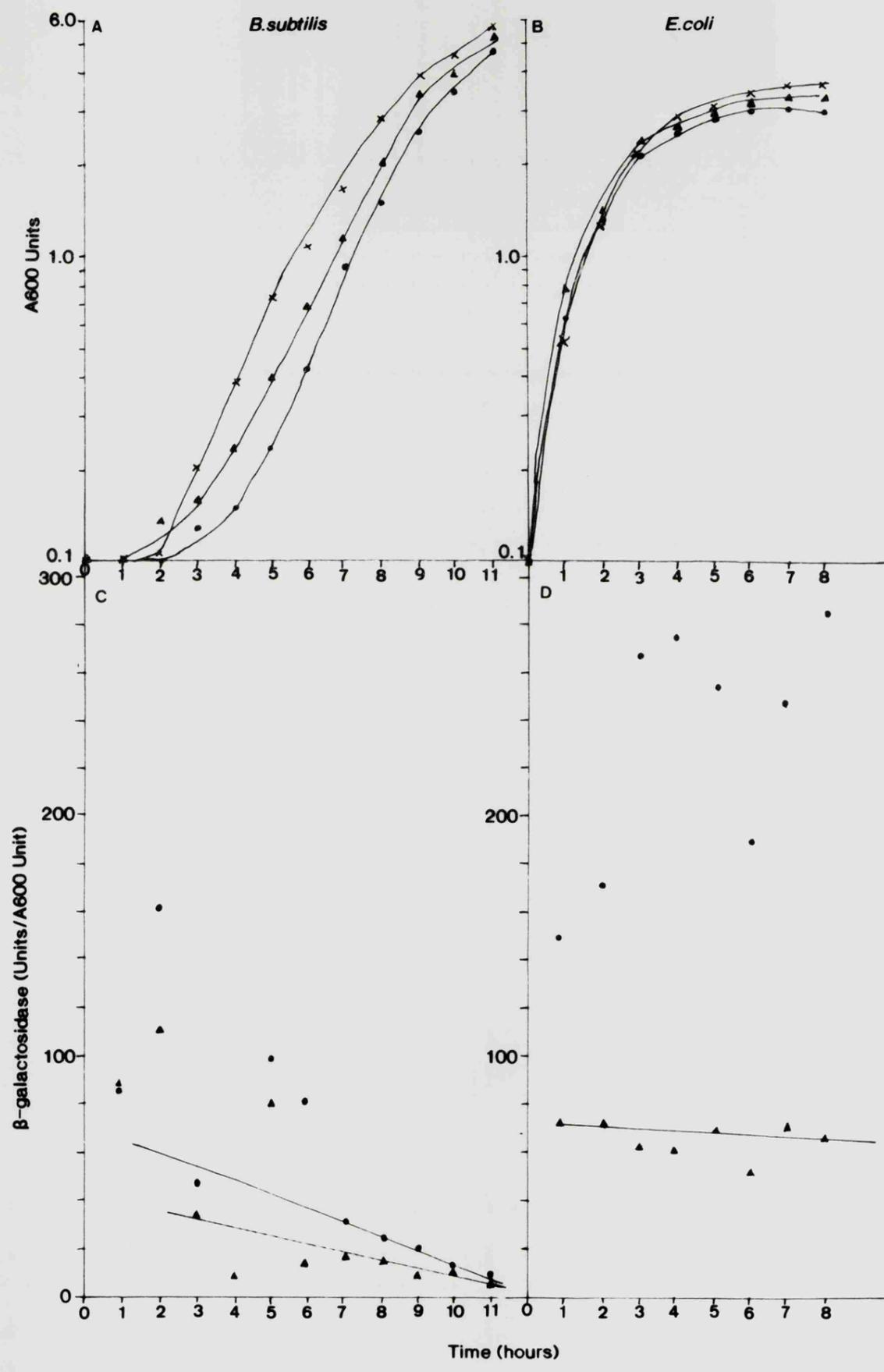
### 7.3. Analysis of the expression of the 41KDa-LacZ and 59KDa-LacZ fusions in *E.coli* and *B.subtilis*.

*B.subtilis* BR151 and *E.coli* MC4100 transformants containing the plasmids pHV41-LacZ and pHV59-LacZ were grown in Luria broth containing 5µg/mL chloramphenicol. In the case of *B.subtilis*, the plasmid-harboring strains were grown for 2 days to obtain fully sporulated cultures. The *E.coli* cultures containing the plasmids were grown overnight. Before inoculation in fresh Luria broth to a final A<sub>600</sub>=0.05, the sporulated *B.subtilis* cultures were synchronized by heat shock at 78°C for 10min (Kalfon et al., 1984) in order to kill growing cells and to activate dormant spores. Optical density readings and samples for the estimation of β-galactosidase levels were taken every hour. The amount of β-galactosidase produced, and Units of β-galactosidase/A<sub>600</sub> Unit of cells were estimated by the method of Miller (1972). The graph presented in Figure 7.5 shows the results obtained from *E.coli* and *B.subtilis* containing the plasmids pHV33, pHV41-LacZ or pHV59-LacZ. In the case of *E.coli*, the strain harbouring the plasmid pHV41-LacZ produced β-galactosidase in a manner largely unrelated to the phase of growth of the *E.coli* strain, i.e. the amount of protein synthesized was constant at all the stages of growth. For the *E.coli* strain containing pHV59-

Figure 7.5 Analysis of the expression of the 41-LacZ and 59-LacZ fusion proteins in *E.coli* and *B.subtilis*.

*E.coli* and *B.subtilis* transformants containing the plasmids pHV33 (control), pHV41-LacZ and pHV59-LacZ were grown at 37°C in Luria broth from an  $A_{600}=0.05$ . The *B.subtilis* strains were inoculated from a culture grown into sporulation and then heat-shocked to activate spores. The *E.coli* strains were inoculated from an overnight culture. Samples of cells were taken at 1 hour intervals for optical density measurements (Panel A) and  $\beta$ -galactosidase assays (Panel B; Miller, 1972).  $\beta$ -galactosidase levels for cells containing the pHV41-LacZ and pHV59-LacZ are shown in Units of  $\beta$ -galactosidase/ $A_{600}$  Unit.

- x = *E.coli* or *B.subtilis* cells containing pHV33
- ▲ = *E.coli* or *B.subtilis* cells containing pHV41-LacZ
- = *E.coli* or *B.subtilis* cells containing pHV59-LacZ



LacZ, no overall pattern was found, although the amount of LacZ activity tended to increase during growth. In addition, the amount of  $\beta$ -galactosidase produced by *E.coli* containing pHV59-LacZ was two-fold higher than in strains containing the plasmid pHV41-LacZ.

In *B.subtilis*, the amount of  $\beta$ -galactosidase produced by the cells harbouring the plasmids pHV41-LacZ and pHV59-LacZ decreased with the increasing age of the culture, with the amount of  $\beta$ -galactosidase produced by the cell undergoing a 10-fold reduction. As in the *E.coli* strain, the amount of  $\beta$ -galactosidase produced by *B.subtilis* cells containing the plasmid pHV59-LacZ was higher than cells containing the plasmid pHV41-LacZ. The  $\beta$ -galactosidase values for strains containing pHV33 approached zero, and are not shown.

The results clearly indicated some form of regulation of both genes in *B.subtilis*, but it is not clear how this might relate to its normal regulation in *B.sphaericus*. An important consideration in these experiments is that the plasmids containing the fusions are present in multiple copies in the cells, and this high copy number could affect the control of expression of the gene (see Chapter 8, Section 8.5). Therefore, these experiments should ideally be repeated with the fusion present in a single copy in the cell.

#### 7.4. Analysis, using antibodies of the expression of the 59KDa protein in *B.sphaericus*.

Antibodies were obtained to the S10-59SER fusion protein as described in Chapter 6, Section 6.7. These antibodies were used in the work described here.

Heat shocked spores of *B.sphaericus* 1593 were inoculated into Luria broth and MSB sporulation medium in order to determine which medium supported better growth and sporulation. Spores of *B.sphaericus* 14577 were inoculated into Luria broth, and grown to sporulation phase as determined graphically (Kalfon et al., 1984), and by phase contrast microscopy for the appearance of spores. Thus, stage t=0 of sporulation can be determined as the stage at which the optical density of the culture stops increasing at the rate

seen in exponentially growing cells. Samples of cells containing identical  $A_{600}$  Units were taken at various points during growth (Figure 7.6, Panel A). These cell samples were resuspended in SDS loading buffer and heated to 100°C for three minutes to lyse the cells. *B.sphaericus* total cell proteins were then separated on an 8.5% SDS polyacrylamide gel and transferred by electroblotting onto nitrocellulose paper (see Chapter 2, Section 2.6.3). The nitrocellulose paper was probed with antibodies to the S10-59SER fusion protein, which had previously been adsorbed to acetone powder of *E.coli* BL21-DE3 containing pET3a, to remove non-specifically binding antibodies. The nitrocellulose paper was then incubated sequentially with 1:1000 dilution of goat anti-rabbit antibodies and 1:1000 dilution of rabbit peroxidase anti-peroxidase antibodies. The nitrocellulose paper was then developed as described in Chapter 2, Section 2.6.3. The Western immunoblot is shown in Figure 7.6, Panel B and demonstrates that a specific cross-reacting protein of 59KDa was not present in the strain 14577, which is non-toxic, but was, as expected, present in strain 1593 which is toxic to mosquito larvae. A similar cross-reacting protein was also detected by the antibodies in cell lysates of *B.sphaericus* 2013-4, a second toxic strain, whose crystals do not appear to contain the 59KDa protein (data not shown; see also Chapter 6, Section 6.8).

A low molecular weight protein migrating at 31KDa was also recognized in cells of strains 14577 and 1593, with an additional protein at 26KDa also recognized in strain 1593. These proteins may correspond to proteins distinct from the toxin protein itself, but bearing an epitope recognized by the anti-S10-59SER antibodies.

A protein migrating at the expected size of exactly 59KDa was clearly detected in these experiments in the later stages of growth in strain 1593 accompanying sporulation. In electron microscope studies, Kalfon *et al.* (1984) reported that the crystal in *B.sphaericus* 2297 appeared at  $t=2$  of sporulation, and our results correlate well with this observation, since the 59KDa protein has been shown to be a constituent of the crystal in *B.sphaericus* 1593, 2362 and 2297. This result would be consistent with specific

Figure 7.6 Analysis of the presence of the 59KDa protein during the growth cycle of *Bacillus sphaericus* strains 14577 and 1593 with antibodies to the S10-59SER fusion protein.

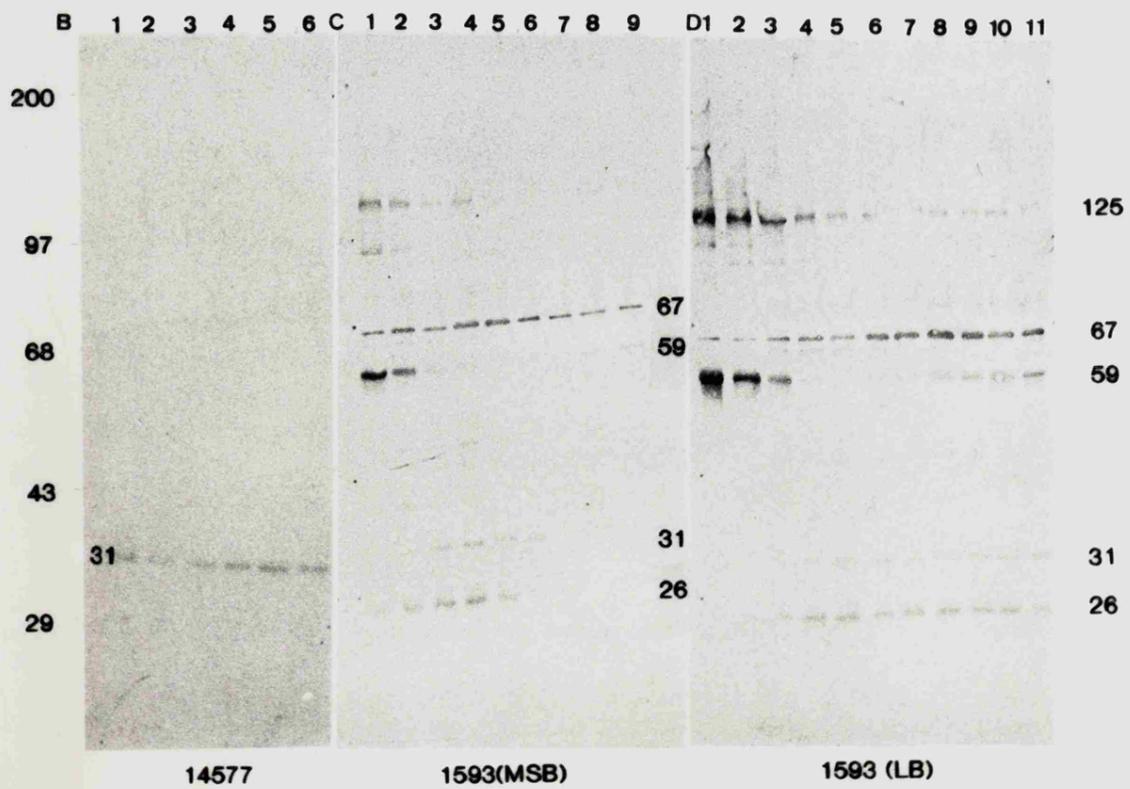
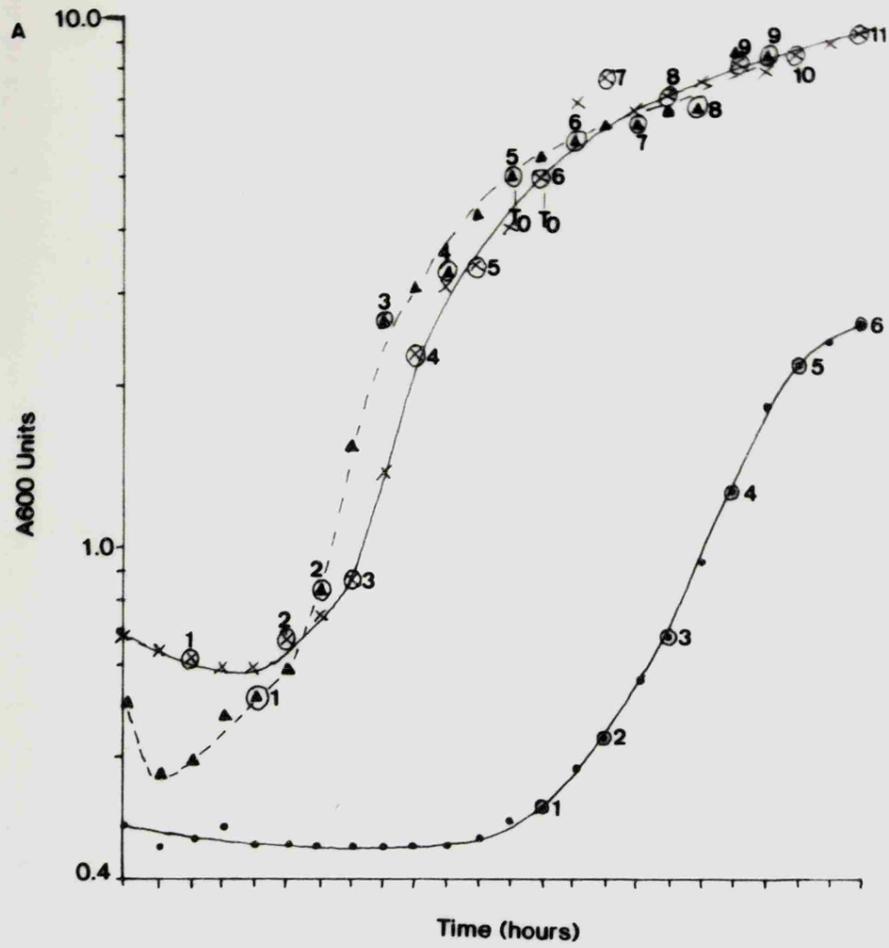
Heat-activated spores of *B.sphaericus* 14577 were inoculated into Luria broth, and heat-shocked spores of *B.sphaericus* 1593 were inoculated into MSB sporulation broth (Kalfon et al., 1984) and into Luria broth to an  $A_{600}=0.05$ . The cells were grown at 30°C, and samples were taken for measurement of the optical density (Figure 7.6A) and for Western immunoblot analysis (Figure 7.6B, C, D).

The 0.1  $A_{600}$  Units of cells were heated to 100°C for 3min in SDS loading buffer, and the proteins were separated on 8.5% separating, 5% stacking SDS polyacrylamide gels. The proteins were then electrophoretically transferred onto nitrocellulose paper. Non-specific binding sites on the paper were blocked by incubation in 5% (w/v) Marvel milk in 1X PBS. The blots were then incubated in 1:100 dilution of antibodies to the S10-59SER fusion protein. This was followed by sequential incubations in 1:1000 dilution of goat anti-rabbit antibodies and 1:1000 dilution of rabbit peroxidase anti-peroxidase antibodies (Chapter 2, Section 2.6.3).

Figure 7.6B shows the results of the Western immunoblot assays with antibodies raised to the S10-59SER protein, using total cell proteins from strain 14577 grown in Luria broth (Figure 7.6B), strain 1593 grown in MSB sporulation broth (Figure 7.6C) and strain 1593 grown in Luria broth (Figure 7.6D). The lane numbers refer to the sample numbers shown on the corresponding growth curve of the strain in Figure 7.6A.

The sizes of the protein standards and the major proteins in the cell recognized by the antibodies are shown in KDa.

- x = *B.sphaericus* 1593 in Luria broth
- ▲ = *B.sphaericus* 1593 in MSB sporulation broth
- = *B.sphaericus* 14577 in Luria broth



transcription and translation and/or translation only during the sporulation phase of growth. A strong signal was, however, also detected as a 59KDa protein in the lag and very early log phase of growth. This presumably reflects carry over with the inoculum as it appears to be rapidly diluted out. This would have to be confirmed.

However, the results indicated in Figure 7.6 appeared more complex and interesting, since the antibody also detected other proteins which migrated at 67KDa and 125KDa. The 67KDa protein was detected at the start of the exponential stage of growth and its synthesis continued, perhaps at a maximum during mid-exponential phase, when the 59KDa protein was barely detectable. This 67KDa protein may be a cellular, i.e. non-crystal associated, and posttranslationally modified form of the 59KDa protein, which may be converted into the "active" 59KDa toxin during crystal formation. The results obtained in Chapter 6, Section 6.8, using the S10-59SER antibodies clearly demonstrated that only the 59KDa species was present in the crystal preparations (Chapter 6, Section 6.8). The synthesis of the 125KDa protein mimicked that of the 59KDa protein, and may represent a dimer of the 59KDa protein. The results raised interesting questions therefore about complex control of the expression and or modification of the product of the gene encoding the 59KDa protein when the cells entered sporulation phase. In attempts to clarify some of the issues, I decided to analyze total RNA from *B.sphaericus* cells for the presence of gene specific transcripts during the growth phase. The results described in the next section, obtained with *B.sphaericus* cells grown in MSB sporulation broth or in Luria broth were identical, and therefore only the data for cells grown in Luria broth are presented.

7.5. Preparation of RNA from *B.sphaericus* at various stages of growth, and analysis of expression of the 41 and 59KDa protein encoding mRNA.

This section describes the analysis of gene-specific mRNA synthesized in the *B.sphaericus* strain 1593 in order to determine whether growth stage-related transcription of the 41 and 59KDa genes occurs. Total RNA was prepared by taking samples of *B.sphaericus* cells at various stages of growth (Figure 4.6, Panel A) and processing them for RNA as described in Chapter 2, Section 2.9.1. This RNA was quantified spectrophotometrically, and approximately equal quantities were spotted onto Hybond-N paper using a "Slot-blotter" apparatus. Since the quantity of cells processed for RNA was very small (2 A<sub>600</sub> Units), the consequent yield of RNA was very low (30-50µg). This precluded accurate spectrophotometric quantification of the RNA and this is reflected in the fluctuations of RNA loadings in the slot-blot. The RNA on the Hybond-N filter was hybridized to the 1.35Kb *SphI-HincII* fragment and 1.64Kb *BspHI-PstI* DNA fragments, as probes for the genes encoding the 41 and 59KDa proteins respectively, described in Chapter 5, Section 5.3.1, and Chapter 6, Section 6.5. Since the amounts of RNA in the slots were not equal, we required a constitutively expressed gene as a control to compare the levels of RNA in the blots. For this purpose, DNA encoding a *B.subtilis* rRNA operon (on a 5.3Kb *BamHI* fragment from the plasmid p14B1) was used. Figure 7.7 shows the results obtained. The signal obtained for each slot was quantified using an LKB laser densitometer. The values obtained for each slot hybridized to the probes for the genes either encoding the 59KDa or the 41KDa protein were normalized against the corresponding value for the rDNA probe. It was assumed for this purpose that the transcription of the rDNA operon was essentially constant during the growth phase. The values thus obtained were plotted and the result is shown in Figure 7.7, Panel A.

The results showed that the transcription of both the 59 and 41KDa mRNA's occurred in two phases, one phase commenced in mid-logarithmic phase and was terminated well before the time of initiation of the sporulation phase ( $t=0$ , where  $t=n$  indicates the hours after the culture enters sporulation

Figure 7.7 Slot-blot analysis of mRNA, corresponding to the genes encoding the 41 and 59KDa proteins, prepared at different stages of the growth cycle of *B.sphaericus* 1593.

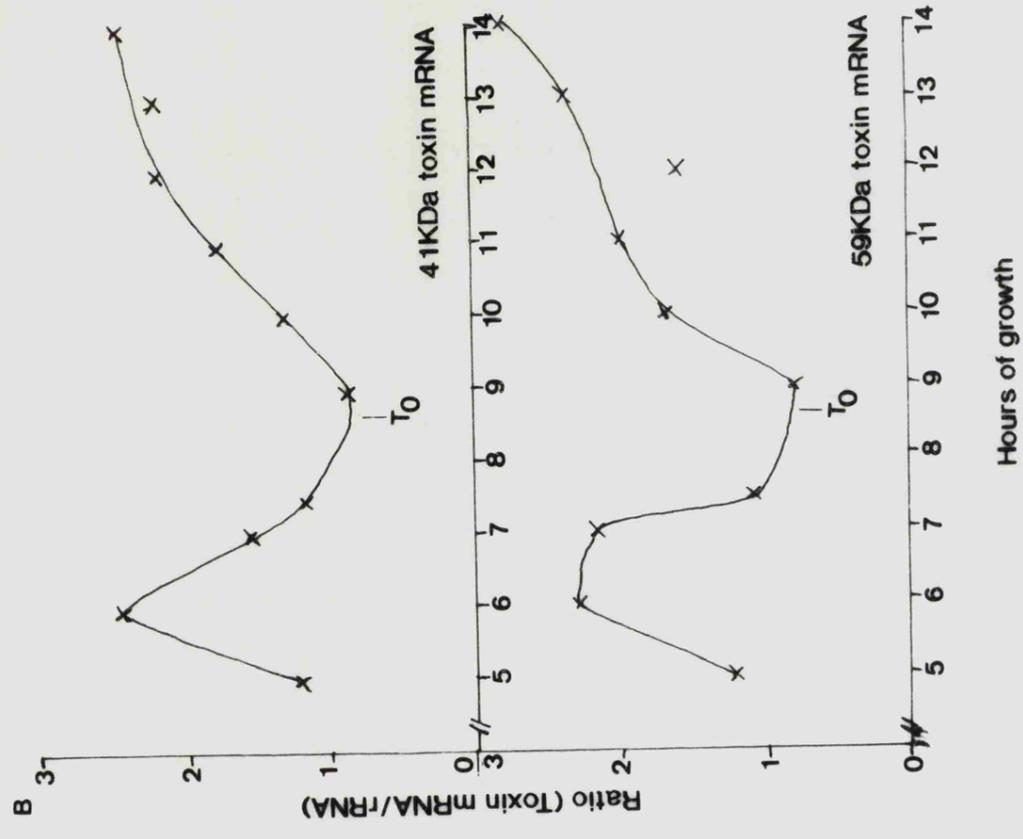
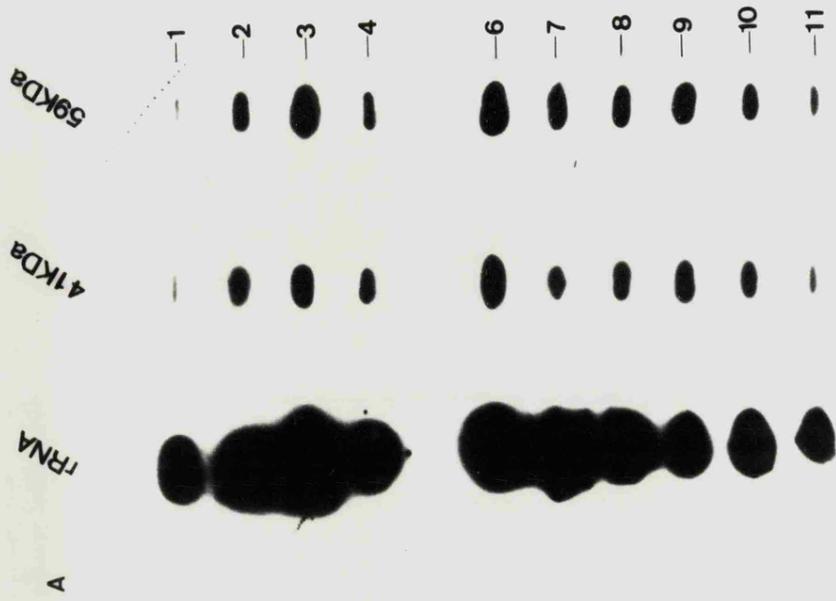
Heat-activated spores of *B.sphaericus* 1593 were inoculated into Luria broth to an  $A_{600}=0.05$ . The cells were grown at 30°C and samples of cells taken at intervals for the optical density measurements (Figure 7.7A). Total cellular RNA was prepared from the cells as described in Chapter 2, Section 2.9.1. Approximately 2µg of each RNA sample was heated to 70°C for 10min and transferred to Hybond-N membrane using a slot-blot apparatus. The RNA was bound to the membrane by exposure to long wave ultraviolet illumination for 4min. The membrane was then subjected to prehybridization and hybridization conditions described in Chapter 2, Section 2.3.12.1 for Southern hybridization analysis.

The radiolabelled probes used were:

- (A) a 5.3Kb fragment isolated by *Bam*HI restriction enzyme digestion of the plasmid p14B1, which contains a *Bacillus subtilis* rRNA operon (Bott et al., 1981).
- (B) a 1.35Kb fragment containing the gene encoding the 41KDa protein obtained by *Sph*I-*Hinc*II restriction enzyme digestion of M13-41M (Chapter 5, Section 5.3.1).
- (C) a 1.64Kb fragment containing the gene encoding the 59KDa protein isolated by *Bsp*HI-*Pst*I restriction enzyme digestion of the plasmid M13-59M (Chapter 6, Section 6.5).

Hybridization was conducted overnight at 60°C exactly as described in Chapter 2, Section 2.3.13. Washes were in 1X SSC; 0.1% (w/v) SDS; 10µg herring sperm DNA/mL at 60°C.

The graph below the slot-blot analysis shows the results obtained after normalizing the values from the Laser densitometric scan of each slot hybridized to the probes encoding the 41 and 59KDa proteins, to the value obtained for the corresponding rDNA hybridization. The rRNA synthesis is assumed to be constitutive. The values shown on the Y-axis are arbitrary. For laser scanning, a lower exposure of the RNA hybridized to rDNA was used.



phase). The second phase of transcription commenced early in sporulation ( $t=2$ ) and continued into the sporulation phase. These results are consistent with the synthesis of the 59KDa protein resulting from the second phase of transcription, when correlated with protein expression data obtained in the previous section (Section 7.4), showing maximum levels of the 59KDa protein in cells entering the second to third hour of sporulation. The early phase of transcription detected in Figure 7.7 presumably emanates from the vegetative promoters of the genes encoding the 41 and 59KDa proteins. The mRNA in this case is presumed then to be poorly translated or perhaps, translated into the modified 67KDa form. The second phase of mRNA transcription which would therefore be expected to emanate from an unidentified, presumably sporulation-controlled promoter, is translated, with consequent accumulation of 59KDa protein in the cell.

#### 7.6. Summary.

The regulation of the expression of the 41 and 59KDa toxin genes were investigated in *E.coli* and *B.subtilis* by the use of fusions of these genes, under control of their *B.sphaericus* promoters, to the *lacZ* gene of *E.coli*. The fusions were constructed in the shuttle vector pHV33 to allow expression of the genes in both Gram negative and Gram positive hosts.

Transformation of the *lacZ* fusion constructs into *B.sphaericus* strains was unsuccessful, and therefore the analysis of  $\beta$ -galactosidase expression was limited to the use of *B.subtilis*, with the anticipation that regulation of these genes might be similar in closely related species. The data obtained show that the synthesis of  $\beta$ -galactosidase, fused to the 41KDa protein encoding gene promoter, was relatively constant at all stages of growth when present in *E.coli*, although no pattern could be distinguished for the 59KDa protein encoding gene. However, when the fusions were transformed into *B.subtilis*, the specific activity of the protein appeared to decrease with time, i.e. translation of the protein was not constant at all the stages of growth, but decreased as the cells got older.

Antibodies to the S10-59SER fusion protein were then used

to probe whole cell samples of *B.sphaericus* 1593 and 14577 at various stages of growth in a Western immunoblot. The results showed that a protein of 59KDa was accumulating in the cells two to three hours after the *B.sphaericus* 1593 cells had entered the sporulation phase, which correlates with the observation that the *B.sphaericus* crystal appears in morphological studies at this stage of growth (Kalfon et al., 1984). No 59KDa protein was recognized by the antibodies in the non-toxic strain 14577. Other proteins at 125, 67, 31 and 26KDa in *B.sphaericus* 1593, and 31KDa in *B.sphaericus* 14577 were also seen. The identity of these proteins is not known. These proteins may be dimers, posttranslationally modified or breakdown products respectively. It is also possible that they simply represent proteins with epitopes recognized by the 59SER antibodies. It would be necessary to carry out pulse chase experiments, and immunoprecipitation coupled with Cleaveland digestion analyses, to determine whether these proteins are the different forms of the 59KDa protein.

An analysis of total RNA from *B.sphaericus* cells at different stages of growth was carried out using the cloned 41 and 59KDa protein encoding genes as probes. The control used to normalize RNA levels in the various slots was an rDNA probe of *B.subtilis*. Although levels of rRNA in vegetative and sporulating cells were assumed to be constant to normalize the values obtained for the toxin gene-specific mRNA, the levels of rRNA in sporulating cells is usually 25% of that present in vegetative cells of *B.subtilis* (Pero et al., 1975). This would imply that the relative amount of toxin gene-specific transcripts is overestimated by approximately 4-fold in sporulating *B.sphaericus* cells. Nevertheless, sporulation specific transcription does occur, at levels lower than observed in vegetative cells. To quantitate gene-specific transcripts, it would thus be preferable to carry out competition-hybridization in solution.

Analysis of the blot indicated that both the 59 and the 41KDa mRNA were being synthesised in a bi-phasic manner. There was a falling off in synthesis of the first phase well before the onset of sporulation, followed by another phase which began at  $t=1$  of sporulation and continued into sporulation phase. Although exclusive translation of the

second phase of sporulation correlates well with the accumulation of the 59KDa protein during this period, this result could also imply that the vegetative phase mRNA was not translated, or that it was a long lived-species that was preferentially translated in the sporulation phase of growth. Thus, the nature of the vegetative and sporulation mRNAs should be investigated to determine whether it is the second phase of transcription of the toxin genes that is alone translated to protein.

## Chapter 8.

### Discussion.

#### 8.1. Introduction.

The bacterium *Bacillus sphaericus* produces a larvicidal toxin to mosquitoes. Over a decade of biochemical analysis of the toxin extracted from spore-crystal complexes has resulted in several different estimates of the molecular weight of the toxin (Davidson, 1982; Tinelli and Bourgoquin, 1982; Baumann et al., 1985; de Marsac et al., 1987). In order to precisely characterize toxic components of the crystal, several research groups have cloned genes from *B.sphaericus* that confer larvicidal activity to *Escherichia coli*, when it is used as the cloning host. Three groups have cloned DNA from the *B.sphaericus* strains 1593, 1593M and 2362. All these show a strong similarity of restriction enzyme sites (Figure 1.2), and thus appear to represent an equivalent fragment of DNA (Baumann et al., 1987; Hindley and Berry, 1987; Souza et al., 1988). Another larvicidal gene cloned by Souza et al. (1988) has recently been shown to have a homologue in the non-crystal forming, weakly toxic *B.sphaericus* strain, SSII-1 (Thanabalu et al., 1991). In contrast, a larvicidal gene cloned by Ganesan et al. (1983) from the strain 1593M appears to be unique. All of the above cloned genes have been investigated in order to discover the nature of the larvicidal toxin(s). In this thesis I have studied the genes encoded on a 3.6Kb *HindIII* restriction enzyme fragment from *B. sphaericus* 1593M cloned by Souza et al. (1988).

#### 8.2. Identification of toxin proteins encoded by DNA cloned from *Bacillus sphaericus*.

The plasmid pAS233HA contains a 3.6Kb DNA insert from *B.sphaericus* 1593M which encodes toxicity to the larvae of *Culex* and *Anopheles* mosquitoes (Souza et al., 1988). *B.sphaericus* is distinct from the larvicidal dipteran-toxic *B.thuringiensis* subspecies *israelensis*, by showing no toxicity to the larvae of another dipteran, *Aedes aegyptii*. It was interesting to determine whether sequences present on the 3.6Kb DNA fragment were common to all strains of *Bacillus sphaericus*, or unique to toxic strains. Using Southern

hybridization analysis, I was able to show that although chromosomal DNA from the toxic strains 1593, 2297 and 2013-4 hybridized to the 3.6Kb probe, DNA from the weakly toxic strain, SSII-1, and the non-toxic strain, 14577, did not hybridize. This indicated that the 3.6Kb DNA fragment was present in highly toxic strains only, and could be responsible for the level of toxicity exhibited by these strains. The observation that the strain SSII-1 is weakly toxic, but does not hybridize to the 3.6Kb DNA fragment indicated the presence of other genes involved in toxicity in *B.sphaericus* strains, such as the gene recently cloned by Thanabalu et al. (1991). Moreover, the second toxin gene cloned by Souza et al. (1988), and the unrelated gene encoding larvicidal activity cloned by Ganesan et al. (1983), confirms the presence of a number of genes that confer varying levels of toxicity on *B.sphaericus* strains. There is a precedent for such a situation with *Bacillus thuringiensis* strains, which often contain more than one type of toxin gene (Schnepf and Whiteley, 1985; Delecluse et al., 1988; Widner and Whiteley, 1989; Klier and Rapaport, 1987). In fact, Louis and Szulmajster (1985) showed that the larvicidal gene cloned from *B.sphaericus* 1593M (Ganesan et al., 1983), was present in a single copy in poorly toxic strains and in multiple copies in highly toxic strains. Thus, more than one mechanism could exist to define the level of toxicity in *B.sphaericus*. It is also evident that toxins present in the cell, as distinct from the crystal, are also active, since the strain SSII-1 possesses a larvicidal gene, but no crystal.

The nature and size of the toxin proteins encoded by the 3.6Kb DNA fragment of *B.sphaericus* at the outset of this study were unknown. Previous analyses of the plasmid pAS233HA in *E.coli* maxicells had failed to demonstrate any protein product encoded by this DNA fragment (Souza et al., 1988). As an alternative, I decided to use *E.coli* minicells for the identification of plasmid-encoded proteins. Expression studies in this system, combined with deletion analysis, led to the unequivocal identification of the genes encoding a 41 and a 59KDa protein, respectively. The deletion analysis also indicated that each gene was probably transcribed from its own promoter. The sequence of the genes encoding the 41 and 59KDa proteins, obtained subsequently, predicted the presence of

Table 8.1 Larvicidal toxins of *B.sphaericus*.

The table shows the toxins from five *B.sphaericus* strains identified as being involved in pathogenesis of mosquito larvae. Figure 3.2 shows the hybridization of the chromosomal DNA from larvicidal strains of *B.sphaericus* to the 3.6Kb insert originally cloned from *B.sphaericus* 1593M. The data of protein present in the crystal are compiled from Chapters 4 and 6. The data on the toxin in *B.sphaericus* SSII-1 are reported in Thanabalu et al., 1991.

Table 8.1 Larvicidal proteins of *B.sphaericus*.

Strain	Toxin (KDa)	DNA hybridization to 3.6Kb probe	Proteins in crystal
1593	41/59	Yes	125, 62, 59, 43, (41?)
2362	41/59	Yes	125, 62, 59, 43, (41?)
2297	41/59	Yes	62, 59, 43, (41?)
2013-4	41/59	Yes	125
SSII-1	100	No	no crystal

strongly hydrophobic regions likely to be involved in toxin action in target membranes (Hindley and Berry, 1987; Baumann et al., 1988; Figure 6.4B). Nevertheless, from the fractionation analysis carried out in this study, in which the 41 and 59KDa proteins were found in the soluble fractions, it appears that these hydrophobic regions are either insufficient to insert the protein non-specifically into *E.coli* membranes, or else are hidden within the proteins.

Ellar et al. (1985) have shown that the 27KDa, CytA, cytolytic toxin of *Bacillus thuringiensis* subspecies *israelensis*, requires unsaturated fatty acids containing dipolar ionic headgroups, such as phosphatidyl choline and phosphatidyl ethanolamine, for binding to eukaryotic membranes.

Since the toxins from *B.sphaericus* are specific for the larvae of mosquitoes, and do not affect recombinogenic *E.coli*, there could be a corresponding requirement for the presence of specific lipid groups or other receptors on the target membrane for the toxin proteins to be active. Alternatively, the polarity of the membrane potential may be the determining factor in whether a toxin opposed to the cytoplasmic face of the *E.coli* membrane will be active.

We required a rapid and sensitive assay to determine the role of the 41 and 59KDa proteins in larvicidal activity. This assay system could also be used subsequently in binding studies of the proteins to cell membranes, and to estimate the toxicity of various crystal components. I therefore attempted to set up an *in vitro* assay system using mosquito larval cells in culture. The 41 and 59KDa proteins were synthesized at very low levels in *E.coli*, and an efficient assay system would therefore require that these low concentrations should exert a visible cytopathic effect on cultured mosquito larval cells. In fact, the *E.coli* extracts used showed cytotoxicity towards these cultured cells, even in the absence of the toxins. By contrast, in my hands, both *Anopheles gambiae* and *Culex quinquefasciatus* cell tissue cultures did not show visible cytopathic effects (cell swelling and lysis), as described by Davidson et al. (1987), following the addition of solubilized

crystal isolated from *B.sphaericus* strains. However, I did not assay cell viability following addition of the crystal to the tissue cultured cells.

Hofte and Whiteley (1989) have cautioned that toxicity tests performed *in vitro* on cultured cell lines should be confirmed by larval bioassays, since cells in culture are largely derived from trypsinized larval tissue of uncertain origin. Sensitivity of tissue cultured cells to toxin also requires 100-fold higher concentrations than required for whole larvae. In addition, the specificity for cell lines derived from different insect species does not necessarily correlate with the *in vivo* host range of the crystal protein (Knowles and Ellar, 1986; Davidson, 1986). Finally, Davidson (1986) has observed that *C.quinquefasciatus* cells in culture showed variable levels of sensitivity to *B.sphaericus* spore-crystal extracts. For these reasons, and because of my inability to establish a rapid assay without the need for extensive purification of *E.coli* derived toxins, I decided to use larval bioassays to estimate the toxicity of recombinant *E.coli*.

Bioassays were carried out using second to third instar larvae of *C.quinquefasciatus*, and showed that both the 41 and 59KDa proteins were required to confer larvicidal activity on *E.coli*. Importantly, mixtures of *E.coli* strains expressing each toxin separately were toxic, whilst each strain alone was non-toxic. This result confirmed other results (Baumann *et al.*, 1987) that the 41 and the 59KDa proteins were together required for larvicidal activity. To date, *B.sphaericus* is the only entomopathogen shown to require two separate proteins for larvicidal activity.

Paralleling my own studies, de la Torre *et al.* (1989) reported that the 41 and 59KDa proteins are together required for toxicity when synthesized by *E.coli*, but that the 41KDa protein alone is sufficient for toxicity when synthesized by *Bacillus subtilis*. They suggested that the 59KDa protein may act as a "maturation" enzyme, activating the 41KDa protein into the toxic form in *E.coli*, but that this "maturation" step was performed by another enzyme in *B.subtilis*. These results were not supported by Broadwell *et al.* (1990a), who reported

that 41KDa protein synthesized in the non-toxic strain *B.sphaericus* strain 718, required the presence of the 59KDa protein for toxicity to mosquito larvae. This result contradicted their own earlier suggestion that the 41KDa protein synthesized in *B.sphaericus* was toxic by itself, but when synthesized in heterologous hosts, required the presence of the 59KDa protein in order to exhibit toxicity (Baumann *et al.*, 1988). Broadwell *et al.* (1990a) in fact suggested that the 59KDa protein acts as a "chaperone" to allow the 41KDa protein to fold into its active form. This model implies that, in the absence of the 59KDa protein, the 41KDa protein in *B.sphaericus* is improperly folded. However, this is inconsistent with the observation that the 59KDa protein can be added from a separate source to reconstitute larvicidal activity. This still leaves open the possibility that the 59KDa protein allows the 41KDa protein to refold, following denaturation in the larval gut. However, in view of the predicted membrane activity of both proteins, and indeed the substantial homology of the two proteins, it seems much more reasonable to suppose that both the 41 and 59KDa proteins are involved in pore formation, as has been shown for *B.thuringiensis* toxins. The availability of large quantities of the 59KDa protein, and prospects for overproducing the 41KDa protein for studies with gut epithelial membranes should help to answer this question.

### 8.3. Analysis of the 125KDa crystal protein of the toxic *B.sphaericus* strain 1593.

Baumann *et al.* (1985) had earlier concluded that the 41 and 59KDa proteins formed higher order oligomers to high molecular weight forms, since antibodies raised to the corresponding 43 and 63KDa proteins purified from crystals of *B.sphaericus* 2362 cross reacted with high molecular weight proteins present in the crystal. As a specific test of this hypothesis, antibodies were raised in this study to one of the high molecular weight forms in the crystal, a 125KDa protein, in order to determine possible cross-reaction with the larvicidal 41 and 59KDa proteins.

With this and related approaches, several lines of evidence were in fact obtained which demonstrated that the 41

and 59KDa proteins were completely unrelated to the major protein species found in association with the crystal, the 125KDa protein. There was no cross-reaction between the antibodies raised to the gel-purified 125KDa protein and the 41 and 59KDa proteins. A second preparation of antibodies, raised to total crystal proteins isolated on NaBr density gradients, gave the same result in both immunoprecipitation and Western immunoblot experiments. These results might also have been interpreted to mean that the 41 and 59KDa proteins were not present in the crystal, or were present as minor components, compared to the 125KDa polypeptide. An alternative explanation for these observations was that the 41 and 59KDa proteins, even if present in substantial amounts in the crystal, are not sufficiently antigenic to elicit a significant antibody response. This suggestion is supported by the observation that the 125KDa protein was highly immunogenic in rabbits, whereas the 59KDa protein was not (Chapter 4, Section 4.3; Chapter 6, Section 6.7).

The above studies left the identity of the 125KDa crystal protein as a complete mystery. Baumann *et al.* (1985) reported that this protein was toxic to mosquito larvae, and therefore it was a possibility that this protein was a second, high molecular weight larvicidal toxin in *B.sphaericus*, as often seen in *B.thuringiensis* species. However, the finding in this study that this protein was in fact the processed form of the Surface (S)-layer protein of *B.sphaericus* as deduced from its N-terminal sequence was not expected in relation to this hypothesis.

The S-layer protein of *B.sphaericus* 2362 is cleaved during translocation to the surface (Bowditch *et al.*, 1990) and the processed protein is predicted to have a molecular weight of 122KDa. Some other species like *Myxococcus xanthus* have S-layer proteins that are translocated to the cell surface in the absence of a cleavable signal sequence (Nelson and Zusman, 1983). S-layer proteins are present on the surface of both Gram positive and Gram negative cells (Sleyter and Messner, 1988), but the role of such proteins is not clear. Koval and Hynes (1991) suggested that the S-layer proteins have a protective role in Gram negative cells. For example, they apparently serve to protect these cells from infection by

*Bdellovibrio bacteriovorus* by masking the binding sites on the Gram negative bacterium for *B.bacteriovorus*.

Antigenically distinct S-layer proteins have been isolated from various strains of *B.sphaericus*, and they are involved in phage attachment in this organism (Word et al., 1983). S-layer proteins isolated from 21 different *B.sphaericus* strains were classified into 8 antigen groups (Lewis et al., 1987). Interestingly, non-toxic strains (14577; DNA homology groups I and III) show a regularly structured tetragonal or hexagonal pattern of arrangement of the S-layer. Surprisingly, all the toxic strains (DNA homology group IIA) show no such repeat assembly, their S-layers being non-regularly structured (Word et al., 1983; Lewis et al., 1987). Effects on the surface patterns of the S-layers are presumably due to differences in the sequence of the S-layer proteins present in different strains of *B.sphaericus*. Similarly, this would constitute the simplest explanation for the inability of the *B.sphaericus* 1593 anti-125KDa antibodies to recognize the S-layer protein of strain 14577 in Western immunoblots experiments (Figure 4.2). This variation in the S-layer protein is also consistent with its having larvicidal activity in the toxic strains, either as a genuine crystal component or independently.

Bowditch et al. (1990) used antibodies raised earlier to the 125KDa protein purified from the *B.sphaericus* 2362 crystal, to label whole cells of *B.sphaericus* containing crystals in immunogold assays. This experiment revealed that although the cell surface of *B.sphaericus* bound the antibodies, the crystal did not. Thus, Bowditch et al. (1990) concluded that the crystal of the *B.sphaericus* strain 2362 did not usually contain the 125KDa protein, and that the presence of this protein in the crystal constituted contamination. In contrast, crystals purified from several sources by independent groups (Baumann et al., 1985; F. Rajamohan, personal communication and work reported in this thesis) and by different methods (Chapter 2, Section 2.1.5.1 and 2.1.5.2) invariably contained substantial quantities of the 125KDa protein. Whether this is simply due to inadequate purification therefore appears questionable, since the crystal preparations were homogeneous when examined under a light

microscope. It has been shown that the crystals from strain 2297, in contrast to those of strains 1593, 2362 and 2013-4, have no associated 125KDa protein. In addition, the 125KDa protein isolated from the crystal was present in the processed form, i.e. it had lost its signal sequence and therefore must already have been translocated to the surface of the cell. Since the crystal in strain 1593 is contained within the cell together with the spore, the 125KDa protein must associate with the crystal following cell breakage. Indeed, Sleytr and Messner (1988) suggest that large amounts of the S-layer protein are deposited on the cell surface during growth, which are then redistributed following cell breakage. It is possible that the S-layer proteins stick very tightly to the crystal even under the conditions required for purification of the crystals, such as passages through NaBr or sucrose density gradients.

Bowditch *et al.* (1990) have recently examined the possible role of the 125KDa S-layer protein as a toxin, and have reported that the protein, although they suggest not part of the crystal, is a precursor of a 110KDa protein, which is toxic for the larvae of mosquitoes. Other reports have noted the entomocidal properties of disulfide-rich spore coat proteins (Somerville and Pockett, 1975). Such toxicity, however, may be due to a non-specific effect due to the formation of lysinoalanines, caused by disulphide bond cleavage (Nickerson, 1980). In fact, alkali-treated lysozyme, under conditions that generate lysinoalanines to 4.82% of the component amino acids, is toxic to tobacco hornworm larvae at 100µg/mL, whereas untreated lysozyme is not toxic, even at 50mg/mL (Hagesgawa *et al.*, 1981). The presence of S-layer proteins in the alkaline environment of the larval gut may serve to potentiate the toxicity of the *B.sphaericus* strain. Thus, S-layers may have an accessory role in the larvicidal activity of the cell. It would, however, be necessary to purify the 125KDa protein for use in bioassays in order to estimate its contribution to the toxicity of the cell.

The stoichiometry of the protein composition of the crystal is still not known. It does appear, however, that the crystal also contains the 41KDa protein (Baumann *et al.*, 1985) and the 59KDa protein (Chapter 6, Section 6.8), in addition to

the "contaminating" 125KDa protein. Again careful analysis of the structure and content of the strain 2297 crystal would be instructive here in view of its apparently simple composition.

#### 8.4. Analysis of the gene encoding the 41KDa protein and the attempted high level expression of this gene.

The 3.6Kb DNA fragment cloned from *B.sphaericus* 1593M was shown by minicell analysis to encode two proteins of 41 and 59KDa. Moreover, both these proteins were required for toxicity of recombinant *E.coli* for mosquito larvae. Antibodies raised to gel-purified 125KDa protein, once thought to be an oligomeric form of the 41 and 59KDa proteins in the crystal, did not recognize these proteins in Western immunoblots and immunoprecipitation assays. In order to study the role of the 41KDa protein in the toxicity process, and the expression of the protein in *B.sphaericus*, sufficient quantities of the 41KDa protein to use as antigen for the production of antibodies were required. This was initially attempted by the construction of translational fusions of the gene encoding the 41KDa protein to the 23KDa C-terminal signal region of haemolysin, in order to facilitate its subsequent purification from the medium.

Fusion of a part, or of all of the 41KDa protein to the 23KDa C-terminal signal region of haemolysin did not lead to the export of large quantities of the protein to the medium. In addition, some fusion molecules appeared to be cleaved prior to translocation. In the case of the full length fusion of the 41KDa protein to the haemolysin signal region, which was successfully secreted at low levels to the medium, Western immunoblots also demonstrated the presence of small amounts of the fusion protein within cells containing the export proteins HlyB and HlyD. Interestingly, this was not observed in cells lacking the export proteins, indicating that the fusion could be rescued from degradation, perhaps by interaction with the translocation machinery for haemolysin secretion. In both cases, the 41KDa fusion proteins were transcribed from poorly expressed *B.sphaericus* promoters, and this may have led to low levels of synthesis of the fusion proteins, and the consequent low levels of export observed.

Since secretion via the Hly system did not lead to a simple method for purification, an alternative procedure for the production of large amounts of the 41KDa protein in *E.coli* was examined. Surprisingly, in the case of the recombinant plasmid pJLA504-41, expressing the 41KDa protein from the heat-inducible  $\lambda$  P<sub>L</sub>, P<sub>R</sub> promoters, overexpression *in vivo* was not detected, although *in vitro*, using the coupled transcription-translation assay, the protein was expressed from the  $\lambda$  promoters both at 30°C and at 42°C. Moreover, biological activity of the 41KDa protein expressed for this construct *in vivo* was detected by bioassay. There were several possibilities to account for absence to visualize the 41KDa protein *in vivo*, such as that the protein was unstable *in vivo*, or that transcription or translation of the 41KDa mRNA was inefficient *in vivo* for some reason.

Whilst my experiments did not rule out the possibility that the 41KDa protein was labile *in vivo*, the Northern blotting experiments surprisingly demonstrated that mRNA transcript synthesis from the  $\lambda$  P<sub>R</sub>, P<sub>L</sub> promoters of the 41KDa protein gene was not enhanced after induction, suggesting some disturbances of the cI<sub>857</sub> gene of the construct. In fact a more detailed analysis of total RNA purified from induced cells containing the plasmid pJLA504-41 in addition indicated that there were three major mRNA species of 7.8Kb, 4.6Kb and 3.5Kb, that hybridized to the gene probe encoding the 41KDa toxin. My inference from these results was that the transcript of the gene encoding the 41KDa protein was not terminated efficiently, and this was confirmed when it was shown that probes specific for the vector hybridized to the two larger transcripts of 7.8 and 4.6Kb in cells containing the plasmid pJLA504-41.

Thus, transcription emanating from the  $\lambda$  promoters continued to read into the vector in the majority of transcripts. The secondary structure of the resultant mRNA molecules might preclude efficient initiation and completion of translation (Ganoza *et al.*, 1987). Thus, lack of high level expression *in vivo* of the 41KDa protein in these experiments could be ascribed to inefficient induction and termination of transcription. Other factors that could affect translation of protein, such as secondary structure of the

mRNA transcripts of the gene encoding the 41KDa protein *per se* cannot be entirely dismissed, but have not been investigated in this context.

#### 8.5. DNA sequence analysis of the gene encoding the 59KDa protein and the high level expression of this gene.

In my initial studies, the gene encoding the 59KDa protein was clearly shown to reside on the 3.6Kb DNA insert of the plasmid pAS233HA. Consequently, it was of interest to establish the relationship, if any, of this protein to the crystal. Purification of the protein was a first step to this, and again initially, the approach taken was to secrete the 59KDa protein, or a portion of it, fused to the 23KDa signal region of haemolysin. The failure to recover high levels of protein in this way could simply be due to the presence of an inefficient *B.sphaericus* promoter. The fusion protein, however, also appeared to be specifically cleaved within the cell, as observed with the 41KDa protein fused to the 23KDa signal sequence of haemolysin.

In the case of the 59KDa protein, high level secretion was subsequently re-examined using a construct expressing the protein from the T7 promoter. Secretion of this overexpressed protein into the medium as a fusion to the 23KDa C-terminal signal region of haemolysin was very inefficient. Western immunoblotting analysis indicated that the protein was being degraded within the cell either in the presence or the absence of the export functions. The 59KDa protein may also possess sequences that inhibit or retard secretion, as have been seen in the case of  $\beta$ -galactosidase either translocated via the *secA*, *secY* pathway (Lee *et al.*, 1989), or when fused to HlyA (B.Kenny, personal communication). Mapping these sequences could lead to a method of predicting proteins that may be suitable for future export by the haemolysin pathway.

Since the relatively simple procedure of exporting the 59KDa protein to the medium proved to be unsuitable, it was necessary to explore the use of high level promoters for conventional overproduction, and this necessitated the nucleotide sequence of the gene being determined. At the

outset of my studies, the DNA sequence of the 59KDa toxin gene was not known, and therefore this was desirable in its own right. Although this sequencing was obtained successfully and relatively speedily, the sequence of the corresponding gene was reported from the *B.sphaericus* strain 2362 (Bowditch *et al.*, 1988), followed by the sequence from the isolate examined here, 1593M (Arapinis *et al.*, 1988). In all cases, the amino acid sequences predicted a size of 51.4KDa. The DNA sequence determined in my analysis corresponded exactly to the coding region to the gene reported by Bowditch *et al.* (1988) and Arapinis *et al.* (1988). However, in contrast to both studies, I observed 3 nucleotide changes at positions 1890, 1891 and 1892, downstream of the coding sequence. In fact, the sequence I determined in these positions agreed with that reported for the 5' non-coding region of the gene encoding the 41.9KDa protein from *B.sphaericus* 1593, sequenced by Hindley and Berry (1987).

Examination of the nucleotide sequence immediately upstream of the coding sequence of the gene encoding the 59KDa protein revealed a -10 sequence 5'TATAAT3', for the  $\sigma 55$  ( $\sigma A$ ) subunit of *B.subtilis* RNA polymerase. A further 18 nucleotides upstream of this putative -10 sequence is a sequence 5'ATGAAC3' that resembles the canonical -35 sequence 5'TTGACA3' for the  $\sigma 55$  subunit of *B.subtilis* RNA polymerase (Ishihama, 1988). The -35 sequence was identical to the canonical sequence in 3 out of 6 nucleotides. Moreover, the spacing between the -10 and -35 sequences (18 nucleotides) is within the optimal range of 16-18 nucleotides. In addition, a possible Shine-Dalgarno sequence was identified 25 nucleotides downstream of the -10 sequence. Thus, these two -10 and -35 sequences may correspond to the promoter for the gene encoding the 59KDa protein.

In contrast to the promoters previously identified for crystal protein genes of *B.thuringiensis*, the promoter for the 59KDa protein encoding gene identified here has the characteristics of a vegetative promoter, when compared with other *Bacillus* promoters. In addition, a similar vegetative-like promoter was identified for the 41KDa protein gene from *B.sphaericus* 1593 (Hindley *et al.*, 1987) and the *mtx* larvicidal toxin from *B.sphaericus* SSII-1 (Thanabalu *et al.*,

1991). Thus, in contrast to *B.thuringiensis* toxin proteins, which are associated with crystals normally produced during the onset of sporulation of the cell (Wong et al., 1983; Ward and Ellar, 1986), the situation for the 41 and 59KDa toxins from *B.sphaericus* may be more complex. However, the use of antibodies showed that the 41KDa protein was synthesized in cells upon the onset of sporulation and accumulated in parallel with the maturation of the spore (de la Torre et al., 1989). In results presented in Chapter 7, the 59KDa protein was also apparently synthesized upon the onset of the sporulation phase in *B.sphaericus* 1593. Clearly, these results are not consistent with the presence of vegetative-phase translation of the toxin gene mRNA's. On the other hand, the mRNA may be transcribed in the vegetative phase, but may not be translated until the sporulation phase.

An alternative explanation may be that there is also an as yet unidentified sporulation-specific promoter for regulation of the expression of the toxin genes. Unfortunately, there is no strongly conserved consensus evident for sporulation promoters, and S1 nuclease protection assays will be required to identify promoter sequences. However, it is possible that the toxin encoding genes could involve promoters that functionally resemble the BtI and BtII promoters of the *cryIA* gene of *Bacillus thuringiensis* subspecies *kurstaki* (Wong et al., 1983), whose temporal control is, however, more complex. Thus, the gene for the 59KDa protein may be expressed in the vegetative phase, as non-translated mRNA. Translation then may occur later from this long-lived mRNA species, or from a second round of transcription during the sporulation phase, which would be consistent with the observed bimodal transcription of the 59KDa gene observed in Chapter 7, Section 7.5. This point will be discussed in detail subsequently in Section 8.6.

Other interesting features of the coding region of the 59KDa toxin encoding gene include the presence of several inverted repeat sequences, between nucleotides 2064-2087 and 2158-2183 nucleotides downstream of the coding sequence of the gene for the 59KDa protein. These were identified as lying within the coding sequence of the gene for the 41KDa protein. The inverted repeat sequence at 2158-2183 nucleotides is

followed by a stretch of T's that could serve as a rho-independent transcription terminator for the gene encoding the 59KDa protein (Figure 6.3).

From the sequence of the gene for the 59KDa protein, it was possible to design constructs in the expression vector pET3a, in which a translational fusion to the *s10* gene was expressed from the T7 RNA polymerase recognized  $\Phi 10$  promoter. In contrast to the results with the 41KDa protein, there was no difficulty in expressing the 59KDa protein from the plasmid pET3a-59SER at high levels.

The antibodies subsequently raised to the S10-59SER fusion protein were then available to carry out the experiment using Western immunoblots to investigate the possible presence of the 59KDa protein in purified crystals from *B.sphaericus* strains. The 59KDa protein was indeed detected in this way in purified crystals of *B.sphaericus* strains 1593, 2297 and 2362, but not in that of strain 2013-4. This strain also does not contain the 43 and 62KDa proteins identified earlier in the crystal of the other *B.sphaericus* strains 1593, 2297 and 2362 (Chapter 4, Section 4.2). The 59KDa protein, although readily detected by the use of antibodies, was not present in quantities detectable by Coomassie staining of the crystal preparation. This therefore demonstrated that the 59KDa protein exists in the crystal preparation in relatively small quantities as compared to, for example, the 125KDa protein. Baumann *et al.* (1985) demonstrated that the 41KDa protein was present in the crystal, although they did not study the stoichiometry. Interestingly, if it is assumed that indeed the 43KDa protein detected by Coomassie staining of the crystal proteins from *B.sphaericus* strains separated by SDS-PAGE (Chapter 4, Section 4.2) corresponds to the 41KDa protein, the results indicated that the 41KDa protein is present in larger amounts in the crystal than the 59KDa protein.

Larval bioassays, conducted using whole cells containing the plasmid pET3a-59SER expressing the S10-59SER fusion protein from the  $\Phi 10$  promoter, confirmed that the S10-59SER

protein by itself was incapable of causing the death of mosquito larvae. In contrast, when these cells were mixed with cells expressing the 41KDa gene under the control of a  $\lambda$  P<sub>R</sub>, P<sub>L</sub> promoter, toxicity was observed. The toxicity shown by this mixture of cells, independently containing the plasmids pET3a-59SER and pJLA504-41, was substantially higher than that observed when expressed from their *B.sphaericus* promoters in *E.coli* (Table 6.1) demonstrating that enhancement of biological activity due to overproduction of the 59KDa protein had been obtained.

The presence of 13 amino acids of the S10 protein at the N-terminus of the 59KDa protein, and the presence of a serine at the 2nd position in the sequence encoding the 59KDa protein in the S10-59SER fusion, rather than the native cysteine, did not abolish its toxicity. These findings were supported independently by the results of Clark and Baumann (1990), who have made deletions in the gene encoding the 59KDa protein. Thus, the absence of the first 21 amino acids at the N-terminus and 53 amino acids from the C-terminus resulted in the production of a protein that had the same mobility as a 44KDa degradation product of the 59KDa protein, identified in the guts of larvae fed the toxin (Broadwell et al., 1990b). This truncated 59KDa protein in fact retained toxicity to mosquito larvae in the presence of the 41KDa toxin. However, a deletion of 32 amino acids from the N-terminus coupled with the 53 amino acid C-terminal deletion led to loss of toxic activity of the 59KDa protein in the presence of the 41KDa protein (Clark and Baumann, 1990).

Davidson (1986) has shown that an alkaline extract of spore-crystals of *B.sphaericus* 1593 can cause cytotoxicity to susceptible *C.quinquefasciatus* cell lines. Davidson (1986) also reported cytotoxicity by the alkaline extracts, even in the absence of activation by larval gut proteases, although the activity of the extract was increased by such a treatment. Since both the 41 and 59KDa proteins are required for larvicidal activity, assuming the presence of the 41 and 59KDa proteins in these alkaline extracts of the spore-crystal

complex, Davidson's experiment indicates that the toxin(s) can in principle bind to the cell membrane of susceptible cells in the absence of prior processing with larval enzymes. The *in vivo* processing step may therefore be required to trim the protein to a protease-resistant core, rather than to expose an active site or cause other modifications to the toxin. It must be borne in mind, however, that toxins other than the 41 and 59KDa toxins present in the *B.sphaericus* crystal could have been responsible for the observed cytotoxic activity in Davidson's study. The availability of milligram amounts of the S10-59SER and S10-59CYS fusion proteins expressed from the  $\Phi$ 10 promoter, could now facilitate studies involving treatment of the protein with different proteases and chemical agents. This would then allow the monitoring of the binding of the protein to cell membranes in the presence of the 41KDa protein and other studies to directly analyze the possible channel forming properties of the 59KDa protein.

#### 8.6. Regulation of expression of the toxin encoding genes in *E.coli*, *B.subtilis* and *B.sphaericus*.

Fusions of promoters of various genes have been constructed to the *lacZ* gene in order to determine the pattern of regulation of the gene and its product *in vivo* (Chapman and Piggot, 1987; Vasil *et al.*, 1989; Zuber and Losick, 1983). Analysis of the expression of the  $\beta$ -galactosidase reporter, fused to the 41 and 59KDa proteins expressed under the control of their *B.sphaericus* promoters, was carried out in *B.subtilis* and *E.coli*. *B.sphaericus*, the host of choice for the regulation studies, was difficult to transform. *B.sphaericus* is reported to have a highly efficient restriction-modification system in the cell (Koncz *et al.*, 1978). The presence of recognition sites for *B.sphaericus* restriction enzymes in the plasmids could have led to the difficulties experienced in isolating transformants. I therefore decided to use *B.subtilis* to carry out the reporter gene assays. There were two reasons for choosing *B.subtilis*. Firstly, although transformation of *B.sphaericus* has been reported, transformation protocols for *B.subtilis* are much better established. Secondly, since *B.subtilis* and *B.sphaericus* belong to the same genus, genetic controls operating in *B.sphaericus* probably have allied patterns in *B.subtilis*.

Synthesis of the 41 and 59KDa proteins determined by  $\beta$ -galactosidase assays in *B.subtilis* demonstrated that production of the proteins was reduced with increasing age of the culture. It must be noted that the plasmids containing the *lacZ* gene fusions were present in multiple copies in these *B.subtilis* cells. From Southern hybridization analysis data of total DNA prepared from *B.sphaericus* strains, it appears that the toxin genes are probably present in a single copy in these strains (Chapter 3, Section 3.2). The introduction on multicopy plasmids of some genes, like *spoOF* and *spoVG*, which are usually present in a single copy, has been shown to interfere with normal control of cell events such as sporulation (Chapman and Piggot, 1987; Zuber and Losick, 1983). However, when the promoter of the crystal gene encoding a 130KDa toxin of *B.thuringiensis* subspecies *kurstaki* HD-1-Dipel was present on a multicopy plasmid in *B.subtilis*, it was still expressed under sporulation control. Interestingly, the onset of sporulation of the cell was considerably delayed, compared to identical strains lacking the crystal promoter (Whiteley et al., 1986). In contrast, the presence of a *cry* gene encoding a 135KDa product from *B.thuringiensis* subspecies *kurstaki* on the multicopy plasmid pBD64 led to the expression of the Cry protein in the vegetative phase of growth in *B.subtilis* (Shivakumar et al., 1986). The effect of single copy promoters, and the timing of the onset of sporulation in these cases was not determined. High copy number effects are nevertheless better avoided, and such experiments should be repeated with the *lacZ* fusion targeted, so as to cause an homologous replacement of the resident gene in *B.sphaericus* in a single copy. The value of the use of such a heterologous host, even one as closely related as *B.subtilis*, for such regulation studies must however be questioned, and is no substitute for analysis of crystal regulation in the original host.

Baumann et al. (1987) suggested that the genes encoding the 41KDa and 59KDa proteins are transcribed as an operon. Subsequently, Baumann and Baumann (1989) subcloned a 1.9Kb *EcoRI-HindIII* fragment containing the gene encoding the 41KDa protein into *B.subtilis*, and probed the cells with antibodies raised to the 41KDa protein. No expression of the 41KDa

protein was seen in *B.subtilis*. The authors concluded on this basis, that the 41KDa protein did not contain a functional promoter. However, this interpretation may be too simplistic. Sequences upstream of the crystal genes in *B.thuringiensis* have been shown to negatively regulate transcription of the genes (Thorne *et al.*, 1986; Schnepf *et al.*, 1987). Hindley and Berry (1987) have shown that these regions (-177 to -235 relative to the translation start site of the 41KDa protein) are present in *B.sphaericus*. These negative regulatory regions may have contributed to Baumann and Baumann's inability to detect the 41KDa protein. In this study with minicell analysis of deletion derivatives of pAS233HA, incorporating the same region 5' to the 41KDa protein gene as Baumann and Baumann (1989), and with the  $\beta$ -galactosidase fusion, I have, in fact, demonstrated that there was a promoter within this region that supported transcription of the 41KDa protein encoding gene. My suggestion for Baumann and Baumann's failure to detect expression of the 41KDa toxin with the antibodies is that their test was not sensitive enough.

The analysis of toxin mRNA synthesis during the growth phase in *B.sphaericus* indicated that the mRNA transcripts for the genes encoding the 41 and 59KDa proteins were both synthesized in a bimodal manner. The levels of rRNA in the vegetative and sporulating *B.sphaericus* cell were assumed to be equal for this experiment. However, it has been shown that although the nature and sequences of the rRNA in the vegetative and sporulating cells of *B.subtilis* were the same (Edgell *et al.*, 1975), sporulating *B.subtilis* cells contain approximately only 25% of the rRNA present in vegetative cells (Pero *et al.*, 1975). This experiment, therefore initially overestimates the amount of gene specific transcripts of the toxin proteins in the sporulating cell. Thus, adjusted for the lower rRNA levels in sporulating cells, the amount of gene-specific transcript would be 25% of the value observed. This still indicates the presence of a sporulation-associated promoter whose transcription leads to the accumulation of the toxin gene mRNA. This result is substantially different from results obtained with the *cryIA* gene of *B.thuringiensis* subspecies *kurstaki*, and the *cytA* gene of *B.thuringiensis* subspecies *israelensis*, which indicate that regulation of

expression occurs primarily at the transcription level (Wong *et al.*, 1983; Ward and Ellar, 1986). In *B.sphaericus*, regulation appears to occur at both the transcriptional and at the translational level. The results obtained indicated that the mRNA transcripts for the 41 and 59KDa genes were both synthesized in a bimodal manner. Synthesis of the 41 and 59KDa transcripts peaked at 4 hours following the start of the exponential phase of growth. This high level expression was then reduced during late exponential growth (6 hours after the start of exponential growth;  $t=0$  of sporulation). It is unlikely that this early RNA could be carried over from the spores used as inoculum because prokaryotic mRNA is normally relatively short-lived (Goodenough, 1978). If, indeed, the mRNA was an effect of carry over from the spore inoculum, the results should show high levels of RNA at the start of the assay, with a reduction in levels due to turnover as growth continued. This is not the case. A second, weaker, round of transcription started at 7 hours after the start of exponential growth ( $t=1$  of sporulation) and continued to increase to  $t=5$  of sporulation, which was the end of the assay. These results, therefore, probably mirror events occurring in the cell during the transition from vegetative to sporulation growth.

The earlier peak of expression of the mRNA could correlate with the activity of vegetative promoters for the genes. This mRNA does not appear to be translated at least directly into the 59KDa protein, in view of my inability to detect the 59KDa protein in the vegetative cell using antibodies. The translated product, on the other hand, may correspond to the 67KDa species in vegetative cells that reacts strongly with antibodies to the S10-59SER protein. The identity of the 67KDa protein could be established by immunoprecipitation, followed by Cleaveland analysis to determine whether it is indeed a posttranslationally modified form of the 59KDa species, present predominantly in vegetative cells, and performing a physiological role.

The second round of transcription is the more interesting. Presumably, the 59KDa protein synthesized at this stage would correspond to that accumulating in association with the crystal, and this was apparently confirmed by the Western

immunoblot analysis of whole *B.sphaericus* cells expressing these proteins, and primary increase in the larvicidal activity of the cell (Chapter 7, Section 7.4; de la Torre et al., 1989; Kalfon et al., 1984). No sporulation-associated promoters have yet been identified in the case of either of the *B.sphaericus* species toxin genes. The localization of such promoters if they exist would require detailed S1 nuclease mapping and footprinting experiments during the second phase of mRNA synthesis observed in my experiments.

As indicated above, the accumulation of the 59KDa protein in strain 1593 appeared to start at a late stage of growth, approximately 8 hours after the start of the exponential phase of growth; t=2 of sporulation, and about 1 hour after the onset of the second phase of transcription of the toxin gene mRNA. The amount of the 59KDa protein increased, per A<sub>600</sub> Unit of cells, with increasing age of the culture. This observation correlates well with the increase in the amount of mRNA present in the cell during sporulation phase.

#### 8.7. Future work.

*Bacillus sphaericus* has been shown to exhibit considerable potential for application to integrated programmes for the control of dipteran vectors of human disease. Work carried out so far has helped to identify several genes responsible for the toxic activity of the organism (Ganesan et al., 1983; Hindley and Berry, 1987; Baumann et al., 1987; Souza et al., 1988; Thanabalu et al., 1991).

Although preliminary work has served to establish the nature of the toxic proteins, very little is known about the nature and mechanism of toxicity. Work by Souza et al. (1988), Ganesan et al. (1983) and Thanabalu et al. (1991) has shown that more than one toxin exists in *B.sphaericus* 1593. Thus the physiological data obtained so far using whole cells or purified crystals of *B.sphaericus* would have to be assumed to be caused by more than one toxin. Thus, cloned genes could serve a very important role in establishing the part played by each larvicidal toxin in pathogenesis. The 59KDa component of the bipartite 41/59KDa toxin has been overproduced in large

quantities in this study. The S10-59SER protein has been shown to be active in bioassays, or the S10-59CYS fusion protein, could be used in tissue culture studies, with and without proteolytic activation to determine whether they bind to cells *in vitro*. Further work could focus on the overproduction, in a similar manner, of the 41KDa component, using a suitable vector. The purified proteins would be invaluable in providing an insight into *in vitro* studies on the mechanism of toxin action.

Elegant deletion, fusion and site-directed mutagenesis experiments on *B.thuringiensis* cry genes by several workers (Haider and Ellar, 1989; Raymond *et al.*, 1990; Widner and Whiteley, 1990) have helped to map the region of the *B.thuringiensis* protein involved in toxic activity and to identify the epitope determining specificity. Experiments causing single amino acid changes in the sequence of the 27KDa CytA cytolytic and haemolytic toxin of *B.thuringiensis* subspecies *israelensis*, have led to the prediction of a secondary structure model for the toxin (Ward *et al.*, 1988), although this model has yet to be vindicated by X-ray crystallography. Such methods have been applied to *B.sphaericus* toxins to a small extent by Clark and Baumann (1990) and by Broadwell *et al.* (1990b), who created N- and C-terminal deletions of the 41 and 59KDa proteins in order to identify the regions involved in toxicity and specificity. Site-directed mutagenesis based on predictions to disrupt membrane binding domains, or other secondary structures probably involved in toxin binding or activity could yield considerable information in further studies.

The *B.thuringiensis* toxins have been shown to bind a specific receptor on the membrane of target cells (Knowles and Ellar, 1986; Haider and Ellar, 1987b). The *B.sphaericus* toxin has been predicted to bind to a similar glycosylated receptor on mosquito larval gut cells (Davidson *et al.*, 1987). Similar biochemical techniques could help to define the receptors in the case of mosquito larvae for the *B.sphaericus* toxins.

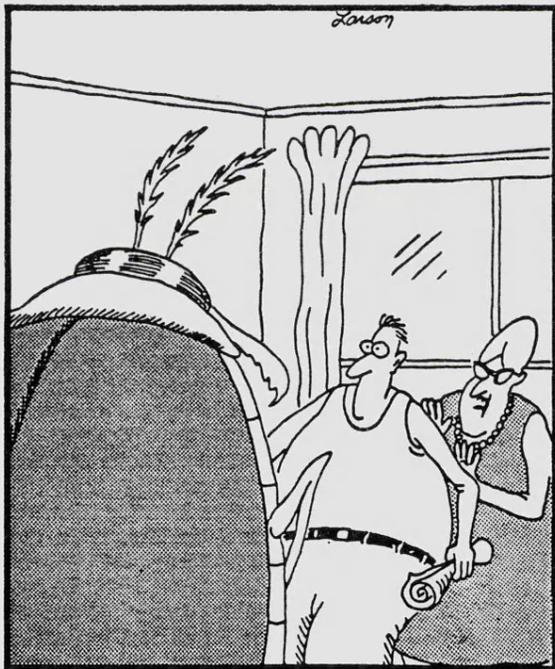
Further analysis of the genetic controls involved in the expression of the 41 and 59KDa toxins in *B.sphaericus* is required to understand the factors involved in their control. Brown and Whiteley (1988, 1990) have succeeded in identifying novel  $\sigma$  factors in *B.thuringiensis* involved in the control of transcription of *cry* genes. Such controls may also be operational in the case of the genes encoding the 41 and 59KDa proteins in *B.sphaericus*, which appear to be transcribed at two stages of cell growth, but are perhaps only translated during the sporulation stage.

It is also feasible that the 67KDa species is a posttranslationally modified form of the 59KDa protein translated during the vegetative phase of cell growth. In this context, it would be necessary to determine whether it is the mRNA synthesized in the vegetative phase is also translated, or whether the sporulation phase mRNA is preferentially translated. The half-life of the vegetative phase mRNA could be determined to establish whether it is a long- or short-lived species. The relative levels of toxin gene-specific mRNA in the cell could be more accurately established by solution hybridization. In addition, pulse chase analysis and Cleaveland digestion analysis could establish whether the 67KDa species is a posttranslationally modified form of the 59KDa protein.

The haemolysin signal sequence provides a very useful tool for the export of heterologous proteins from *E.coli* (Holland et al., 1990). The limitation of this technique is the unpredictability of the nature of proteins that can be exported efficiently, and those that cannot. The 59KDa protein of *B.sphaericus* provides an example of the latter. The portion of the molecule that interferes with efficient export is unclear. Mapping for sequences that limit export, for example, the presence of cysteine bonds that presumably interfere with any unfolding of the protein required for passage through the HlyB/HlyD pore, could lead to a method of

predicting proteins amenable for transport to the medium by this pathway.

Finally, although the picture is now considerably clearer, the composition of the *B.sphaericus* crystal and in particular, the stoichiometry of the proteins in the crystal have still to be elucidated. The specific activity of the crystal components and that of cellular toxins have also to be established. In conclusion, channel forming studies, such as have been carried out with *B.thuringiensis* toxins would help to determine whether the *B.sphaericus* toxins act in a similar manner.



"Step on it, Arnold! Step on it!"

## References.

- Adams, L. F., Brown, K. L. and Whiteley, H. R. (1991) Molecular cloning and characterization of two genes encoding sigma factors that direct transcription from a *Bacillus thuringiensis* crystal protein gene promoter. *J. Bacteriol.*, 173 (12), 3846-3854
- Adams, L. F., Visick, J. E. and Whiteley, H. R. (1989) A 20-kilodalton protein is required for efficient production of the *Bacillus thuringiensis* subsp. *israelensis* 27-kilodalton crystal protein in *Escherichia coli*. *J. Bacteriol.*, 171(1), 521-530
- Adang, M. J., Idler, K. F. and Rochleau, T. A. (1987) Structural and antigenic relationships among the insecticidal crystal proteins of *Bacillus thuringiensis* subsp. *kurstaki*. In: *Biotechnology in invertebrate pathology and cell culture* (K. Maramorosch, ed.). Academic Press Ltd., San Diego, California. pp. 85-99
- Adler, H. I., Fischer, W. D., Cohen, A. and Hardigree, A. A. (1967) Miniature *Escherichia coli* cells deficient in DNA. *Proc. Natl. Acad. Sci. USA*, 57, 321-326
- Andrews Jr., R. E., Bechtel, D. B., Campbell, B. S., Davidson, L. I. and Bulla Jr., L. A. (1981) Solubility of parasporal crystal and presence of toxin protein during sporulation, germination and outgrowth. In *Spores VIII* (Levinson, H.S., Sonenshein, A. L. and Tipper, D. J., eds.) American Society for Microbiology, Washington D. C. pp. 174-177
- Arapinis, C., de la Torre, F. and Szulmajster, J. (1988) Nucleotide and deduced amino acid sequence of the *Bacillus sphaericus* 1593M gene encoding a 51.4kD polypeptide which acts synergistically with the 42kD protein for expression of the larvicidal toxin. *Nucl. Acids Res.*, 16 (15), 7731
- Aronson, A. I., Beckman, W. and Dunn, P. (1986) *Bacillus thuringiensis* and related insect pathogens. *Microbiol. Rev.*, 50 (1), 1-24

Aronson *et al.* (1982) Relationship of the syntheses of spore coat protein and parasporal crystal protein in *Bacillus thuringiensis*. *J. Bacteriol.*, 151 (1), 399-410

Baumann, L. and Baumann, P. (1989) Expression in *Bacillus subtilis* of the 51- and 42-kilodalton mosquitocidal toxin genes of *Bacillus sphaericus*. *Appl. Environ. Microbiol.*, 55 (1), 252-253

Baumann, P., Baumann, L., Bowditch, R. D. and Broadwell, A. H. (1987) Cloning of the gene for the larvicidal toxin of *Bacillus sphaericus* 2362: evidence for a family of related sequences. *J. Bacteriol.*, 169 (9), 4061-4067

Baumann, L., Broadwell, A. H. and Baumann, P. (1988) Sequence analysis of the mosquitocidal toxin genes encoding 51.4- and 41.9-kilodalton proteins from *Bacillus sphaericus* 2362 and 2297. *J. Bacteriol.*, 170 (5), 2045-2050

Baumann, P., Unterman, B. M., Baumann, L., Broadwell., A. H., Abbene, S. J. and Bowditch, R. D. (1985) Purification of the larvicidal toxin of *Bacillus sphaericus* and evidence for the presence of high-molecular weight precursors. *J. Bacteriol.*, 163 (2), 738-747

Benoit, T. G., Wilson, G. R., Bull, D. L. and Aronson, A. I. (1990) Plasmid-associated sensitivity of *Bacillus thuringiensis* to UV light. *Appl. Environ. Microbiol.*, 56 (8), 2282-2286

Berry, C. and Hindley, J. (1987) *Bacillus sphaericus* strain 2362: identification and nucleotide sequence of the 41.9KDa toxin gene. *Nucl. Acids Res.*, 15 (14), 5891

Bialy, H. (1987) Recombinant proteins: virtual authenticity. *Biotechnology*, 5 (9), 883-890

Birnboim H. C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acids Res.*, 7, 1513-1523.

Bolivar, F., Rodreiguez, R. L., Greene, P. J., Betlach, M. C.,

Heynecker, H. L., Boyer, H. W., Crosa, J. H. and Falkow, S. (1977) Construction and characterization of new cloning vehicles, II. A multipurpose cloning system. *Gene*, 2, 95-113

Bone, E. J. and Ellar, D. J. (1989) Transformation of *Bacillus thuringiensis* by electroporation. *FEMS Microbiol. Lett.*, 58, 171-178

Bott, K. F., Wilson, F. E. and Stewart, G. C. (1981) In : Sporulation and Germination (Levinson, H. C., Sonenshein, A. L. and Tipper, D. J., eds.), American Society for Microbiology, Washington D. C. pp. 119-122

Bourgouin, C., Delecluse, A., Ribier, J., Klier, A. and Rapaport, G. (1988) A *Bacillus thuringiensis* subsp. *israelensis* gene encoding a 125-kilodalton larvicidal polypeptide is associated with inverted repeat sequences. *J. Bacteriol.*, 170 (8), 3575-3583

Bourgouin, C., Tinelli, R., Bouvet, J-P. and Pires, R. (1984) *Bacillus sphaericus* 1593-4: Purification of fractions toxic for mosquito larvae. In: Bacterial Protein Toxins (Alouf, J., Fehrenbach, F. J., Freer, J. H. and Jeljaszewicz, J., eds.), Academic Press, London, New York. pp. 387-388

Bowditch, R.D., Baumann, P. and Yousten, A. A. (1990) Cloning and sequencing of the gene encoding a 125-kilodalton surface-layer protein from *Bacillus sphaericus* 2362 and of a related cryptic gene. *J. Bacteriol.*, 171 (8), 4178-4188

Brizzard, B. L. and Whiteley, H. R. (1988) Nucleotide sequence of an additional crystal protein gene cloned from *Bacillus thuringiensis* subsp. *thuringiensis*. *Nucl. Acids Res.*, 16, 4168-4169

Broadwell, A. H., Baumann, L. and Baumann, P. (1990a) The 42- and 51-kilodalton mosquitocidal proteins of *Bacillus sphaericus* 2362: construction of recombinants with enhanced expression and in vivo studies of processing and toxicity. *J. Bacteriol.*, 172 (5), 2217-2223

Broadwell, A. H. and Baumann, P. (1986) Sporulation-

associated activation of *Bacillus sphaericus* larvicide. Appl. Environ. Microbiol., 52, 758-764

Broadwell, A. H., Clark, M. A., Baumann, L. and Baumann, P. (1990b) Construction by site-directed mutagenesis of a 39-kilodalton mosquitocidal protein similar to the larva-processed toxin of *Bacillus sphaericus* 2362. J. Bacteriol., 172 (7), 4032-4036

Broome-Smith, J. and Spratt, B. G. (1986) A vector for the construction of translational fusions to TEM  $\beta$ -lactamase and the analysis of protein export signals and membrane protein topology. Gene, 49, 341-349

Brown, K. L. and Whiteley, H. R. (1988) Isolation of a *Bacillus thuringiensis* RNA polymerase capable of transcribing crystal protein genes. Proc. Natl. Acad. Sci. USA, 85, 4166-4170

Brown, K. L. and Whiteley, H. R. (1990) Isolation of a second *Bacillus thuringiensis* RNA polymerase that transcribes from a crystal protein gene promoter. J. Bacteriol., 172 (12), 6682-6688

Bucher, G. E. (1960) Potential bacterial pathogens of insects and their characteristics. J. Invertebr. Pathol., 2, 172-195

Buluwela, L., Forster, A., Boehm, T. and Rabbitts, T. H. (1989) A rapid procedure for colony screening using nylon filters. Nucl. Acids Res., 17 (1), 452

Caullery, M. (1965) Science in the Nineteenth Century. In: A general history of the sciences (Taton, R., ed.), Thames and Hudson Press, London. pp. 390-396

Chak, K-F. and Ellar, D. J. (1987) Cloning and expression in *Escherichia coli* of an insecticidal crystal protein gene from *Bacillus thuringiensis* var. *aizawai* HD-133. J. Gen. Microbiol., 133, 2921-2931

Chapman, J. W. and Piggot, P. J. (1987) Analysis of the inhibition of sporulation of *Bacillus subtilis* caused by

increasing the number of copies of the spoOF gene. J. Gen. Microbiol., 133, 2079-2088

Chestukhina, G. G., Kostina, L. I., Zalunin, A., Khodova, O. M. and Stepanov, V. M. (1988) *Bacillus thuringiensis* ssp. galleriae simultaneously produces two  $\delta$ -endotoxins differing strongly in primary structure and entomocidal activity. FEBS Lett., 232 (1), 249-251

Chilcott, C. N. and Ellar, D. J. (1988) Comparative toxicity of *Bacillus thuringiensis* var. *israelensis* crystal proteins *in vivo* and *in vitro*. J. Gen. Microbiol., 134, 2551-2558

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem., 162, 156-159

Chunjatupornchai, W., Hofte, H., Seurinck, J., Angouthanasombat, C. and Vaeck, M. (1988) Common features of *Bacillus thuringiensis* toxins specific for Diptera and Lepidoptera. Eur. J. Biochem., 173, 9-16

Clark, M. A. and Baumann, P. (1990) Deletion analysis of the 51-kilodalton protein of the *Bacillus sphaericus* 2362 binary mosquito-cidal toxin. Construction of derivatives equivalent to the larva-processed toxin. J. Bacteriol., 172 (12), 6759-6763

Dalhammar, G. and Steiner, H. (1983) Characterization of inhibitor A, a protease from *Bacillus thuringiensis* which degrades attacins and cecropins, two classes of anti-bacterial proteins in insects. Eur. J. Biochem., 139, 247-252

Davidson, E. W. (1983) Alkaline extraction of toxin from spores of the mosquito-pathogen, *Bacillus sphaericus* strain 1593. Can. J. Microbiol., 29, 271-275

Davidson, E. W. (1981) Bacterial diseases of insects caused by toxin-producing Bacilli other than *Bacillus thuringiensis*. In: Pathogenesis of invertebrate microbial diseases (Davidson, E. W., ed.), Allanheld, Osmun (Totowa, N. J.). pp. 269-291

Davidson, E. W., Bieber, A. L., Meyer, M. and Shellabarger, C. (1987) Enzymatic activation of the *Bacillus sphaericus* mosquito larvicidal toxin. *J. Invertebr. Pathol.*, 50, 40-44

Davidson, E. W. (1986) Effects of *Bacillus sphaericus* 1593 and 2362 spore/crystal toxin on cultured mosquito cells. *J. Invertebr. Pathol.*, 47, 21-31

Davidson, E. W. and Myers, P. (1981) Parasporal inclusions in *Bacillus sphaericus*. *FEMS Microbiol. Lett.*, 10, 261-265

Davidson, E. W. (1982) Purification and properties of soluble cytoplasmic toxin from the mosquito pathogen *Bacillus sphaericus* strain 1593. *J. Invertebr. Pathol.*, 39, 6-9

Davidson, E. W., Shellabarger, C., Meyer, M and Bieber, A. L. (1987) Binding of the *Bacillus sphaericus* mosquito larvicidal toxin to cultured insect cells. *Can. J. Microbiol.*, 33, 982-989

de Barjac, H., Larget-Thierry, I., Cosmao-Dumanoir, V. and Ripoteau, H. (1985) Serological classification of *Bacillus sphaericus* strains on the basis of toxicity to mosquito larvae. *Appl. Microbiol. Biotechnol.*, 21, 85-90

de Barjac, H., Thierry, I., Cosmao-Dumanoir, V., Franchon, E., Laurent, P., Charles, J-F., Hamon, S. and Ofori, J. (1988) Another *Bacillus sphaericus* serotype harbouring strains very toxic to mosquito larvae: serotype H6. *Ann. Inst. Pasteur/Microbiol.*, 139, 363-377

Delannay, X. B. J., Proksch, R. K., Fuchs, R. L., Sims, S. R., Greenplate, J. T., Marrone, P. G., Dodson, R. B., Augustine, J. J., Layton, J. G. and Fischhoff, D. A. (1989) Field performance of transgenic tomato plants expressing the *Bacillus thuringiensis* var. *kurstaki* insect control protein. *Biotechnology*, 7, 1265-1269

de la Torre, F., Bennardo, T., Sebo, P. and Szulmajster, J. (1989) On the respective roles of the two proteins encoded by the *Bacillus sphaericus* 1593M toxin genes expressed in *Escherichia coli* and *Bacillus subtilis*. *Biochem. Biophys.*

Res. Commun., 164 (3), 1417-1422

Delecluse, A., Bourgouin, C., Klier, A. and Rapoport, G. (1988) Specificity of action of *Bacillus thuringiensis israelensis* toxins encoded by two different genes. Mol. Gen. Genet., 214, 42-47

Delecluse, A., Charles, J-F., Klier, A. and Rapoport, G. (1991) Deletion by *in vivo* recombination shws that the 28-Kilodalton cytolytic polypeptide from *Bacillus thuringiensis* subsp. *israelensis* is not essential for mosquitocidal activity. J. Bacteriol., 173 (11), 3374-3381

de Marsac, N. T., de la Torre, F. and Szulamjster, J. (1987) Expression of the larvicidal gene of *Bacillus sphaericus* 1593M in the cyanobacterium *Anacystis niudlans* R2. Mol. Gen. Genet., 209, 396-398

Dimarcq, J-L., Zachary, D., Hoffmann, J. A., Hoffmann, D. and Reichhart, J-M. (1990) Insect immunity: expression of the two major inducible antibacterial peptides, defensin and dipteracin in *Phormia terranova*. EMBO J., 9 (8), 2507-2515

Donovan, W. P., Dancosik, C. and Gilbert, M. P. (1988) Molecular characterization of a gene encoding a 72-kilodalton mosquito-toxic crystal protein from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol., 170 (10), 4732-4738

Dougan, G. and Sherrat, D. (1977) The transposon Tn1 as a probe for studying ColE1 structure and function. Mol. Gen. Genet., 151, 151-160

Dower, W. J., Miller, J. F. and Ragsdale, C. W. (1988) High efficiency transformation of *E.coli* by high voltage electroporation. Nucl. Acids Res., 16 (13), 6127-6145

Drobniewski, F. A. and Ellar, D. J. (1988) Investigation of the membrane lesion induced *in vitro* by mosquitocidal  $\delta$ -endotoxins of *Bacillus thuringiensis*. Curr. Microbiol., 16, 195-199

Drobniewski, F. A. and Ellar, D. J. (1989) Purification and

properties of a 28-Kilodalton haemolytic and mosquitocidal protein toxin of *Bacillus thuringiensis* subsp. *darmstadiensis* 73-E10-2. *J. Bacteriol.*, 171 (4), 3060-3067

Dubois, R. J. (1951) Louis Pasteur, free-lance of science. Victor Gollancz Ltd., London. pp. 209-2332

Edgell, M. H., Hutchinson III, C. A. and Bott, K. F. (1975) Are the same cistrons used to code for ribosomal ribonucleic acid in vegetative and sporulating cells of *Bacillus subtilis*? In: Spores VI (Gerhardt, P., Costilow, R. N. and Sadoff, H. L., eds.), American Society for Microbiology, Washington D. C. pp 195-201

Ellar, D. J., Thomas, W. E., Knowles, B., Ward, S., Todd, J., Drobniowski, F., Lewis, J., Sawyer, T., Last, D. and Nichols, C. (1985) Biochemistry, genetics and mode of action of *Bacillus thuringiensis*  $\delta$ -endotoxins. In: Molecular biology of microbial differentiation (Hoch, J. A. and Setlow, P., eds.), American Society for Microbiology, Washington D. C. pp. 230-240

English, L. H. and Cantley, L. C. (1986) Delta endotoxin as a potent inhibitor of the (Na, K)-ATPase. *J. Biol. Chem.*, 261, 1170-1173

Feinberg, A. P. and Vogelstein, B. (1984) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.*, 137, 266-267

Flyg, C. and Xanthopoulos, K. G. (1983) Insect pathogenic properties of *Serratia marcescens*. Passive and active resistance to insect immunity studied with protease deficient and phage resistant mutants. *J. Gen. Microbiol.*, 129, 453-464

Galjart, N., Sivasubramaniam, N. and Federici, B. A. (1987) Plasmid location, cloning and sequence analysis of the gene encoding a 27.3-kilodalton cytolytic protein from *Bacillus thuringiensis* subsp. *morrisoni* (PG-14). *Curr. Microbiol.*, 16, 171-177

Ganesan, S., Kamdar, H., Jayaraman, K. and Szulmajster, J.

(1983) Cloning and expression in *Escherichia coli* of a DNA fragment from *Bacillus sphaericus* coding for biocidal activity against mosquito larvae. *Mol. Gen. Genet.*, 189, 181-183

Ganoza, M. C., Kafoid, E. C., Marliere, P. and Louis, B. G. (1987) Potential secondary structure at translation initiation sites. *Nucl. Acids Res.*, 15 (1), 345-360

Garduno, F., Thorne, L., Walfield, A. M. and Pollock, T. J. (1988) Structural relatedness between mosquitocidal endotoxins of *Bacillus thuringiensis* subsp. *israelensis*. *Appl. Environ. Microbiol.*, 54 (1), 277-279

Gonzalez, J. M., Brown, B. S. and Carlton, B. C. (1982) Transfer of *Bacillus thuringiensis* plasmids coding for  $\delta$ -endotoxin among strains of *B.thuringiensis* and *B.cereus*. *Proc. Natl. Acad. Sci. USA*, 79, 6951-6955

Goodenough, U. (1978) In: *Genetics*, Second edition. Holt, Rinehart and Winston, New York. pp 290

Gottesman, S. (1989) Genetics of proteolysis in *Escherichia coli*. *Annual Rev. Genet.*, 23, 163-198

Gotz, P., Enderlein, G. and Roettgen, I. (1987) Immune reactions of *Chironimus* larvae (Insecta:Diptera) against bacteria. *J. Insect Physiol.*, 33 (12), 993-1004

Green, B. D., Battisti, L. and Thorne, C. B. (1989) Involvement of Tn4430 in transfer of *Bacillus anthracis* plasmids mediated by *Bacillus thuringiensis* plasmid pX012. *J. Bacteriol.*, 171 (1), 104-113

Griego, V. M. and Spence, K. D. (1978) Inactivation of *Bacillus thuringiensis* spores by ultraviolet and visible light. *Appl. Environ. Microbiol.*, 35, 906-910

Haider, M. Z. and Ellar, D. J. (1987a) Characterization of the toxicity and cytopathic specificity of a cloned *Bacillus thuringiensis* crystal protein using insect cell culture. *Molec. Microbiol.*, 1, 59-66

- Haider, M. Z. and Ellar, D. J. (1987b) Analysis of the molecular basis of insecticidal specificity of *Bacillus thuringiensis* crystal  $\delta$ -endotoxin. *Biochem. J.*, 248, 197-201
- Haider, M. Z. and Ellar, D. J. (1989) Functional mapping of an entomocidal  $\delta$ -endotoxin. Single amino acid changes produced by site-directed mutagenesis influence toxicity and specificity of the protein. *J. Molec. Biol.*, 208, 183-194
- Hagesgawa, K., Okamoto, N., Ozawa, H., Kitajima, S. and Takado, Y. (1981) Limits and sites of lysinoalanine formation in lysozyme, Alpha-lactalbumin and Alpha<sub>S1</sub>- and Beta-caseins by alkali treatment. *Agric. Biol. Chem.*, 45, 1645-1651
- Hanahan, D. (1985) Techniques for transformation of *E.coli*. In: DNA Cloning Volume I (Glover, D. M., ed.), IPL Press, Oxford. pp. 109-135
- Hardy, K. G. (1985) Bacillus cloning methods. In: DNA cloning Volume II (Glover, D. M., ed.), IRL press Ltd., Oxford. pp 1-17
- Hargrove, J. L. and Schmidt, F. H. (1989) The role of mRNA and protein stability in gene expression. *The FASEB J.*, 3, 2360-2370
- Herrnstadt, C., Gilroy, T. E., Sobieski, D. A., Bennett, B. D. and Gaertner, F. H. (1987) Nucleotide sequence and deduced amino acid sequence of a coleopteran-active delta-endotoxin gene from *Bacillus thuringiensis* subsp. *san diego*. *Gene*, 57, 37-46
- Hindley, J. and Berry, C. (1987) Identification, cloning and sequence analysis of the *Bacillus sphaericus* 1593 41.9 kD larvicidal toxin gene. *Molec. Microbiol.*, 1 (2), 187-194
- Hoffmann, C., Vanderbruggen, H., Hofte, H., Van Rie, J., Jansens, S. and Mellaert, H. V. (1988) Specificity of *Bacillus thuringiensis* delta-endotoxins is correlated with the presence of high-affinity binding sites in the brush-border membrane of target insect midguts. *Proc. Natl. Acad. Sci. USA*, 85, 7844-7848

Hofte, H., deGreve, H., Seurinck, J., Jansens, S., Mahillon, J., Ampe, C., Van der Kerckhove, J., Vanderbruggen, H., van Montagu, M., Zabeau, M. and Vaeck, M. (1986) Structural and functional analysis of a cloned delta endotoxin of *Bacillus thuringiensis berliner 1715*. *Eur. J. Biochem.*, 161, 273-280

Hofte, H. and Whiteley, H. R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.*, 53 (2), 242-255

Holland, I. B., Kenny, B., Steipe, B. and Pluckthun, A. (1990) Secretion of heterologous proteins in *Escherichia coli*. In: Guide to protein purification, *Meth. Enzymol.* (Deutscher, M. P., ed.), 182, 132-143

Honee, G., van der Salm, T. and Visser, B. (1988) Nucleotide sequence of crystal protein gene isolated from *B.thuringiensis* subspecies *entomocidus* 60.5 coding for a toxin highly active against *Spodoptera* species. *Nucl. Acids Res.*, 16, 6240

Hurlbert, R. E., Karlinsey, J. E. and Spence, K. D. (1985) Differential synthesis of bacteria-induced proteins of *Manduca sexta* larvae and pupae. *J. Insect Physiol.*, 31 (3), 205-215

Ibarra, J. E. and Federici, B. A. (1986) Isolation of a relatively nontoxic 65-kilodalton protein inclusion from the parasporal body of *Bacillus thuringiensis* subsp. *israelensis*. *J. Bacteriol.*, 165(2), 527-533

Ishihama, A. (1988) Promoter selectivity of prokaryotic RNA polymerases. *Trends in Genetics*, 4 (10), 282-286

Jamuna, R., Kamdar, H. and Jayaraman, K. (1982) Parameters accompanying optimal formation of biocides in Bacilli active against mosquito larvae. WHO-BIOS/BCV Proceedings of the meeting on Microbial insecticides, Geneva.

Johnson, D. E., Niezgodski, D. M. and Twaddle, G. M. (1980) Parasporal crystals produced by oligosporogenous mutants of *Bacillus thuringiensis* (*spo*<sup>-</sup>, *cry*<sup>+</sup>). *Can. J. Microbiol.*, 29, 486-491

Kalfon, A., Charles, J-F., Bourgouin, C. and deBarjac, H. (1984) Sporulation of *Bacillus sphaericus* 2297: an electron microscope study of crystal-like inclusion biogenesis and toxicity to mosquito larvae. *J. Gen. Microbiol.*, 130, 893-900

Kalfon, A., Larget-Thiery, I., Charles, J. F. and de Barjac, H. (1983) Growth, sporulation and larvicidal activity of *Bacillus sphaericus*. *Eur. J. Appl. Microbiol. Biotechnol.*, 18, 168-173

Kaufmann, S. H., Ewing, C. M. and Shaper, J. H. (1987) The erasable Western Blot. *Anal. Biochem.*, 161, 89-95

Khachatourians, G. G. (1986) Production and use of biological pest control agents. *Trends Biotechnol.*, 4, 120-124

Kirschbaum, J. B. and Konrad, E. B. (1973) Isolation of a specialized  $\lambda$  transducing bacteriophage carrying the beta subunit for *Escherichia coli* ribonucleic acid polymerase. *J. Bacteriol.*, 116 (2), 517-526

Klausner, A. (1984) Microbial insect control: using bugs to kill bugs. *Biotechnology*, 2, 408-419

Klier, A., Fargette, F., Ribier, J. and Rapaport, G. (1982) Cloning and expression of the crystal protein genes from *Bacillus thuringiensis* strain berliner 1715. *The EMBO J.*, 1 (7), 791-799

Klier, A., Lerecluse, D., Ribier, J., Bourgouin, C., Menou, G., Lecadet, M-M. and Rapaport, G. (1985) Cloning and expression in *Escherichia coli* of the crystal protein gene from *Bacillus thuringiensis* strain aizawa 7-29 and comparison of the structural organisation of genes from different serotypes. In: *Molecular Biology of Microbial differentiation* (Hoch, J. and Setlow, P., eds.), American Society for Microbiology, Washington D.C. pp. 217-224

Klier, A. and Rapoport, G. (1987) *Bacillus* larval toxin crystal protein. *Microbiol. Sci.*, 4 (9), 274-276

Knowles, B. H. and Ellar, D. J. (1986) Characterization and partial purification of a plasma membrane receptor for *Bacillus thuringiensis* var. *kurstaki* lepidoptera-specific  $\delta$ -endotoxin. *J. Cell Sci.*, 83, 89-101

Knowles, B. H. and Ellar, D. J. (1987) Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis*  $\delta$ -endotoxins with different insect specificity. *Biochem. Biophys. Acta*, 924, 509-518

Knowles, B. H., Thomas, W. E. and Ellar, D. J. (1984) Lectin-like binding of *Bacillus thuringiensis* var. *kurstaki* lepidopteran-specific toxin is an initial step in insecticidal action. *FEBS Lett.*, 168 (2), 197-202

Koncz, C., Kiss, A. and Venetianer, P. (1978) Biochemical characterization of the restriction-modification system of *Bacillus sphaericus*. *Eur. J. Biochem.*, 89, 523-529

Koval, S. F. and Hynes, S. H. (1991) Effect of paracrystalline protein surface layers on predation by *Bdellovibrio bacteriovorus*. *J. Bacteriol.* 173 (7), 2244-2249

Kronstad, J. W., Schnepf, H. E. and Whiteley, H. R. (1983) Diversity of locations for *Bacillus thuringiensis* crystal proteins. *J. Bacteriol.*, 154, 419-428

Kronstad, J. W. and Whiteley, H. R. (1984) Inverted repeat sequences flank a *Bacillus thuringiensis* crystal protein gene. *J. Bacteriol.*, 160, 95-102

Krych, V., Johnson, J. and Youstean, A. (1980) Deoxyribonucleic acid homologies among strains of *Bacillus sphaericus*. *Int. J. Syst. Bacteriol.*, 30, 476-484

Lacey, L. A. and Undeen, A. H. (1986) Microbial control of black flies and mosquitoes. *Annual Rev. Entomol.*, 31, 265-296

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685

Lecadet, M-M. and Dedonder, R. (1971) Biogenesis of the crystalline inclusion of *Bacillus thuringiensis* during sporulation. *Eur. J. Biochem.*, 23, 282-294

Lee, C., Li, P., Brickman, E. R. and Beckwith, J. (1989) Genetic studies on the inability of  $\beta$ -galactosidase to be translocated across the *Escherichia coli* cytoplasmic membrane. *J. Bacteriol.*, 171 (9), 4609-4616

Lewis, L. O., Yousten, A. A. and Murray, R. G. E. (1987) Characterization of the surface protein layers of the mosquito-pathogenic strains of *Bacillus sphaericus*. *J. Bacteriol.*, 169 (1), 72-79

Louis, J. and Szulmajster, J. (1985) Biocidal gene(s) of *Bacillus sphaericus* cloned and expressed in *Escherichia coli*. In: *Molecular biology of microbial differentiation* (Hoch, J. A. and Setlow, P., eds.), American Society for Microbiology, Washington D. C. pp. 241-245

Louis, J., Jayaraman, K. and Szulmajster, J. (1984) Biocide gene(s) and biocidal activity in different strains of *Bacillus sphaericus*. Expression of the gene(s) in *E.coli* maxicells. *Mol. Gen. Genet.*, 195, 23-28

Lovgren, A., Zhang, M., Engstrom, A., Dalhammar, G. and Landen, R. (1990) Molecular characterization of immune inhibitor A, a secreted protease from *Bacillus thuringiensis*. *Molec. Microbiol.*, 4 (12), 2137-2146

Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein estimation with the Folin phenol reagent. *J. Biol. Chem.*, 193, 265-275

Luckevich, M. D. and Bureridge, T. J. (1989) Characterization of a dynamic S-layer on *Bacillus thuringiensis*. *J. Bacteriol.*, 171 (2), 6656-6667

Luthy, P. and Ebersold, H. R. (1981) The entomocidal toxins of *Bacillus thuringiensis*. *Pharmac. Ther.*, 13, 257-283

Lysenko, O. (1985) Non-sporeforming bacteria pathogenic to

insects: incidence and mechanisms. Annual Rev. Microbiol., 39, 673-695

Mackman, N., Baker, K., Gray, L., Haigh, R., Nicaud, J-M. and Holland, I. B. (1987) Release of a chimeric protein into the medium from *Escherichia coli* using the C-terminal secretion signal of haemolysin. The EMBO J., 6 (9), 2835-2841

Mackman, N., Nicaud, J-M., Gray, L. and Holland, I. B. (1985) Identification of polypeptides required for the export of haemolysin 2001 from *E.coli*. Mol. Gen. Genet., 201, 529-536

Mahillon, J. and Seurinck, J. (1988) Complete nucleotide sequence of pG12, a *Bacillus thuringiensis* plasmid containing Tn4430. Nucl. Acids Res., 16 (24), 11827

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) Molecular Cloning: a laboratory manual. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

Marston, F. A. O. (1987) The purification of eukaryotic polypeptides expressed in *Escherichia coli*. In : DNA Cloning Volume III: A practical approach (Glover, D. M., ed.), IRL Press, Oxford. pp. 59-87

McDonald, K. O. and Burke Jr., W. F. (1984) Plasmid transformation of *Bacillus sphaericus*. J. Gen. Microbiol., 130, 202-208

McLean, K. M. and Whiteley, H. R. (1987) Expression in *Escherichia coli* of a cloned crystal protein gene of *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol., 169 (3), 1017-1023

McGaughey, W. (1985) Insect resistance to the biological insecticide *Bacillus thuringiensis*. Science, 229, 193-195

Meenakshi, K. and Jayaraman, K. (1979) On the formation of crystal proteins during sporulation in *Bacillus thuringiensis* var. *thuringiensis*. Arch. Microbiol., 120, 9-14

Miller, J. H. (1972) Experiments in molecular genetics. Cold

Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

Minnich, S. A. and Aronson, A. I. (1984) Regulation of protoxin synthesis in *Bacillus thuringiensis*. *J. Bacteriol.*, 158, 447-454

Minton, N, P. (1984) Improved plasmid vectors for secretion of translational *lac* fusions. *Gene*, 31, 269-273

Murphy, D. W., Sohi, S. S. and Fast, P. G. (1976) *Bacillus thuringiensis* enzyme digested  $\delta$ -endotoxin: effect on cultured insect cells. *Science*, 194, 954-956

Muthukumar, G. and Nickerson, K. W. (1987) The glycoprotein toxin of *Bacillus thuringiensis* subsp. *israelensis* indicates a lectinlike receptor in the larval mosquito gut. *Appl. Environ. Microbiol.*, 53 (11), 2650-2655

Myers, P., Yousten, A. A. and Davidson, E. W. (1979) Comparative studies of the mosquito-larval toxin of *Bacillus sphaericus* SSII-I and 1593. *Can. J. Microbiol.*, 25, 1227-1231

Myers, P. and Yousten, A. A. (1980) Localisation of the mosquito-larval toxin of *Bacillus sphaericus* 1593. *Appl. Environ. Microbiol.*, 39, 1205-1211

Myers, P. and Yousten, A. (1981) Toxic activity of *Bacillus sphaericus* for mosquito larvae. *Dev. Ind. Microbiol.*, 22, 41-52

Myers, P. and Yousten, A. (1978) Toxic activity of *Bacillus sphaericus* SSII-1 for mosquito larvae. *Infect. Immunol.*, 19, 1047-1057

Narasu, M. L. and Gopinathan, K. P. (1986) Purification of larvicidal protein from *Bacillus sphaericus* 1593. *Biochem. Biophys. Res. Commun.*, 141, 756-761

Nelson, D. R. and Zusman, D. R. (1983) Protein S of *Myxococcus xanthus*. *J. Bacteriol.*, 154 (2), 547-553

Nicholls, C. N., Ahmad, W. and Ellar, D. J. (1989) Evidence for two different types of insecticidal P2 toxins with dual specificity in *Bacillus thuringiensis* subspecies. J. Bacteriol., 171 (9), 5141-5147

Nickerson, K. W. (1980) Structure and function of the *Bacillus thuringiensis* crystal protein. Biotechnol. Bioeng., 22, 1305-1333

Oeda, K., Inouye, K., Ibuchi, Y., Oshie, K., Shimizu, M., Nakamura, K., Nishioka, R., Takada, Y. and Okhawa, H. (1989) Formation of crystals of the insecticidal proteins of *Bacillus thuringiensis* subsp. *aizawai* IPL7 in *Escherichia coli*. J. Bacteriol., 171 (6), 3568-3571

Ogaswara, N., Moriya, S. and Yoshikawa, H. (1983) Structure and organization of rRNA operons in the region of the replication origin of the *Bacillus subtilis* chromosome. Nucl. Acids Res., 11, 6301-6318

Old, R. W. and Primrose, S. B. (1985) Cloning in bacteria other than *E.coli*. In: Principles of Gene manipulation. An introduction to genetic engineering. Blackwell Scientific Publications, Oxford. pp 155-182

Pero, J., Nelson, J. and Losick, R. (1975) In vitro and in vivo transcription by vegetative and sporulating *Bacillus subtilis*. In : Spores VI (Gerhardt, P., Costilow, R. N. and Sadoff, H. L., eds.), American Society for Microbiology, Washington D. C. pp, 202-213

Perlak, F. J., Deaton, R. W., Armstrong, T. A., Fuchs, R. L., Sims, S. R., Greenplate, J. T. and Fischhoff, D. A. (1990) Insect resistant cotton plants. Biotechnology, 8, 939-943

Pfannenstiel, M. A., Muthukumar, G., Couche, G. A. and Nickerson, K. W. (1987) Amino sugars in the glycoprotein toxin from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol., 169 (2), 796-801

Pfannenstiel, M. A., Ross, E. J., Kramer, V. C and Nickerson, K. W. (1984) Toxicity and composition of protease-inhibited

*Bacillus thuringiensis* var. *israelensis* crystals. FEMS  
Microbiol. Lett., 21, 39-42

Pratt, J. M. (1984) Coupled transcription-translation in  
prokaryotic cell-free systems. In: Transcription and  
translation: a practical approach, (Hames, B. D. and Higgins,  
S. J., eds.), IRL Press, Oxford. 179-210

Primrose, S. B. and Ehrlich, S. D. (1981) Isolation of  
plasmid stability mutants and study of their instability.  
Plamid, 6, 193-201

Raymond, K. C., John, T. R. and Bulla Jr., L. A. (1990)  
Larvicidal activity of chimeric *Bacillus thuringiensis*  
protoxins. Molec. Microbiol., 4 (11), 1967-1973

Rees, C. (1986) PhD thesis, University of Leicester,  
Leicester, U. K.

Rosenberg, A. H., Lade, B. N., Chui, D-S., Lin, S-W., Dunn, J.  
J. and Studier, F. W. (1987) Vectors for selective expression  
of cloned DNAs by T7 RNA polymerase. Gene, 56, 125-135

Sacchi, V. F., Parenti, P., Hanozet, G. M., Giordana, B.,  
Luthy, P. and Wolfersberger, M. G. (1986) *Bacillus*  
*thuringiensis* toxzin inhibits K<sup>+</sup>-gradient-dependent amino acid  
transport across the brush border membrane of *Pieris brassicae*  
midgut cells. FEBS Lett., 204, 213-218

Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA  
sequencing with chain-terminating inhibitors. Proc. Natl.  
Acad. Sci. USA, 74 (12), 5463-5467

Schauder, B., Blocker, H., Frank, R. and McCarthy, J. E. G.  
(1987) Inducible expression vectors incorporating the  
*Escherichia coli* atpE translational initiation region. Gene,  
52, 279-283

Schnepf, H. E. and Whiteley, H. R. (1981) Cloning and  
expression of the *Bacillus thuringiensis* crystal protein gene  
in *Escherichia coli*. Proc. Natl. Acad. Sci. USA, 78 (5),  
2893-2897

Schnepf, H. E. and Whiteley, H. R. (1985) Endotoxin formation in sporulating organisms. Protein toxins of *Bacillus* spp. In: Molecular biology of microbial differentiation, (Hoch, J. A. and Setlow, P., eds.), American Society for Microbiology, Washington D. C., 209-216

Schnepf, H. E., Wong, H. C. and Whiteley, H. R. (1985) The amino acid sequence of a crystal protein from *Bacillus thuringiensis* deduced from the DNA base sequence. J. Biol. Chem., 260, 6264-6272

Schnepf, H. E., Wong, H. C. and Whiteley, H. R. (1987) Expression of a cloned *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*. J. Bacteriol., 169 (9), 4110-4118

Sgarella, F. and Szulmajster, J. (1987) Purification and characterization of the larvicidal toxin of *Bacillus sphaericus* 1593M. Biochem. Biophys. Res. Commun. 143 (3), 901-907

Shivakumar, A. G. Gundling, G. J., Benson, T. A., Casuto, D., Miller, M. F. and Spear, B. B. (1986) Vegetative expression of the Delta endotoxin genes of *Bacillus thuringiensis* subsp. *kurstaki* in *Bacillus subtilis*. J. Bacteriol. 166 (1), 194-204

Sleytr, U. B. and Messner, P. (1988) Crystalline surface layers in procaryotes. J. Bacteriol., 170, 2891-2897

Somerville, H. J. (1971) Formation of the parasporal inclusion of *Bacillus thuringiensis*. Eur. J. Biochem., 18, 226-237

Somerville, H. J. and Pockett, H. V. (1975) An insect toxin from species of *Bacillus thuringiensis* and *Bacillus cereus*. J. Gen. Microbiol., 87, 359-369

Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Molec. Biol., 98, 503-517

Southern, E. M. (1979) Gel electrophoresis of restriction

fragments. Meth. Enzymol., 68, 152-176

Souza, A. E., Rajan, V. and Jayaraman, K. (1988) Cloning and expression in *Escherichia coli* of two DNA fragments from *Bacillus sphaericus* encoding mosquito-larvicidal activity. J. Biotechnol., 7 (1), 71-82

Souza, A. E. (1987) PhD thesis, Anna University, Madras, India.

Stoker, N.G., Pratt, J. M. and Holland, I. B. (1984) In vivo gene expression systems in prokaryotes. In: Transcription and translation: a practical approach (Hames, B. D. and Higgins, S. J., eds.). IRL Press Ltd, Oxford. pp. 153-177

Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. In: Meth. Enzymol., (Goedell, D, ed.) 185, Academic Press Inc., San Diego. pp. 60-89

Thanabalu, T., Hindley, J., Jackson-Yap, J. and Berry, C. (1991) Cloning, sequencing, and expression of a gene encoding a 100-Kilodalton mosquitocidal toxin from *Bacillus sphaericus* SSII-1. J. Bacteriol., 173 (9), 2776-2785

Thomas, W. E. and Ellar, D. J. (1983a) *Bacillus thuringiensis* var. *israelensis* crystal  $\delta$ -endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*. J. Cell Sci., 60, 181-197

Thomas, W. E. and Ellar, D. J. (1983b) Mechanism of action of *Bacillus thuringiensis* var. *israelensis* insecticidal  $\delta$ -endotoxin. FEBS Lett. 154 (2), 362-368

Thorne, L., Garduno, F., Thompson, T., Decker, D., Zounes, M. A., Wild, M., Walfield, A. M. and Pollock, T. J. (1986) Structural similarity between the Lepidoptera- and Diptera-specific insecticidal endotoxin genes of *Bacillus thuringiensis* subsp. "*kurstaki*" and "*israelensis*". J. Bacteriol., 166 (3), 801-811

Tiedemann, A. A. and Smith, J. M. (1988) *lacZY* gene fusion

cassettes with Kan<sup>R</sup> resistance. Nucl. Acids Res., 16 (8), 3587

Tinelli, R. and Bourguin, C. (1982) Larvicidal toxin from *Bacillus sphaericus* spores. FEBS Lett., 142 (1), 155-158

Towbin, H., Staehlin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA, 76 (9), 4350-4354

Trisrisook, M., Pantuwatana, S., Bhumiratna, A and Pnabangred, W. (1990) Molecular cloning of the 130-kilodalton mosquitocidal  $\delta$ -endotoxin gene of *Bacillus thuringiensis* subsp. *israelensis* in *Bacillus sphaericus*. Appl. and Environ. Microbiol., 56 (6), 1710-1716

Vaeck, M., Reynaerts, A., Hofte, H., Jansens, S., de Beuckleur, M., Dean, C., Zabeau, M., von Montagu, M. and Leemans, J. (1987) Transgenic plants protected from insect attack. Nature, 328, 33-37

Van Rie, J., McGaughey, W. H., Johnson, D. E., Barnet, B. D. and van Mellaert, H. (1990) Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. Science, 247, 72-74

Vasil, M. L., Grant, C. R. and Prince, R. W. (1989) Regulation of exotoxin A synthesis in *Pseudomonas aeruginosa*: characterization of *toxA-lacZ* fusions in wild-type and mutant strains. Molec. Microbiol., 3 (3), 371-381

Visick, J. E. and Whiteley, H. R. (1991) Effect of a 20-kilodalton protein from *Bacillus thuringiensis* subsp. *israelensis* on production of the *cytA* protein by *Escherichia coli*. J. Bacteriol., 173 (5), 1748-1756

Visser, B., Van der Salm, T., van der Brink, W. and Falkers, G. (1988) Genes from *Bacillus thuringiensis entomocidus* 60.5 coding for insect-specific crystal proteins. Mol. Gen. Genet., 212, 219-224

Vogelstein, B. and Gillespie, D. (1979) Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA, 76 (2), 615-619

Waalwijk, C., Dullemans, A. M., van Workum, M. E. S. and Visser, B. (1985) Molecular cloning and the nucleotide sequence of the Mr 28000 crystal protein gene of *Bacillus thuringiensis* subsp. *israelensis*. Nucl. Acids Res., 13 (22), 8207-8217

Wabiko, H., Raymond, K. C. and Bulla, Jr., L. A. (1986) *Bacillus thuringiensis* entomocidal protein gene sequence and gene product analysis. DNA, 5, 305-314

Wakisaka, Y., Masaki, E., Koizumi, K., Nishimoto, Y., Endo, Y., Nishimura, M. s. and Nishii-tsutsuji-Uwo, J. (1982) Asporogenous *Bacillus thuringiensis* mutant producing high yields of  $\delta$ -endotoxin. Appl. and Environ. Microbiol., 43 (6), 1498-1500

Walfield, A. M., Garduno, F., Thorne, L., Zounes, M., Decker, D., Wild, M. A. and Pollock, T. J. (1986) Cloning of a gene that codes for a mosquitocidal toxin from *Bacillus thuringiensis* var. *israelensis*. In: *Bacillus Molecular Genetics and Biotechnology Applications* (Ganesan, A. T. and Hoch, J. A., eds.). Academic Press Inc. pp. 321-333

Wandersman, C. and Delepeliare, P. (1990) TolC, an *Escherichia coli* outer membrane protein required for haemolysin secretion. Proc. Natl. Acad. Sci. USA, 87 (12), 4776-4780

Ward, E. S. and Ellar, D. J. (1986) *Bacillus thuringiensis* var. *israelensis*  $\delta$ -endotoxin nucleotide sequence and characterization of the transcripts in *Bacillus thuringiensis* and *Escherichia coli*. J. Molec. Biol., 191, 1-11

Ward, E. S. and Ellar, D. J. (1988) Cloning and expression of two homologous genes of *Bacillus thuringiensis* subsp. *israelensis* which encode 130-kilodalton mosquitocidal proteins. J. Bacteriol., 170 (2), 727-735

Ward, E. S. and Ellar, D. J. (1987) Nucleotide sequence of a *Bacillus thuringiensis* var. *israelensis* gene encoding a 130kDa  $\delta$ -endotoxin. Nucl. Acids Res., 15 (17), 7195

Ward, E. S., Ellar, D. J. and Chilcott, C. N. (1988) Single amino acid changes in the *Bacillus thuringiensis* var. *israelensis* delta-endotoxin affect the toxicity and expression of the protein. J. Molec. Biol., 202, 527-535

Watson, G. M. F. and Mann, N. H. (1988) Protein phosphorylation in *Bacillus thuringiensis* during growth and  $\delta$ -endotoxin production. J. Gen. Microbiol., 134, 2559-2565

Whiteley, H. R., Kronstad, J. W. and Schnepf, H. E. (1985) Structure and expression of cloned *Bacillus thuringiensis* toxin genes. In: Molecular Biology of Microbial differentiation (Hoch, J. A. and Setlow, P., eds.), American Society for Microbiology, Washington D. C. pp 225-229

Whiteley, H. R., Schnepf, H. E., Widner, W. R. and Wong, H. C. (1986) Regulation of a cloned *Bacillus thuringiensis* crystal protein gene in *Escherichia coli* and *Bacillus subtilis*. In: *Bacillus* Molecular Genetics and Biotechnology Applications (Ganesan, A. T. and Hoch, J. A., eds.). Academic Press Inc. pp. 377-385

Whiteley, H. R., Widner, W. R. and Schnepf, H. E. (1987) Genes encoding peptides of the small cuboidal crystals of *Bacillus thuringiensis*. In: Genetics and Biotechnology of Bacilli Vol. 2 (Ganesan, A. T. and Hoch, S. J., eds.). Academic Press Inc., San Diego, California. pp. 239-244

Widner, W. R. and Whiteley, H. R. (1989) Two highly related insecticidal crystal proteins of *Bacillus thuringiensis* subsp. *kurstaki* possess different host range specificities. J. Bacteriol., 171, 965-974

Widner, W. R. and Whiteley, H. R. (1990) Location of the dipteran specificity region in a lepidopteran-dipteran crystal protein from *Bacillus thuringiensis*. J. Bacteriol., 172 (6), 2826-2832

Wong, H. C. and Chang, S. (1985) Identification of a positive retroregulator that functions in *Escherichia coli* and *Bacillus subtilis*. In: Molecular Biology of Microbial Differentiation (Hoch, J. A. and Setlow, P., eds.), American Society for Microbiology, Washington D. C. pp. 104-109

Wong, H. C. and Chang, S. (1986) Identification of a positive retroregulator that stabilizes mRNAs in bacteria. Proc. Natl. Acad. Sci. USA, 83, 3233-3237

Wong, H. C., Schnepf, H. E. and Whiteley, H. R. (1983) Transcriptional and translational start sites for the *Bacillus thuringiensis* crystal protein gene. J. Biol. Chem., 258, 1960-1967

Word, N. S., Yousten, A. A. and Howard, L. (1983) Regularly structured and non-regularly structured surface layers of *Bacillus sphaericus*. FEMS Microbiol. Lett., 17, 277-282

Yamamoto, T. and McLaughlin, R. E. (1981) Isolation of a protein from the parasporal crystal of *Bacillus thuringiensis* var. *kurstaki* toxic to the mosquito larva, *Aedes taeniorhynchus*. Biochem Biophys. Res. Commun., 103, 414-421

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene, 33, 103-119

Young, F. E., Smith, C. and Reilly, B. E. (1969) Chromosomal location of genes regulating resistance to bacteriophage in *Bacillus subtilis*. J. Bacteriol., 98, 1087-1097

Yousten, A. A. and Davidson, E. W. (1982) Ultrastructural analysis of spores and parasporal crystals formed by *Bacillus sphaericus* 2297. Appl. Environ. Microbiol., 44, 1449-1455

Zhang, H., Scholl, R., Browse, J. and Somerville, C. (1988) Double stranded sequencing as a choice for DNA sequencing. Nucl. Acids Res., 16 (3), 1220

Zuber, P. and Losick, R. (1983) Use of a *lacZ* fusion to study

the role of the *spo0* genes of *Bacillus subtilis* in developmental regulation. *Cell*, 35, 275-283