The isolation and characterisation of conditional (Out^{ts}) and null (Out^{-}) secretion mutants of *Erwinia carotovora* subspecies *carotovora* (SCRI193)

by

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NUMEROUS ORIGINALS IN COLOUR

For my mother and sister

Contents

SUMMARY

Ecc strain SCRI193 secretes a wide range of extracellular degradative enzymes. Examples of these include pectate lyase (Pel), cellulase (Cel) and protease (Prt). Secretion mutants, Out$^-$ (Pel$^-$, Cel$^-$, Prt$^+$) that accumulate Pel and Cel periplasmically have previously been isolated by transposon and chemical mutagenesis. Protease secretion was always unaffected. Genetic and molecular analysis has revealed a cluster of genes (*out*) that are essential for the secretion of Pel and Cel in *Ecc*. In order to eventually investigate the putative periplasmic intermediate in the natural secretion process this study has employed localised mutagenesis, using hydroxylamine, of the *out* cluster, in order to isolate conditional secretory mutants. Tn*5* was successfully linked 3' to the *out* cluster. Using the *Ecc* generalised transducing phage, øKP, localised mutagenesis yielded 17 Out$^-$ mutants (Pel$^-$, Cel$^-$, Prt$^+$) that accumulated Pel and Cel periplasmically. These 17 mutants included 2 conditional secretory mutants (Out^{ts}), HJN1003 and HJN1004, that were shown to accumulate Pel and Cel periplasmically at the restrictive temperature (33°C) but were wild type for secretion at the permissive temperature (26°C) and one auxotroph that was defined as histidine requiring. Each mutation was shown to be linked to the transposon Tn*5* and most were subsequently shown, by cosmid complementation, to be within the *out* cluster. The two Out^{ts} secretion mutants, HJN1003 and HJN1004, were mapped to *out*E and *out*L respectively. PCR amplification, cloning and sequence analysis has revealed two lesions in *out*L, from the Out^{ts} strain HJN1004 and one lesion in the Out$^-$ strain HJN1008. Attempts to perform pulse chase experiments proved to be difficult and suggestions have been made to overcome these problems.

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Most of all I would like to thank Carol for making everything seem worthwhile and for her constant support and encouragement during the compilation of this thesis.

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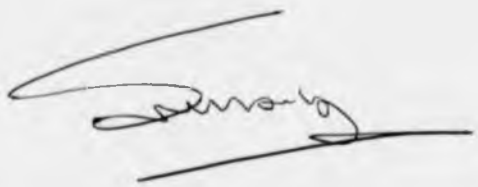
Declaration

This thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself unless stated otherwise. All sources of information have been specifically acknowledged by means of reference.



Abbreviations

pfu	plaque forming units
NBA	nutrient broth agar
LBA	Luria broth agar
DDA	double Difco agar
UV	ultraviolet light
w/v	weight/volume
v/v	volume/volume
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
APS	ammonium persulphate
EDTA	diaminoethanetetraacetic acid
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N' - tetramethylethylenediamine
CTAB	cetyltrimethyl ammonium bromide
PGA	polygalacturonic acid
PMB	pectate lyase minimal broth
IPTG	isopropyl-β-galactopyranoside
Tris	Tris(hydroxymethyl)aminoethane

CHAPTER 1

INTRODUCTION

1.1 Preface

Both prokaryotic and eukaryotic biological systems have complex mechanisms for targeting proteins, either from one organelle to another, as in eukaryotes, or from the cytoplasmic compartment of prokaryotes to the external milieu. Many bacteria secrete proteins such as toxins, pathogenicity factors and enzymes. Much research has been done on these processes for the study of pathogenicity and for biotechnological reasons.

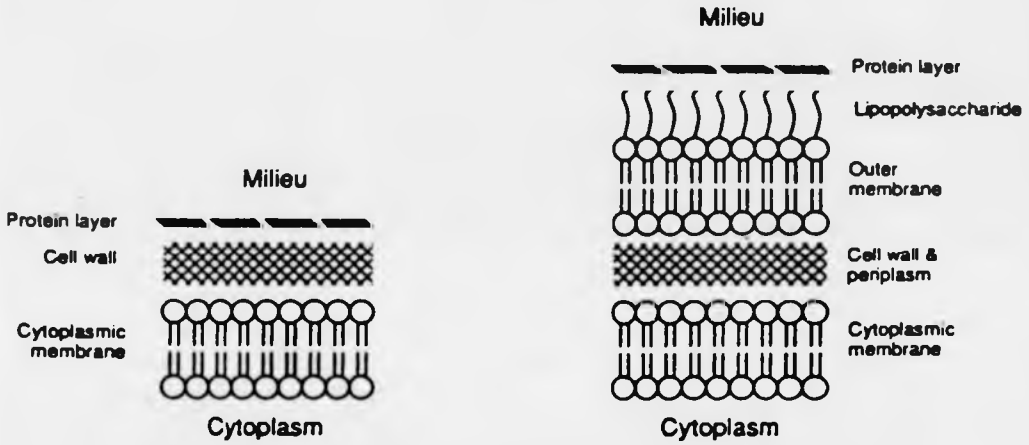
There are interesting questions to ask about protein targeting in prokaryotic and eukaryotic systems. How can proteins "release" themselves into the external milieu of Gram positive bacteria or translocate across both membranes of Gram negative bacteria ? How can a protein be targeted to the matrix of the mitochondrion or the lumen of the endoplasmic reticulum ? How are extracellular proteins sorted from integral membrane proteins, periplasmic or cytoplasmic ones?

This chapter will attempt to answer some of these questions by discussing recent advances in the topic of protein targeting and how this information is pertinent to the work described in this thesis.

1.2 Targeting of proteins

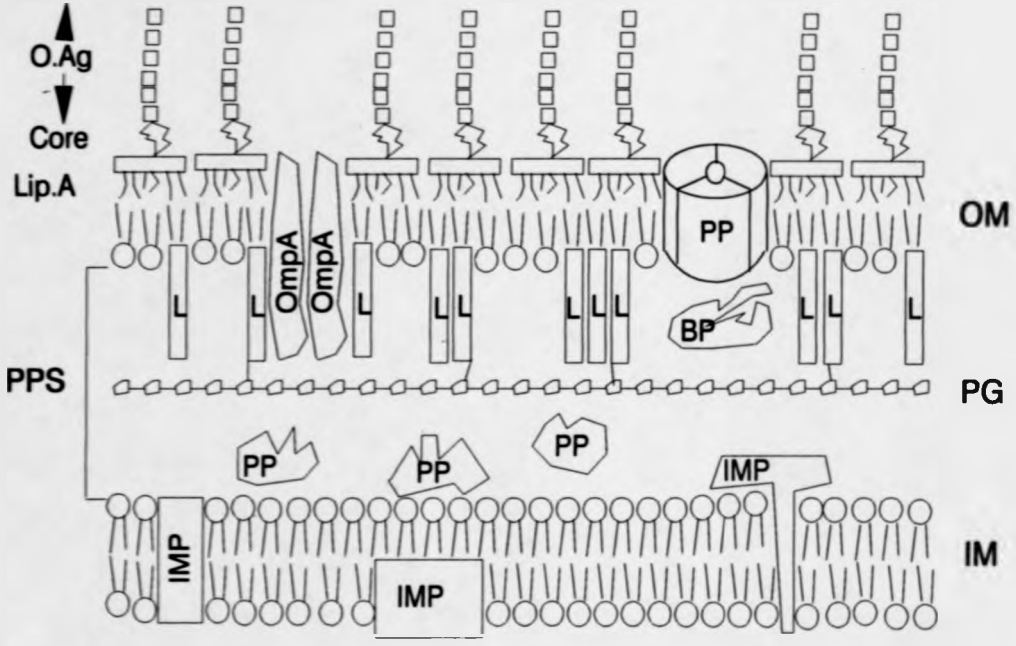
The targeting of proteins has been shown to follow similar conserved mechanisms in both prokaryotes and eukaryotes. The secretion of proteins across the Gram negative bacterial cell envelope (see figure 1.1) involves a number of translocation steps. Firstly, the extracellular protein has to traverse the cytoplasmic membrane in order to cross from the cytoplasm to the periplasm. Secondly, once in the periplasm, the protein has to traverse the outer membrane before reaching the extracellular milieu. During this translocation procedure the protein will face a number of obstacles; hydrophobic lipid bilayers, periplasmic proteins, lipopolysaccharide and protein layer (see figure 1.2). In order to clarify the terminology associated with translocation of the Gram negative cell envelope, I shall use "export" to define translocation from the cytoplasm to the periplasm and "secretion" to determine translocation from the

Figure 1.1 A simplified diagram of the Gram positive and Gram negative cell envelopes.



Based on Pugsley (1993).

Figure 1.2 A diagrammatic representation of the complex gram negative cell envelope.



Key:
PPS - Periplasm, PP/BP - Periplasmic proteins, L - Lipid, OmpA - Outer membrane protein A, IMP - Inner membrane protein, PP - Protein pore complex, OM - Outer membrane, PG - Peptidoglycan layer, IM - Inner membrane, Lip. A - Lipid A, Core - Core oligosaccharide, O.Ag - "O" antigen.

periplasm to the external milieu. There, therefore, has to be a well defined mechanism of recognition, and targeting procedures, to sort proteins for export from those that have been targeted elsewhere (Eg. cytoplasmic membrane, outer membrane, periplasmic space).

The first step in the translocation of an extracellular protein across the cytoplasmic membrane of Gram negative bacteria is referred to as Sec-dependent, and has been discussed, in detail, in many recent reviews (Lory, 1992; Randall *et al.*, 1990; Schatz and Beckwith, 1990; Wickner *et al.*, 1991; Salmond and Reeves, 1993; Pugsley, 1993). This stage in translocation has been shown to be analogous to translocation of the single membrane of Gram positive bacteria and the endoplasmic reticulum (ER) of eukaryotes. The following sections will discuss the mechanisms of Sec-dependent translocation in *Escherichia coli* (*E. coli*).

1.3 The study of protein secretion

Extracellular protein export requires the interaction of the immature or pre-protein with various other proteins that constitute the export machinery. Pre-proteins are recognised by the export machinery by an N-terminal signal or leader sequence of the pre-protein. In order to investigate exactly how this happens many workers have used a genetic approach (for a review see Schatz and Beckwith, 1990). Signal sequence mutants were isolated by fusing ß-galactosidase, which cannot be fully exported across the inner membrane, to cell envelope proteins, that as a consequence block the export pathway and are lethal to the cell. Fusions of the maltose inducible proteins LamB and maltose binding protein (MBP) were constructed with *lacZ* (*lam*B-*lacZ* and *mal*E-*lacZ*). In the presence of maltose these fusion proteins were lethal to the cell (Ito *et al.*, 1981). Selecting survivors directly enriches for signal sequence mutations that relieve the lethality phenotype. These mutations could also be selected for on the basis of a change of a Lac^- phenotype to that of Lac^+ due to the location of the *lacZ* hybrid protein. If the hybrid protein was cytoplasmic, due to a signal sequence mutation that prevented export, then the strain would be Lac^+. If the hybrid protein did not have a signal sequence mutation the export machinery would be jammed resulting in the improper

folding of ß-galactosidase and a Lac$^-$ phenotype. In identifying specific secretion genes, export defective mutants were selected on their ability to recognise specific portions of secreted proteins. The localisation of proteins in mutant strains compared to wild type strains, was different, indicating export defective mutations. Various mutations that suppress defects in signal sequences were also isolated, *prl* mutations (Emr *et al.*, 1981; Stader *et al.*, 1989).

By performing localised mutagenesis of *prl*A Shiba *et al.* (1984) identified a conditional mutation in a gene that they called *sec*Y, which caused the intracellular accumulation of pre-protein (OmpA and OmpF). *sec*Y and *prl*A were found to be allelic. Similar allelic properties were found for *sec*A (*prl*D) and *sec*E (*prl*G). The discovery of signal sequence suppressors suggested that there was no rate limiting step in export, but instead several proteins can act to reduce rate limitation caused by a defective signal sequence.

Oliver and Beckwith (1981) isolated conditional lethal secretion mutants of *sec*A that were Lac$^+$ using the MBP-ß-galactosidase fusion technology described above. *sec*B was identified using the same technology without selecting the conditional lethality phenotype (Kummamoto and Beckwith, 1983). *sec*D was identified by selecting cold sensitive lethality mutants using *pho*A-*lac*Z and *lam*B-*lac*Z fusions, in a similar vein to that of *sec*A isolation (Gardel *et al.*, 1987).

A simpler way of isolating export defective mutants was based on the induction of the SecA protein by the presence of mutations in *sec*Y or *sec*D, or by jamming the secretion apparatus with fusion proteins. Riggs *et al.* (1988) selected for export mutants by the over-expression of *sec*A-*lac*Z fusions. In this way Riggs *et al.* (1988) identified a new secretion gene (*sec*E) as well as *sec*A, D and Y mutations.

Biochemical analysis of the export system using purified protein products of the *sec* genes, and membrane vesicles, has been reviewed by Wickner *et al.* (1991). This approach has defined the minimum requirements for pre-protein translocation. The

components of the export apparatus and their predicted functions and interactions will now be discussed.

1.3.1 Signal peptides

The N-terminal signal sequence of pre-proteins has been thought to be important in the recognition of the correct targeting pathway (Sec) via a signal peptide receptor (Pugsley *et al.*, 1990(a)) with which it interacts and undergoes insertion into the membrane and subsequent translocation. This is followed by cleavage of the signal peptide and consequent release of the protein. The protein is then either released or enters further secretory mechanisms, as in Gram negative bacteria (see section 1.4.2.2).

Signal peptides are of obvious importance and have been shown to be required for Sec dependent secretion (see above). Signal peptides have been compared between eukaryotes and prokaryotes to reveal common regions (Von Heijne, 1985), firstly, a short hydrophilic N-terminal region (n) that is highly variable and contains at least 1 positively charged residue. Secondly, there is a long hydrophobic central region (h) that is ≥ 8 residues in prokaryotes or ≥ 7 residues in eukaryotes. Thirdly, there is a carboxy terminal region (c) that is ≥ 5 residues in eukaryotes or ≥ 6 residues in prokaryotes, that contains the cleavage site. Upon translocation the signal sequence is cleaved off by signal peptidase (see section 1.3.3).

1.3.2 The Sec proteins

The *sec* genes that encode the export machinery are listed in table 1.1. The genetic analysis, biochemistry and possible functional roles of each Sec protein have been described (Pugsley, 1993; Schatz and Beckwith, 1990; Wickner *et al.*, 1991). The major features of each protein will be discussed briefly here.

1.3.2.1 SecB

SecB is a soluble cytosolic protein that has been shown to act at an early stage of export, before SecA (Kummamoto and Beckwith, 1985), and acts on only a subset of

Table 1.1 *sec* genes that encode the export machinery in *Escherichia coli*.

Gene	Gene product
secA(*prlD*)	102 Kda peripheral membrane protein, ATPase
secB	16.6 Kda cytoplasmic protein, antifolding factor
secD	67 Kda inner membrane protein
secF	35 Kda inner membrane protein
secE(*prlG*)	13.6 Kda inner mebrane protein
secY(*prlA*)	49 Kda inner mebrane protein
lepB	36 Kda inner membrane protein signal (leader) peptidase I
lspA	18 Kda inner membrane protein, signal peptidase II

Information taken from Schatz and Beckwith (1990). See text for details of the biochemical and possible functional roles of the gene products.

cell envelope proteins. Its importance in export has been shown to be with helping to present pre-proteins to the translocation machinery in a "translocation competent" state. SecB is a member of a family of molecular chaperones that recognise pre-proteins for targeting and folding (Landry and Gierasch, 1991). Although SecB is not essential for export of pre-ribose binding protein it was necessary for export of pre-MBP (Kummamoto and Beckwith, 1985). Thus SecB was thought to be a specific molecular chaperone for export of some proteins, whereas other molecular chaperones, such as GroEL, may act as a more general folding modulator that can bind to many proteins (Kummamoto, 1991). SecB might therefore act to block folding so as to stabilise the structure for translocation, as mutations in *sec*B slow the rate of export of preMBP and increase the folded amount of preMBP in the cytoplasm (Kummamoto and Gannon, 1989).

How do molecular chaperones recognise certain proteins ? It was thought that the leader or signal sequence was recognised by SecB (Watanabe and Blobel, 1989) but Randall *et al.* (1990) have shown that SecB binds to a part of partially folded protein (preMBP), modulated by the leader sequence, to reveal specific recognition elements for SecB binding. SecB has also been shown to bind to non-exported proteins such as part of the tail fibre protein of phage T4 and binds to ß conformation structures exposed on partially folded protein (MacIntyre *et al.*, 1991). SecB does not bind to pre ß-lactamase *in vivo* or *in vitro* (Laminet *et al.* 1991) confirming its specificity for some proteins.

GroES and GroEL are heat shock proteins of *E. coli* and have been shown to be required for the export of ß-lactamase (Crooke *et al.*, 1988; Crooke and Wickner, 1987). GroEL has been shown to bind to preß-lactamase and therefore may be a molecular chaperone that can bind to Sec independent proteins (Laminet *et al.*, 1990). The role of SecB and other molecular chaperones has been discussed by Kummamoto, (1991).

1.3.2.2. Sec proteins A, Y, D, E, F

These proteins have been well reviewed in terms of their structure and function in protein export (Schatz and Beckwtih, 1991; Wickner *et al*, 1991; Pugsley, 1993; Oliver, 1993). Only the location and the interaction of these proteins in the secretion process will be discussed here.

1.3.2.2.1 SecA

SecA is a peripheral cytosolic protein that has been indicated in dual roles of synthesis and secretion of cell envelope proteins (Reviewed by Oliver, 1993; Liss and Oliver, 1986). It is thought to be required for precursor binding to membrane vesicles (Cabelli *et al.*, 1988). SecA has been shown to have a high affinity for SecB (Hartl *et al.*, 1990) and binds and hydrolyses ATP in the presence of the leader peptide, mature portions of the pre-protein, acidic phospholipids and SecY which is required for translocation (Lill *et al*, 1990).

An interesting feature of SecA is its ability to regulate its own translation. Translation is increased when translocation is blocked (Schmidt *et al.*, 1991). Pugsley (1993) suggests that this might compensate for temporary jamming or saturation of export that may occur naturally.

1.3.2.2.2 SecY, SecE

SecY/SecE is an integral membrane protein complex. SecY is predicted to span the inner membrane ten times (Akiyama and Ito, 1987) and SecE three times (Schatz *et al.* 1989). There is a third subunit of the SecY/SecE complex termed Band 1 (Brundage *et al.* 1992) that, as yet, has no defined function. The SecY/SecE complex binds SecA (Hartl *et al.*, 1990) and interacts to translocate the pre-protein. Functional SecY is necessary for SecA ATPase activity (Lill *et al.* 1989). The energy supplied from ATP hydrolysis is predicted to be involved in translocation of the pre-protein through the SecY/SecE complex or to be needed to actively unfold proteins for translocation (Wickner *et al.*, 1991). Further experiments to determine SecY/SecE interactions, using purified proteins, will need to be performed in order to test these predictions.

1.3.2.2.3 SecD/SecF

The *sec*D and *sec*F genes map together and encode two highly similar integral inner membrane proteins (Gardel *et al*, 1990). Both proteins are thought to span the membrane 6 times with large periplasmic domains (Pugsley, 1993). The proteins probably have similar functions, due to their similarity, but have not been implicated in early export stages of secretion but may bind to proteins emerging from the translocation complex SecY/E (Gardel *et al.*, 1990). Wickner *et al.* (1991) suggest that SecD and F may clear the SecY/E periplasmic surface for further translocation or catalyse protein folding.

Pugsley (1993) suggests that SecY, D, E, F and another protein, Ydr, form a pentamer. Ydr protein has been implicated in complexing with SecY (Shimoike, 1992).

1.3.3 Signal peptidase

Once proteins have translocated the membrane via the Sec machinery, the leader peptidase is cleaved, in order to release the protein from the surface of the membrane (Dalbey and Wickner, 1985). This also showed that leader peptides can act as membrane anchors for pre-proteins. Two leader peptidases have been implicated in the processing of nearly all *E. coli* cell surface proteins. These peptidases are lipoprotein peptidase (signal peptidase II) and leader peptidase (signal peptidase I) (reviewed by Dalbey, 1991). Leader peptidase (LepB) is the major peptidase, and is associated with the inner membrane of *E. coli*. It spans the membrane twice with a large carboxy terminal portion in the periplasm, which is thought to contain the catalytic site (Dalbey, 1991). A pre-protein leader peptide must have small amino acid residues at -1 and -3 for cleavage (see Pugsley, 1993).

Two other peptidases are known to function in *E. coli* to cleave leader peptides. Firstly, a lipoprotein signal peptidase (LspA) which cleaves diacylglycerol modified N-proximal cysteine residues only (Pugsley and Schwartz, 1985). Secondly, a type IV prepilin peptidase that cleaves leader peptides in the cytoplasm and has been found in

both Gram positive and Gram negative bacteria (Nunn and Lory, 1992; Pugsley, 1993), see also section 1.5.3.1.

1.3.4 Translocation - how do proteins cross lipid bilayers ?

It is still unknown how a hydrophilic protein can translocate a nonpolar lipid bilayer environment. The helical loop model of Engleman and Steitz (1981) is strongly favoured because of the discovery that leader peptides can anchor pre-proteins to the membrane (see previous section). The leader peptide is thought to orientate itself in the membrane translocation machinery, such that the N-terminus is cytosolic and the C-terminus and mature protein form a loop structure in the membrane complex. The protein translocates the membrane while the leader peptide spans the membrane.

Singer *et al.* (1986) favour a model based upon the formation of an aqueous channel to make a hydrophilic environment for the translocating polypeptide. Simon and Blobel (1992) suggest that a channel within the translocation complex opens laterally to capture an export initiation domain (signal peptide ?) that triggers the opening of a channel perpendicular to the membrane. The models of protein translocation through a pore-like structure have been argued against because of the possibility of an electrochemical dissipation across the membrane due to protons crossing through the pore. Further work has to be done to determine if any of these models for lipid bilayer translocation is correct.

1.3.5 Energy requirements for translocation

It has been shown that both ATP and an electrochemical membrane potential are required for the export and subsequent processing of pro-OmpA (outer membrane protein) in *E. coli* (Geller *et al.*, 1986). These energy requirements for protein translocation have been reviewed by Geller (1991) who suggests that SecA binding of ATP allows limited translocation. Further translocation is facilitated by the release of SecA from the precursor, which requires ATP hydrolysis. Pugsley (1993) suggests that ATP hydrolysis may also be involved in the release of SecB, the induction of channel opening (see previous section) or other unfolding reactions. SecA mediated ATP

hydrolysis may occur at an early stage of protein translocation, with the addition of an electrochemical potential initiating the translocation of the rest of the protein (Schiebel *et al.*, 1991; Driessen, 1992; Geller, 1991). SecA could then be released (see above) and recycled to bind to further C-terminal portions of the pre-protein (see figure 1.3). Interestingly Oliver (1993) proposes that this is not the case and suggests that SecA actually penetrates partially into the cytoplasmic membrane and is not released.

1.3.6 Sec-independent export

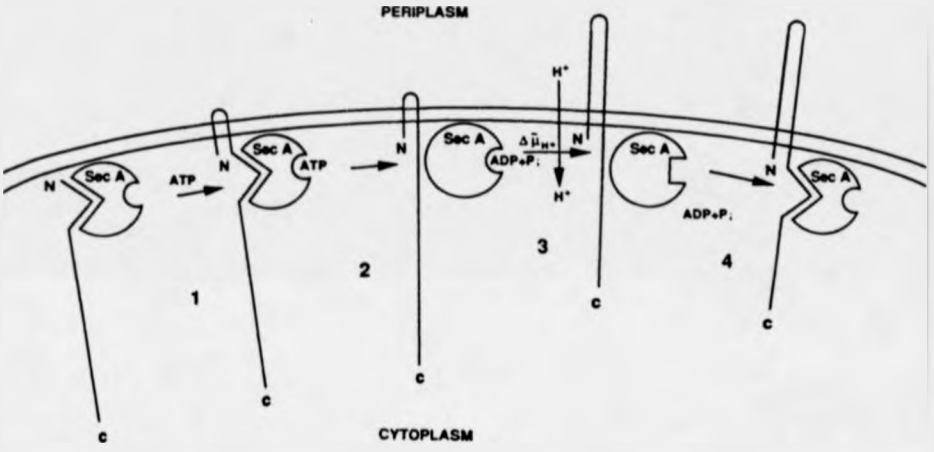
Translocation across membranes can be initiated without the Sec machinery, even though the pre-protein has a typical leader peptide sequence, as is the case for M13 procoat protein (Sugimoto *et al.*, 1977; Wolfe *et al.*, 1985). Other proteins that exhibit Sec-independent translocation include the eukaryotic pre-protein honeybee prepromelittin (Cobet *et al.*, 1989) and a derivative of the LepB signal peptidase (Von Heijne, 1989).

1.3.7 Comparisons to other systems

In eukaryotes the translocation system has been well documented. In the early 1980's two important proteins were identified; the docking protein (DP) (Mayer *et al.*, 1982) and the signal recognition particle (SRP) (Walter and Blobel, 1981). The SRP is a complex of six polypeptides and 1 molecule of RNA which may act as the structural backbone of the particle (Walter *et al.*, 1984). The SRP binds to signal sequences of pre-proteins as they emerge from the ribosome, and retards translocation. This whole complex (SRP-pre-protein-ribosome) then binds to the docking protein (Meyer *et al.*, 1982) whereupon the SRP is released and translation is restored. SRP release from the signal sequence has been shown to require GTP hydrolysis (Connoly *et al.*, 1991). The polypeptide is then translocated across the ER membrane in an as yet unidentified manner. The targeting and recognition cycle is depicted in figure 1.4 (Geller, 1991).

One intriguing question that researchers are asking is whether there is a bacterial form of the SRP ? A number of minor comparisons have been found. Firstly, there has

Figure 1.3 A possible SecA recycling model for protein translocation across the inner membrane of *Escherichia coli*.



Taken from Geller (1991)

Key:

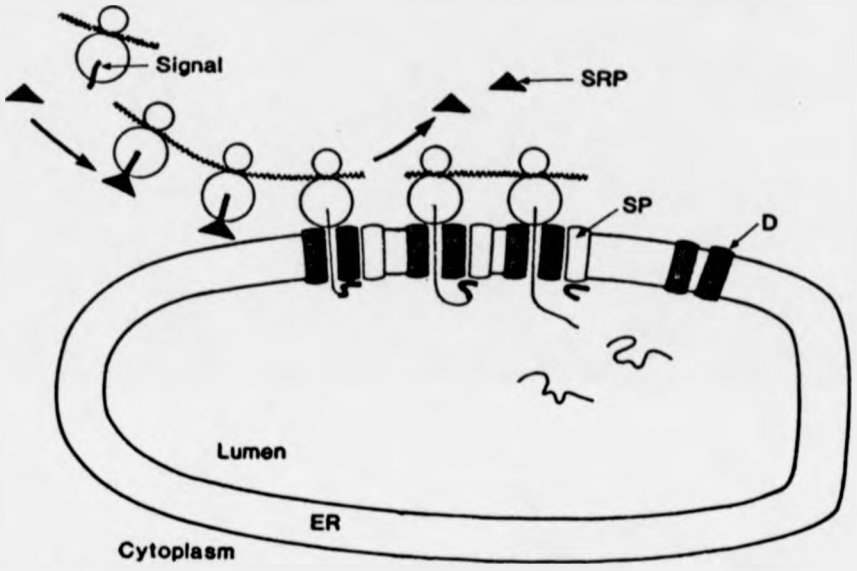
1. SecA binding of ATP enables the insertion and partial translocation of the preprotein.

2. ATP hydrolysis enables the dissociation of SecA from the preprotein.

3. An electrochemical potential drives further translocation.

4. SecA reassociates with a more C-terminal part of the protein, and steps 1-4 are repeated.

Figure 1.4 A proposed model of signal recognition particle (SRP) mediated protein translocation across the endoplasmic reticulum (ER).



Taken from Holland *et al.*, (1986)

The signal recognition particle (SRP) engages with the N-terminal signal sequence of a ribosome-nascent polypeptide. The SRP-ribosome-polypeptide complex connects with the endoplasmic reticulum docking protein (DP). The SRP is released and the polypeptide is translocated, co-translationally, into the lumen of the ER, through a pore formed by the DP. Signal peptidase cleaves the signal sequence and the polypeptide is released into the lumen.

been shown to be homology between the 7SRNA backbone of the SRP complex and bacterial 4.5SRNA [In *E. coli* (Poritz *et al.*, 1988, 1990), in *Mycoplasma pneumoniae* (Simoneau and Hu, 1992)]. Secondly, there has been shown to be sequence similarity between the *ffh* gene product, a GTP binding protein, and the 54 KDa protein of SRP (Romisch *et al.*, 1989). Thirdly, there has been shown to be homology between the α subunit of the SRP receptor in the ER membrane and the *fts*Y gene product that is associated with *E. coli* cell division (Romisch *et al.*, 1989). To date there has been no bacterial SRP found and it is thought that although basic similarities between the eukaryotic and prokaryotic models exist, there seems to be a much more complex system in eukaryotes.

1.4 Gram negative bacterial secretion - a one or two step process ?

As discussed in section 1.2, for extracellular proteins to be targeted to the external milieu is like attempting to cross an "assault course" of obstacles ; 2 lipid bilayers, periplasmic proteins, lipoproteins, lipopolysaccharide, peptidoglycan and phospholipids. Although there are many difficulties in translocating the Gram negative cell envelope many proteins are secreted by various bacteria, see table 1.2. In order to cross these obstacles, there appears to be two general types of secretion pathway, see figure 1.5. Each model will now be discussed.

1.4.1 One step secretion

This secretion pathway does not require the pre-protein to possess a leader sequence for translocation. Many proteins from different bacteria are secreted in this way (see table 1.3). The best studied of these is that of α haemolysin toxin secretion by *E. coli*.

α haemolysin is encoded by the structural gene *hly*A and is translocated, in a Sec-independent fashion, via the secretory proteins HlyB and HlyD (Holland, 1989). HlyA in initially synthesized as a non-toxic prohaemolysin (proHlyA) which is activated to the mature toxin by the product of *hly*C (Nicaud *et al.*, 1986). It is now known that HlyC acts with a low molecular weight cytosolic polypeptide (acyl-ACP), to fatty acylate proHlyA thereby activating it to HlyA (Hardie *et al.*, 1991; Hughes *et al.*,

Table 1.2 Extracellular proteins secreted by some Gram negative bacteria

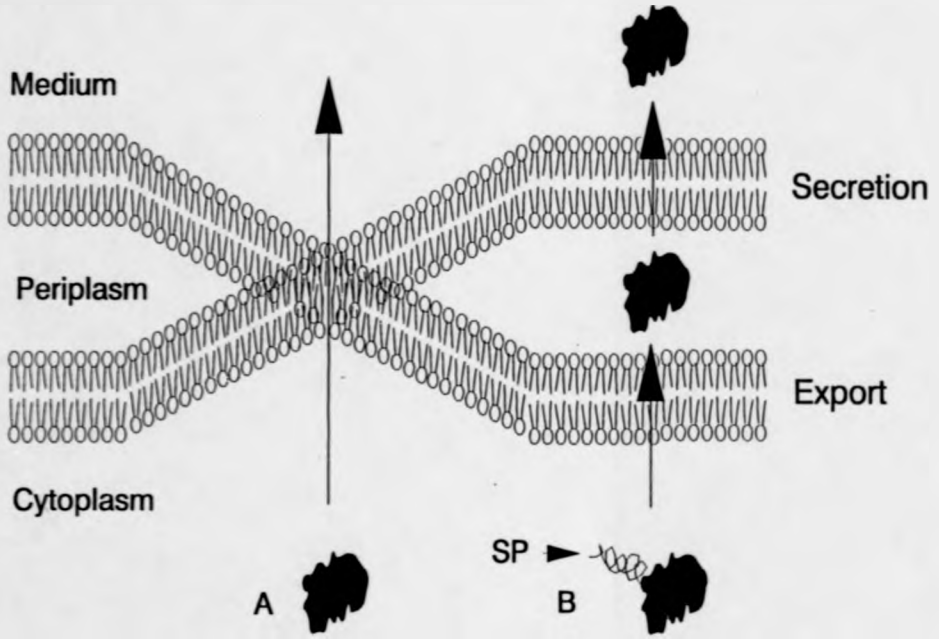
Bacterium	Secreted protein(s)
Escherichia coli	Heat labile enterotoxin
	Heat stable enterotoxin
	α-Hemolysin
	Colicins
Salmonella typhimurium	Colicins
Citrobacter freundii	Endo-ß-D-galactosidase
Klebsiella oxytoca	Pullulanase
Serratia species	Proteases
	Nucleases (RNA and DNA)
	Lipase
	Colicin L
Vibrio cholerae	Cholera toxin (CT)
	α-Hemolysin
Vibrio species	Protease
	Collagenase
Erwinia species	Proteases
	Cellulases
	Pectinases
Pseudomonas aeruginosa	Phospholipase C (hemolysin)
	Toxin A
	Alkaline phosphatase
	Staphylolytic enzyme
	Protease
	Elastase
	Pyocins (S type)

Table 1.2 (cont.)

Bacterium	Secreted protein(s)
Pseudomonas aeruginosa (cont.)	Alginase
Pseudomonas species	Agarase
	Protease
	Poly-ß-hydroxybutarate depolymerase
Haemophilus influenzae	IgA protease
Neisseria gonorrhoea	IgA protease
Aeromonas hydrophila	Protease
	Hemolysin
	Acyltransferase
Yersinia enterolytica	Heat-stable enterotoxin
Bacteroides fragilis	Endo-ß-D-galactosidase
Bordatella pertussis	Adenylate cyclase

Information taken from Pugsley and Schwartz (1985).

Figure 1.5 A simple model of one or two step secretion.



Key:

A - This depicts a protein translocating both membranes from the cytoplasm to the external medium in one complete step. Translocation is facilitated, in this model, by the fusion of both membranes. This could create a pore to enable protein translocation.

B - In this model a cytoplasmic protein translocates both membranes in two steps. Firstly, the protein translocates the inner membrane via a signal peptide (SP) (export). Secondly, once exported to the periplasm the signal peptide is cleaved and the protein translocates the outer membrane (secretion).

Table 1.3 Secretion systems that do not require a typical signal sequence

Bacterium	Secreted protein	Reference
Escherichia coli	Colicin V	Gilson *et al.*, (1990)
	Haemolysin A (HlyA)	Felmlee *et al.*, (1985)
Pasteurella hemolytica	Leukotoxin (LktA)	Felmlee *et al.*, (1985)
Bordatella pertusis	Adenylate cyclase (CyaA)	Glaser *et al.*, (1988)
Proteus vulgaris	Haemolysin A (HlyA)	Koronakis *et al.*, (1987)
Morganella morganii	Haemolysin A (HlyA)	Koranakis *et al.*, (1987)
Actinobacillus actinomycetemcomitans	Leukotoxin (AaLtA)	Lally *et al.*, (1989)
Actinobacillus pleropneumoniae	Haemolysin A HlyA	Frey and Nicolet (1988)
Rhizobium leguminosarum	NodO	Economou *et al.*, (1990)
Erwinia chrysanthemi	Protease (PrtA, B, C)	Létoffé *et al.*, (1990); Ghigo and Wandersman (1992)
Serratia marcescens	Protease (PrtS, M)	Nakahama *et al.*, (1986)

Information taken from Stanley *et al.*, (1991) and Ghigo and Wandersman (1992).

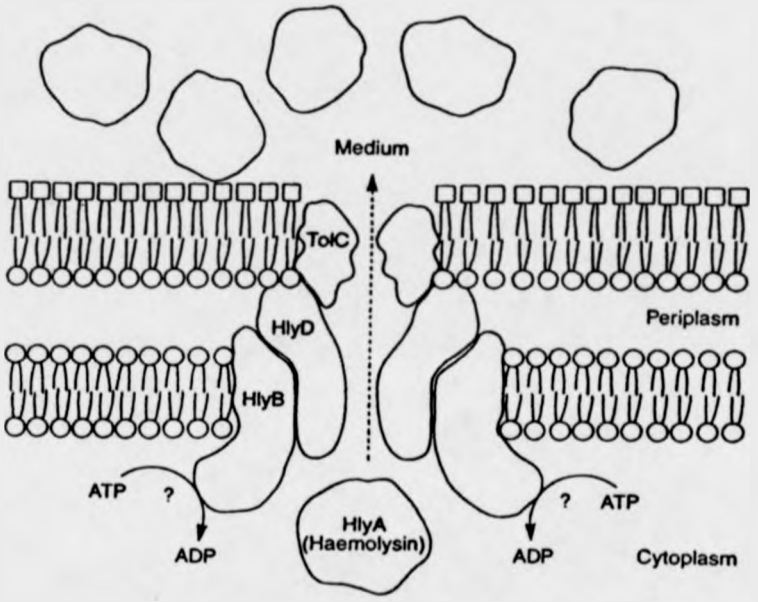
1992).

The four genes *hly*C, A, B, and D are found adjacent to each other, and map to a 7.5Kb DNA fragment (Mackman *et al.*, 1985; Welch *et al.*, 1983). Wandersman and Delepelaire (1990) showed that the *tol*C gene encoding an outer membrane protein of *E. coli*, which maps outside the *hly* cluster, is also required for haemolysin secretion. No periplasmic secretion intermediates of HlyA have been found (Felmlee and Welch, 1988; Koronakis *et al.*, 1989), which suggests that secretion may be via a pore or channel formed by the interaction of HlyB, D and TolC.

HlyB and HlyD are associated largely with the inner membrane (Wang *et al.*, 1991; Mackman *et al.*, 1985). These proteins have also been shown to fractionate with the outer membrane (Wang *et al.*, 1991) which indicates possible membrane adhesion zones. Early topology studies of HlyB, using ß-lactamase fusions, indicated that the protein consisted of a large cytoplasmic domain containing a predicted ATP binding site, three or four periplasmic loops and two cytoplasmic loops (Higgins *et al*, 1986). More recent studies using Tn*lac*Z and Tn*pho*A fusions suggest that HlyD has one membrane spanning domain (Wang *et al.*, 1991) and HlyB has eight membrane spanning domains (Wang *et al.*, 1991).

HlyB is known to have a predicted ATP binding site (Higgins *et al.*, 1986) which resides in the cytoplasmic domain of the protein. HlyB has been shown to be one of a number of proteins, from quite a variety of transport processes, that utilise ATP (Blight and Holland, 1990). It is the C-terminal part of HlyB that contains the targeting signal for translocation, interacting with the membrane associated HlyB and D proteins (Koronakis *et al.* 1989, see figure 1.6). Koronakis *et al.* (1989) mapped the signal sequence to within the last 53 amino acid residues. Recently, Kenny *et al.* (1992) identified 4 residues, by point mutational analysis, within the last 46 amino acid residues, that were essential for maximum secretion. Kenny *et al.* (1992) proposed that at least 4 residues form multiple contact points with the HlyB, D complex. This was in agreement with work by Stanley *et al.* (1991) who also showed large secretion

Figure 1.6 A predicted model for the secretion of hemolysin toxin by *Escherichia coli*.



Taken from Salmond and Reeves (1993)

defects (50-80%) with various, scattered, single, missense mutations in the C-terminal region. Koronakis *et al.* (1989) have shown that the C-terminal region between different haemolysins of *E. coli* and *Proteus* species is well conserved, whereas the C-terminal regions of distantly related HlyB, D type secretory systems is not.

1.4.2 Two step secretion

So far I have discussed mechanisms by which proteins can traverse the whole Gram negative cell envelope in one complete step, or can translocate across the inner membrane, to the periplasm (export), by a Sec dependent route. How does an extracellular protein translocate the outer membrane ? One type of secretion appears to be self promoted and includes the secretion of several bacterial proteases found in pathogenic species of *Serratia*, *Haemophilus* and *Neisseria* (Lory, 1992). Pohlner *et al.* (1987) have suggested a model for the secretion of IgA protease which will be briefly discussed here.

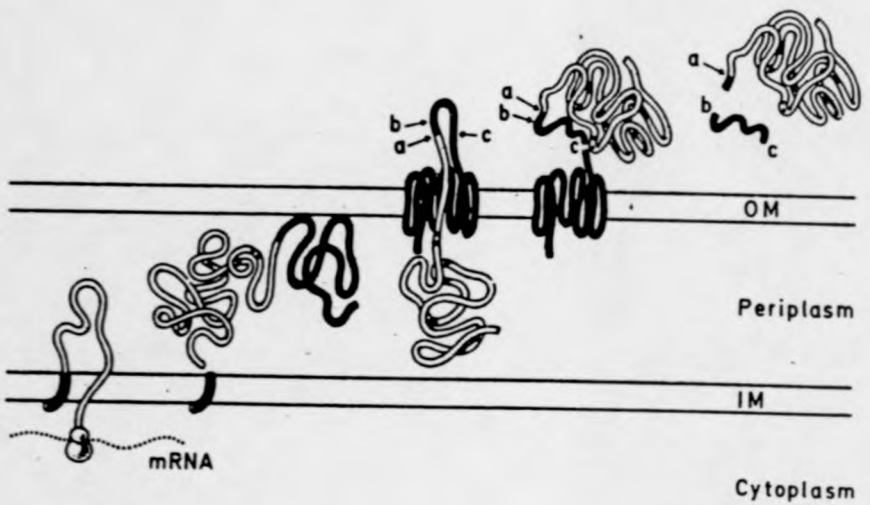
1.4.2.1 Secretion of IgA protease by *Neisseria gonorrheae*

The IgA protease precursor (69KDa) is initially directed to the periplasm with an N-terminal signal peptide (see section 1.3.1) where it is cleaved, probably by a LepB type signal peptidase. The cleaved protein has a protease domain, α domain and a ß domain (IgA_{β}). The ß domain (IgA_{β}) integrates into the outer membrane and probably forms a channel through which the N-terminal end is threaded (Klauser *et al.*, 1992). Once the protein is on the external face of the outer membrane it is autoproteolyticaly cleaved, three times, to release the mature form IgA protease (106KDa), see figure 1.7.

Experiments fusing the non toxic B subunit of cholera toxin (CtxB) to the IgA_{β} domain promotes the translocation and exposition to the external face of the bacterial cell envelope of *Salmonella typhimurium*, but this is dependent on the folded nature of the protein (Klauser *et al.*, 1990; Klauser *et al.*, 1992).

Pugsley (1993) suggests that this type of secretory pathway may require periplasmic chaperones to keep the protein "translocation competent".

Figure 1.7 Proposed model of secretion for IgA protease.



Taken from Pohlner *et al.*, (1987).

The initial export of IgA protease from the cytoplasm to the periplasm is signal peptide dependent (see text for details). The second stage, secretion across the outer membrane, is by the formation of a pore by the C-terminal domain of the immature protein. After translocation of the outer membrane the immature protein is autoproteolytically cleaved three times (a-c) before a mature protein conformation can be achieved.

Not all proteins need to be unfolded/partially unfolded prior to translocation, as is the case for cholera toxin. Cholera toxin is composed of 5 B subunits and one A subunit, which is fully assembled as a holotoxin, in the periplasm, prior to translocation (Hirst and Holmgren, 1987).

Other, more widespread mechanisms, are now known to exist. These other systems have been termed the general secretion pathway (GSP) (Pugsley, 1992). The first step of this pathway has been discussed, see section 1.3. It is the second stage of secretion that is currently under intensive research.

1.4.2.2 The general secretory pathway (GSP)

Several clusters of genes have been identified as having a secretory role (also see sections 1.4.2.3 and 1.5.4). These GSP systems have been shown to share significant homology at the protein level, see section 1.5.3. Mutations within these clusters of genes cause pleiotropic secretion defects, see table 1.4. The intracellular accumulation of normally extracellular enzymes has been localised, in most cases, to the periplasm.

It is interesting to note here that there appears to be no similar system in *E. coli*. When the structural genes for some extracellular enzymes have been cloned and expressed in *E. coli*, the enzyme(s) have been localised to the periplasm (Collmer *et al.*, 1985; Pugsley *et al.*, 1990(a); Pugsley *et al.*, 1990(b)). This indicated that *E. coli* was lacking certain secretion factors necessary for the final secretion step across the outer membrane. The secretion of the periplasmically accumulated protein in *E. coli* could be restored by adding the essential accessory proteins to the strains, in only a few cases (D'Enfert *et al.*,,1987; He *et al.*, 1991(a)). These results suggested a two step process was required for extracellular protein trafficking across both Gram negative cell membranes. The export step was shown to be Sec dependent in both *Klebsiella oxytoca* (Pugsley *et al.*, 1991(a); Pugsley *et al.*, 1991(b); Pugsley *et al.*, 1991(c)) and *Erwinia chrysanthemi* (He *et al.*, 1991(a)). One of the best documented examples of the GSP is that for pullulanase secretion by *K. oxytoca*.

Table 1.4 Pleiotropic secretion defects in various bacteria caused by mutations within specific clusters of genes.

Bacterium	Secretion defect	Reference(s)
Aeromonas hydrophila	Aerolysin	Jiang and Howard, (1991)
	Protease	
	Amylase	
Pseudomonas	Exotoxin A	Filloux *et al.*, (1987)
aeruginosa	Lipase	Lindgren and Wretlind (1987)
	Phospholipase	
	Elastase	
Xanthomonas	Cellulase	Hu *et al.*, (1992)
campestris	Proteases	Dums *et al.*, (1991)
pv. *campestris*	Polygalacturonate Lyase	
	Amylase	
	Endoglucanase	
Klebsiella oxytoca	Pullulanase	D'Enfert *et al.*, (1989)
		D'Enfert and Pugsley, (1989)
		Reyss and Pugsley, (1990)
		Pugsley and Reyss, (1990)
Erwinia carotovora	Pectate lyase	Andro *et al.*, (1984)
subspecies	Cellulase	Ji *et al.*, (1987)
carotovora and	Polygalacturonase	Thurn and Chaterjee,
(1985)		
Erwinia chrysanthemi		Gibson *et al.*, (1988)
		Reeves *et al.*, (1993)
		Murata *et al.*, (1990)
		He *et al.*, (1991)
		This study

For a discussion of the secretion systems involved, see section 1.5.4.2 and 1.5.4.3.

1.4.2.3 Pullulanase secretion by *Klebsiella oxytoca*

Secretion of pullulanase by *Klebsiella oxytoca* has been the subject of two recent reviews (Pugsley *et al.*, 1990(a); Pugsley, 1993). This section will discuss the main features of pullulanase secretion and the current research progress.

Pullulanase is a 117KDa maltose inducible lipoprotein that acts as a starch debranching enzyme on the substrate pullulan. Pullulanase is anchored to the cell surface before slowly being released into the external medium, probably in the form of protein micelles (Pugsley, 1993). The expression and secretion of pullulanase is regulated by maltose and is dependent upon the transcriptional activator protein MalT (Van-Ingligliardi, 1991).

In order to study the secretion process the structural gene for pullulanase (*pul*A) was cloned and expressed in *E. coli* (Pugsley *et al.*, 1990(b)) whereby pullulanase was routed to the periplasm. d'Enfert *et al.* (1987) cloned a 18.8Kb chromosomal DNA fragment that enabled *E. coli*, harbouring *pul*A, to efficiently secrete pullulanase. Mutations in genes flanking *pul*A affected secretion (d'Enfert *et al.*, 1987; Kornacker *et al.*, 1989). Pugsley *et al.* (1986) showed that the pullulanase precursor is synthesized with an N-terminal signal peptide, which is cleaved by a lipoprotein peptidase and fatty acylated prior to secretion (Pugsley *et al.*, 1990(b)). Recent studies have shown that the initial step in pullulanase secretion follows the Sec dependent pathway, as described earlier (Pugsley *et al.*, 1991(a); Pugsley *et al.*, 1991(b); Pugsley *et al.*, 1991(c)). After translocation of the inner membrane, pullulanase remains anchored to the cytoplasmic membrane in an, as yet, undetermined manner (Pugsley *et al.*, 1991(b)). This sorting to the cytoplasmic membrane is thought to be due to the Asp residue at position +2 (Yamaguchi *et al.*, 1988), which, when replaced by asparagine is sorted to the periplasmic face of the cytoplasmic membrane (Kornaker and Pugsley, 1991). Pugsley *et al.* (1991(b)) suggested that pullulanase is transported in a partially folded configuration, using the accessory proteins. This configuration was predicted because DTT did not reduce a putative disulphide bond, unless the protein

was denatured by heating.

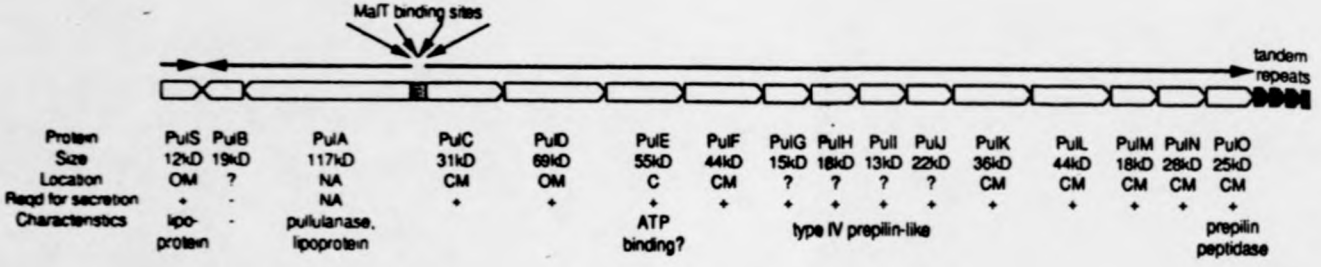
The *pul* cluster has been shown to contain a total of 16 genes, see figure 1.8. Various molecular techniques including subcloning, deletion analysis, sequencing and Tn*tac*-1 and Tn*pho*A mutagenesis have revealed that 13 of these (*pul*C-O) are essential for the secretion of pullulanase (d'Enfert and Pugsley, 1989; Reyss and Pugsley, 1990; Reyss and Pugsley, 1990; Possot *et al.*, 1992).

PulS has been shown via Tn*5* insertional inactivation, to be required for full pullulanase exposition and has been shown by sequence analysis to be transcribed in the opposite direction to *pul*A (d'Enfert and Pugsley, 1989). In the same study, it was shown that *pul*S was not part of the MalT regulon and was unaffected by mutations within the *mal*T regulator gene. PulB was found to be transcribed in *trans* with *pul*A and has the same promoter. Tn*5* insertions in this region did not affect pullulanase secretion and as yet, has no known function (d'Enfert and Pugsley, 1989).

1.4.2.3.1 *pul*C-O

The pulC-O gene products have been expressed using the T7 bacteriophage promoter system (see section 6.3.3 for details) in order to identify each of the proteins and to localise them by subfractionation. The results of this are shown in figure 1.8. Topology studies using Tn*pho*A and *lac*Z fusions have shown that PulO spans the membrane many times (Pugsley and Reyss, 1990). PulF spans the membrane 3 times with the N-terminal and large central loop in the cytoplasm (Possot *et al.*, 1992) and PulL spans the membrane once (Pugsley and Reyss, 1990). The rest of the PulC-O proteins, except PulE, are inner membrane associated with only PulD outer membrane associated (Pugsley *et al.*, 1990(b)). PulG-J have been implicated in the formation of a pilus like structure between the two membranes (see next section and also section 1.5.4.3) and can be found to fractionate with outer membrane vesicles under osmotic lysis (Pugsley and Dupuy, 1992; Possot *et al.*, 1992).

Figure 1.8 The organisation of the *pul* gene cluster and some of the characteristics of the protein products.



Taken from Pugsley (1993).

Four MalT binding sites are present in the promoter region between *pul*A and *pul*C. These regions control the transcription of *pul*C-O and *pul*A-B. The expression of *pul*C-O and *pul*A-B is induced in the presence of maltodextrins. *pul*S transcription is independent of the MalT regulon and is not induced in the presence of maltodextrins. The location of the proteins was determined by subcellular fractionation of cells induced in the T7 bacteriophage promoter system or by the production of Tn*pho*A or *lac*Z fusion proteins. PulG-J were initially though to associated with the cytoplasmic membrane but have now been shown to fractionate with outer membrane vesicles under osmotic lysis. See text for further information and discussion.

NA, not applicable.

1.4.2.3.2 Functions of Pul proteins ?

It is still unknown exactly what each of the accessory proteins role is in the secretory process. Some of their putative roles are discussed later, in section 1.5.4.3.

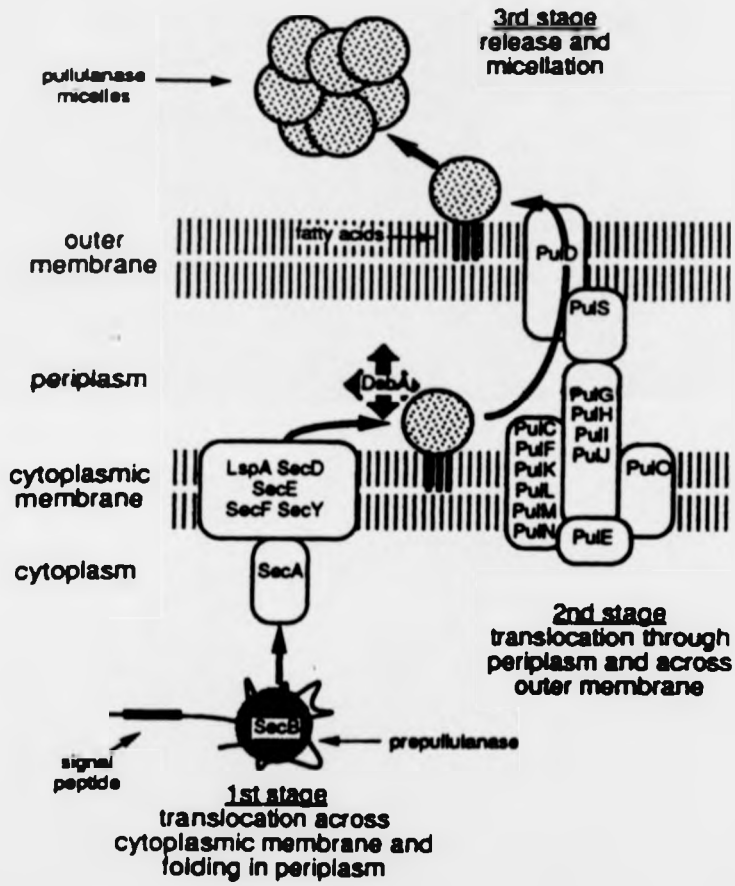
Pugsley (1993) proposed a putative working model for pullulanase secretion, figure 1.9, based on work discussed above and some of the following; PulE is depicted as being associated with the cytoplasmic face of the inner membrane because of its predicted ATPase/kinase activities which may be needed for the interaction with inner membrane proteins of the system (Possot *et al.*, 1992; see sections 1.5.4.3 and 5.5.3). PulO is depicted to be in close proximity with PulG-J because of its type IV prepilin peptidase activity. This has been demonstrated in the processing of PulG-J that have N-terminal type IV signal peptides (Pugsley and Dupuy, 1992). Further discussion of these two features and others will be in section 1.5.4.3.

1.5 Protein targeting in *Erwinia*

1.5.1 Why study protein secretion in *Erwinia* ?.

Erwinia is a good organism with which to study secretion because it secretes a variety of extracellular enzymes, see table 1.4, which can be easily assayed by spectrophotometry. *Ecc* SCRI193 is amenable to a variety of *E. coli* techniques. One major development in the manipulation of *Ecc* was the introduction of the *lam*B plasmid (pHCP2) to SCRI193 (HC131) which enabled infection by Lambda phages (Salmond *et al.*, 1986). This sytem was used as a delivery system for transposons and cosmids to *Ecc*. More recently, the isolation of the *Ecc* generalised transducing phage øKP (Toth *et al.*, 1993) has enabled other genetic techniques such as localised mutagenesis (this study).

Figure 1.9 A model for pullulanase secretion by *Klebsiella oxytoca*.



Taken from Pugsley (1993).

PulE is shown as being associated with many Pul proteins on the cytoplasmic face of the inner membrane. PulG-J are shown as a complex spanning the periplasm and pullulanase is shown to translocate the outer membrane via PulD. None of these points have been proven experimentally but are discussed in the text.

Key:

DsbA - disulfide oxidoreductase.

1.5.2 What is *Erwinia*?

In the early 1970's there was much debate as to whether or not to classify the erwinias as members of the Enterobacteriaceae as they were mainly associated with plant disease. Starr and Chatterjee (1972) attempted to clarify the situation and confirmed the erwinias association with the Enterobacteriaceae. The erwinias are Gram negative, facultatively anaerobic, motile, non-spore forming short rods. Members of the erwinias are pathogenic to plants and saprophytes. In this section I shall only be discussing the group of erwinias that have strong pectolytic capacities and are termed the "soft-rot" group.

One member of the soft-rot group is *Erwinia carotovora* which can be divided into the following subspecies; *carotovora* (*Ecc*), *atroseptica* (*Eca*) and *betavasculorum* (*Ecb*). Other members of the soft-rot group are *Erwinia chrysanthemi* (*Ech*), and the restricted host range erwinias *Erwinia cypripedii* and *Erwinia rhapontici* (Lelliot and Dicky, 1984; Pérombelon and Kelman, 1980).

The soft-rot group characteristically produce large amounts of pectolytic enzymes that break down plant tissue causing soft-rot (Pérombelon, 1985). The soft-rot group are both biochemically and serologically quite similar but differ, quite markedly, in their growth temperature optima; *Eca* <25°C, *Ech* <39°C, *Ecc* <36°C (Pérombelon, 1987). These factors affect the geographical distribution of the soft-rot erwinias. *Ecc* can be found in temperate and tropical zones infecting both stored and field plants including; potato, celery, turnip and cucumber. *Ech* can be found in more subtropical and tropical climates but can also be found in temperate regions infecting greenhouse crops such as philodendron and carnation. *Eca* is restricted to cooler climates and is found mainly associated with potatoes.

1.5.3 Pathogenicity

Eca causes blackleg which usually occurs as a sequel to the mother tuber rotting, and subsequent invasion of the stem. The stem rot causes yellowing of the leaves and wilting. *Ecc* and *Ech* cause soft-rot of stored and "in field" crops, such as potato, by

the production of tissue macerating enzymes (Pérombelon, 1987). The development of the disease is influenced by many environmental factors, such as anaerobic conditions, high bacterial numbers, water and temperature (Pérombelon and Kelman, 1980; Pérombelon, 1985).

Many mechanisms have been shown to influence the dispersal of the soft-rot erwinias (Harrison and Brewer, 1982). The most significant factors appear to be; aerosols, soil and water movement, soil fauna (earthworms and nematodes) and agricultural practices.

1.5.3.1 Factors that affect pathogenicity in "soft-rot" *Erwinia*

Some of the less well studied factors that are involved in pathogenicity include; iron aquisition, lipopolysaccharide (LPS) and motility. Many pathogenic miro-organisms compete with their host for available iron, early on in the infection. This is thought to be the critical period for the outcome of the infection. The hypoferraemic response to infection, by the plant, is thought to combat the pathogenic response of the micro-organism (Kotoujansky, 1987). The sequestration of iron by micro-organisms is facilitated by siderophores or specific receptors that bind lactoferrin or transferrin (Williams and Roberts, 1989). Siderophores are iron specific ligands that are secreted in response to iron limitation. Mutations that cause a depletion in iron assimilation in *Ech*3937, exhibited reduced virulence on Saintpaulia plants, even though they had wild type levels of the degradative extracellular enzymes Pel and Cel (Ennard *et al*, 1988; Expert and Toussaint, 1985). These mutants were thought to be defective in the receptor for siderophores or the siderophore biosynthetic pathway.

Lipopolysaccharide is composed of a high molecular weight heterologous chain ("O" antigen), core oligosaccharide and lipid A. Mutants of *Ech* that are defective in the core oligosaccharide are avirulent and are also resistant to bacteriophage øEC2 infection, even though they secrete wild type levels of extracellular degradative enzymes (Schoonejans *et al.*, 1987). Similar findings of avirulent strains of *Ecc* with altered LPS have been isolated in this laboratory (I. Toth and C. Thorpe, pers.

comm.). This data suggests that the core oligosaccharide is essential for virulence.

Motility minus (Mot$^-$) strains of *Ecc* have been isolated that exhibit reduced virulence (Rvi$^-$) by transposon mutagenesis (Pirhonen *et al.*, 1991; V. Mulholland, pers. comm.). Other characteristics of the mutant strains resembled the wild type, except for motility and a reduced spreading of soft-rot symptoms.

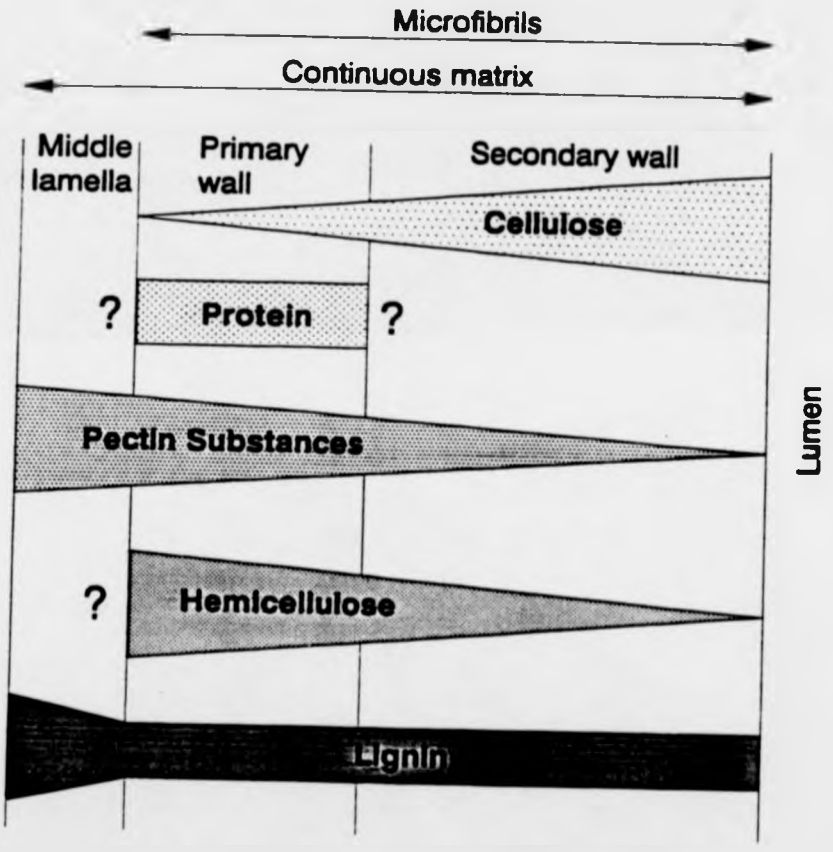
The major pathogenicity determinant of the soft-rot erwinias is thought to be their capacity to secrete a wide range of degradative extracellular enzymes (for reviews see; Daniels *et al.*, 1988; Kotoujansky, 1987). Examples of these include; cellulase (Cel), protease (Prt), pectate lyase (Pel) and polygalacturonase (Peh). It has been shown that mutants of *Ecc* or *Ech* defective in the secretion of Pel, Cel and Peh are avirulent (Andro *et al.*, 1984; Murata *et al.*, 1990). Multiple Pel isozymes are thought to be the major pathogenic determinants in soft-rot (Collmer and Keen, 1986; Payne *et al*, 1987; Boccara *et al.*, 1988). The most important features of some of these enzymes will now be discussed.

1.5.3.2 Plant degradative enzymes of soft-rot erwinias

1.5.3.2.1 Pectate lyase

Ech secretes into the external medium 5 pectate lyases and 1 pectin methylesterase (Pme) that attack and degrade pectin in the plant cell wall (Collmer and Keen, 1986; see figure 1.10). Two forms of Pel enzymes are present in *Erwinia* spp., exo and endo, that cleave α-1,4 glycosidic bonds by ß elimination. This is random for endo-Pel or specific, at chain termini, for exo-Pel (Collmer and Keen, 1986). *Ech* then utilizes the degradation products of pectin as a carbon source for growth and propagation in planta (Reverchon *et al.*, 1991). The Pel isoenzymes (PelA-E) have been characterised by electrofocusing, see table 1.5. The genes encoding PelA-E have been cloned and characterised (Hugouvieux-Cotte-Pattat *et al.*, 1989). The genes have been mapped to two gene clusters on the *Ech* chromosome. One cluster contains *pel*B and C and the other *pel*A, D and E, which, in the case of *Ech* 3937, also contains the *pme* gene.

Figure 1.10 The structure of the plant cell wall.



Taken from Bateman and Basham (1986).

Table 1.5 Characteristics of Pel isoenzymes

Strain	Isoenzyme	Isoelectric point	pI	Reference
Erwinia chrysanthemi				
Ech 3937				
	Pel A	Acidic	4-5	Kotoujansky, 1987
	Pel B	Neutral	7-8	"
	Pel C	Neutral	7-8	"
	Pel D	Alkaline	9-10	"
	Pel E	Alkaline	9-10	"
Erwinia carotovora				
Ecc ECRI193				
	Pel A	Neutral	7-8	Hinton *et al.*, (1989(a))
	Pel B	Neutral	7-8	"
	Pel C	Alkaline	10-11	"
	Pel D	Alkaline	10-11	"

These genes are not arranged in operons and can be individually transcribed (Reverchon *et al.*, 1986; Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1989).

Four *pel* genes were isolated by Plastow *et al.* (1986) from *Ecc* SCRI193. PelA and B have subsequently been shown to be localised to the periplasm while PelC and D were shown to be extracellular (Hinton *et al.*, 1989). The characteristics of each protein is described in table 1.5. The structural gene arrangement is not confirmed but investigations suggest that *pel*C and D are linked and *pel*A and B are thought to map elsewhere.

Boccara *et al.* (1988) have suggested three reasons for *Ech* to produce so many isozymes of Pel. Firstly, several Pel's may be needed for the complete degradation of pectin. Secondly, it is possible that several Pel's are differentially induced during the disease. Thirdly, Pel isozymes may be required for degradation in different parts of the plant. It is thought that the more alkaline Pel's have a more significant effect on the pathogenicity than for the neutral or acidic Pel's (Boccara *et al.*, 1988; Keen and Tamaki, 1986; Payne *et al.*, 1987).

1.5.3.2.2 Pectin lyase (Pnl)

The pectin lyase structural gene (*pnl*A) of *Ecc*71 has been cloned and sequenced (McEvoy and Chatterjee, 1990; Chatterjee *et al.*, 1991). No typical signal sequence was found.

1.5.3.2.3 Pectin methylesterase (Pme)

This enzyme acts by de-esterifying pectin by removing methoxyl groups to yield polygalacturonic acid (PGA) and methanol. The structural gene is located 3' to *pel*D in *Ech*3937 (see earlier). Reduced systemic infection on Saintpaulia plants was seen in *pnl*$^-$ mutants (Boccara and Chatain, 1990). The exact role of the enzyme in pathogenicity is not yet known.

1.5.3.2.4 Polygalacturonase (Peh)

Peh specifically catalyses the generation of galacturonic oligomers by hydrolysis of pectin α-1,4-glycosidic bonds and is found in *Ecc* and *Eca* but not *Ech* (Kotoujansky, 1987). Endo-Peh genes have been cloned from *Ecc* and *Eca* (Lei *et al.*, 1985; Plastow *et al.*, 1986; Saarilahti *et al.*, 1990(a); Hinton *et al.*, 1990). One exo-Peh gene has been cloned and sequenced from *Ech* (He and Collmer, 1990) and a putative exo-Peh gene has now been sequenced in *Ecc* (Wharam, 1992).

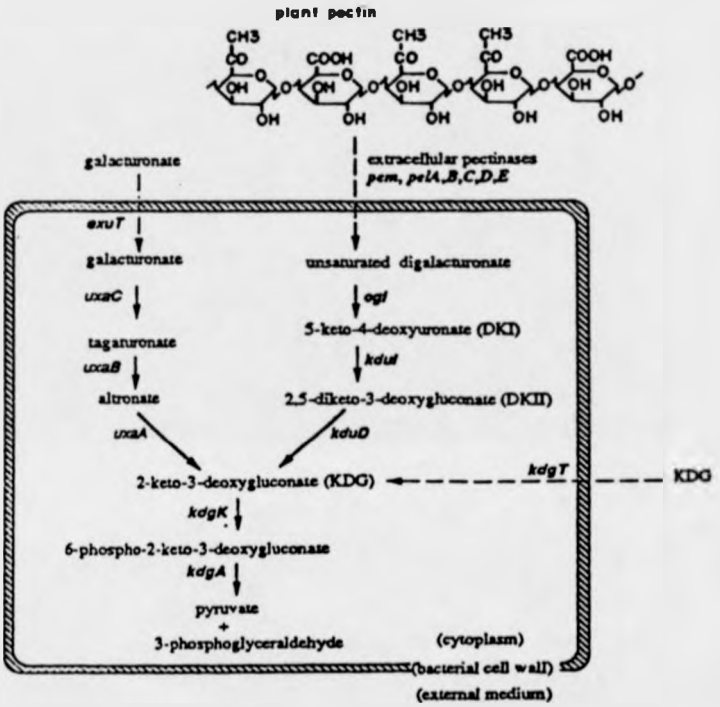
1.5.3.2.5 Regulation of pectinases

It has been suggested that the success or failure of infection is controlled by Pel induction (Kotoujanski, 1987). Pel synthesis is induced in the presence of PGA and oligogalacturonates. The "real" inducers though appear to be the breakdown products of oligogalacturonates, in particular 2-keto-3-deoxygluconate (KDG), 2,5-diketo-3-deoxygluconate (DK11) and 5-keto-4-deoxyuronate (DK1), see figure 1.11 (Hugouvieux-Cotte-Pattat *et al.*, 1986; Condemine *et al.*, 1986). It is now known that the KdgR repressor protein is the negative regulator of pectin degradation in *Ech*3937 (Reverchon *et al.*, 1991; Nasser *et al.*, 1992). Many of the genes controlled by KdgR have been found to contain a consensus KdgR binding site (Condemine and Robert-Baudouy, 1991). Pel is also subject to catabolite repression by glucose and induced at the end of exponential growth phase (Hugovieux-Cotte-Pattat *et al.*, 1986). Self catabolite repression has also been demonstrated with high concentrations of inducing molecules (Collmer and Bateman, 1981). Various *pec* genes have also been implicated in regulation in *Ech* which will not be discussed here except to say that the regulation of the pectinolytic degradation pathway is an extremely complex system and is under intense study. Investigation into *Ecc* Pel regulation is currently in progress.

The *peh* gene cloned from *Ecc* SCRI193 was shown by sequence analysis to contain a putative KdgR binding site (Hinton *et al.*, 1990). This could be involved in the regulation of this gene.

McEvoy and Chatterjee (1990) demonstrated that *Ecc*71 *pnl* expression was induced,

Figure 1.11 The pectin and galacturonate degradation pathway in *Erwinia chrysanthemi*.



Taken from Nasser *et al.* (1992)

The steps in the degradation pathways are catalysed by the gene products of the following genes:

Gene Gene product

pem - pectin methyl esterase
pel - pectate lyase (pelA-E)
ogl - oligogalcturonate lyase
*kdu*I - 5-keto-4-deoxyuronate isomerase
*kdu*D - 2-keto-3-deoxygluconate oxidoreductase
*uxa*C - uronate isomerase
*uxa*B - altronate oxidoreductase
*uxa*A - altronate hydrolyase
*kdg*K - 2-keto-3-deoxygluconate kinase
*kdg*A - 2-keto-3-deoxy-6-phosphogluconate aldolase

*kdg*T and *exu*T - the protein products are invoved in the transport of galacturonate and 2-keto-3-deoxygluconate into the bacterium.

along with cell lysis and the bacteriocin carotovoricin, with mitomycin C, a DNA damaging agent. This was subsequently shown to be *rec*A$^+$ dependent but not in *rec*A$^+$ *E. coli* suggesting the absence in *E. coli* of a *rec*A$^+$ mediated positive regulator.

1.5.3.2.6 Cellulase

Cellulase secreted from *Ech* and *Ecc* are ß-1,4-glucanases. They breakdown the ß,1-4 linked glucose polymers that constitute cellulose. Both exo- and endo-glucanases are thought to act in tandem to degrade cellulose (Wu *et al.*, 1988). *Ech* produces two endoglucanases, CelY and Z. CelZ exhibits 95% of the total Cel activity (Boyer *et al.*, 1984) and is synthesized with a cleavable N-terminal signal sequence (Guiseppi *et al.*, 1988). In *Ecc* CelV has been cloned and sequenced to reveal an putative N-terminal signal sequence and is homologous to CelZ (V. Cooper pers. comm.). Expression of CelV in *E. coli* results in the periplasmic accumulation of this enzyme, which is an endoglucanase. CelS has been cloned from SCC3193 (Saarilahti *et al.*, 1990(a)) and is an endoglucanase. In *Ech cel* genes are constitutively expressed and are subject to catabolite repression (Boyer *et al.*, 1984).

1.5.3.2.7 Protease

Proteases cleave peptide bonds and may be endo- or exo-peptidases. They are synthesized, initially, as inactive precursors (zymogens) due to the presence of a propeptide (Wandersman, 1989). In *Ech* three structural protease genes, *prt*A, B and C, have been cloned and sequenced (Wandersman *et al.*, 1987; Ghigo and Wandersman, 1992). *prt*B and C are two distinct transcriptional units with *prt*A 3' to them both (Delepelaire and Wandersman, 1989; Ghigo and Wandersman 1992). *Ech* produces a protease inhibitor that is periplasmic and is thought to protect the cell against incorrectly localised or processed protease (Wandersman *et al.*, 1987; Wandersman, 1989; Létoffé *et al.*, 1989). The secretion pathway for protease in soft-rot *Erwinia* spp. is discussed in the next section.

1.5.4 Investigation of extracellular enzyme secretion in *Ecc* and *Ech*

Andro *et al.* (1984) isolated mutants of *Ech*, by chemical and transposon mutagenesis, that were defective in their ability to secrete Pel and Cel to the external medium, although they were demonstrated to be synthesized normally. These normally extracellular enzymes were localised to the periplasm and the secretion mutants were called Out$^-$. Studies in various other laboratories also yielded Out$^-$ secretion mutants in both *Ecc* and *Ech* using chemical and insertional mutagenesis (Thurn and Chatterjee, 1985; Ji *et al.*, 1986; Gibson *et al.*, 1988; Reeves *et al.*, 1993; Murata *et al.*, 1990; He *et al.*, 1991(a)). Where tested, the secretion of protease to the external medium was unaffected, which suggested an independent mechanism of secretion, which will now be discussed.

1.5.4.1 One step secretion in Erwinia

Létoffé *et al.* (1990) have shown that in *Erwinia chrysanthemi* the three proteins PrtD, E and F are essential for PrtB and C secretion via a signal sequence independent pathway. PrtD, E and F show significant homology to HlyB, D and TolC respectively (Létoffé, 1990). The *prt*D, E and F genes are located upstream of the structural genes *prt*B, C and A (Ghigo and Wandersman, 1992). The gene products, PrtD and E, were shown to be inner membrane associated (Delepelaire and Wandersman, 1991) while PrtF was outer membrane associated. It is worth noting that Wandersman and Létoffé (1993) have now demonstrated that α haemolysin secretion is reduced when there are mutations in the lipopolysaccharide (LPS) biosynthesis genes, *gal*U and *rfa*H. It is thought that these LPS biosynthesis genes might be involved in correctly orientating the outer membrane proteins TolC and PrtF.

The protease secretion system can be reconstituted in *E. coli* to secrete PrtB and PrtC (Wandersman *et al.*, 1987). The secretion genes *prt*D, E and F are also able to facilitate the secretion of the extracellular enzyme alkaline protease (*apr*) of *Pseudomonas aeruginosa* (Guzzo *et al.*, 1991) and the *Serratia marcescens* metalloprotease SM (Létoffé *et al.*, 1991).

Fath *et al.* (1991) have reported that the HlyB, D system will facilitate the secretion of colicin V (ColV) and the *Erwinia* PrtB and PrtC in *E. coli*. This, however, was not reciprocal, as neither the *Erwinia* protease system nor the *E. coli* colicin V system enabled activated α-haemolysin secretion (Fath *et al.*, 1991). These results suggest differences in the signal recognition process, which may have diverged in many species. Interestingly it has also been shown (Scheu *et al.*, 1992) that the nodulation protein, NodO, of *Rhizobium leguminosarum*, can be secreted by the *E. coli* HlyB, D system and the *Erwinia* protease system, reconstituted in *E. coli*. This is unusual because of the degree of specificity already described for *Erwinia* protease and colicin V systems. Further investigations into which regions of the NodO protein interact with the secretion system is currently under study. The results found by Kenny *et al.* (1992) led to the suggestion that these differences could be caused by the alteration of specific "contact" residues.

There also exists in *Erwinia* a GSP type system of extracellular enzyme secretion. This type of secretion mechanism will now be discussed and compared to other systems that also use the GSP to secrete extracellular proteins.

1.5.4.2 Two step secretion in Erwinia

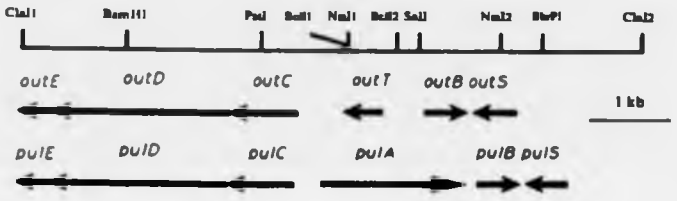
When the structural genes for Pel from *Ech* and *Ecc* are expressed in *E. coli*, the enzyme is exported to the periplasm which suggested that other proteins, accessory proteins, were necessary for the second step in the secretion process. In order to identify these accessory proteins, cosmid libraries of *Ech* or *Ecc* were used to complement Out$^-$ secretion mutants (He *et al.*, 1991(a); Reeves *et al.*, 1993; Murata *et al.*, 1990). For both *Ech* (EC16) and *Ecc* SCRI193, distinct complementation groups were revealed. This suggested the clustering of several genes required for the second stage of secretion. In fact *Ecc*71 DNA was shown to complement Out$^-$ secretion mutants in both *Ecc* and *Ech* as well as *Ecc*71 (Murata *et al.*, 1990). In *Ecc* SCRI193 the insert DNA complementing all the Out$^-$ mutations was subcloned down to a 12.7Kb fragment (Reeves *et al.*, 1993) while that for *Ech* (EC16) was on a 12Kb fragment (He *et al.*, 1991(a)).

The cloned DNA fragment from *Ecc*71 that complemented Out$^-$ mutations in *Ech* and *Ecc* was used in the transformation of *E. coli* harbouring Pel$^+$ and Peh$^+$ plasmids (Murata *et al.*, 1990). This did not enable *E. coli* to secrete Pel or Peh, possibly because *E. coli* lacks specific factors that *Erwinia* has, or the Out proteins were not expressed properly. Reeves *et al.* (1993) also report the unsuccessful attempt to reconstitute the *Ecc* secretion system in *E. coli*. This is interesting because the pullulanase system can be reconstituted in *E. coli* (d'Enfert *et al.*, 1987) and it has now been demonstrated that *Ech out* DNA can enable secretion of *Ech* pectic proteins in *E. coli* (He *et al.*, 1991(a)). He *et al.* (1991(a)) went on to demonstrate that *Ech* PelE could only be secreted with its own *out* DNA *in trans* and not by heterologous hosts such as *Ecc* and *K. oxytoca*. This suggests that there is a species specific secretion factor within the system that facilitates the recognition of host enzymes for targeting.

Is the initial export step Sec dependent as for the pullulanase system? He *et al.* (1991(b)) answered this question for *Ech* (EC16) by culturing SecA(ts) *E. coli* strain harbouring the *Ech out* DNA and PelE$^+$, at the restrictive temperature. This resulted in the accumulation of prePelE and indicated that the process of export is Sec dependent.

Sequencing of the *Ecc* SCRI193 *out* gene DNA revealed that there were 13 open reading frames, *out*C-O (Reeves *et al.*, 1993). Similarly, sequencing of the *Ech* (EC16) *out* DNA revealed 12 ORF's *out*C-M and *out*O (He *et al.*, 1991(a); Lindenberg and Collmer, 1992), see figure 1.12. Neither cluster was shown to be linked to the structural genes for Pel, Cel or Peh. It was interesting to note that *out*N was missing from *Ech*. The 5' end of a similar *out* cluster in *Ech*3937 has also been cloned and sequenced (Ji *et al.*, 1989; Condemine *et al.*, 1992). The sequencing revealed OutS, B, T, C, D and E. OutC, D and E share significant homology to PulC, D and E. The most interesting feature is the homology of OutS and B to PulS and B. This is the first time homologues of PulS or PulB have been found. *out*B is transcribed divergently from *out*C, see figure 1.12, mutations within this gene show only slightly

Figure 1.12 Comparison of the 5' end of the *out* cluster of *Erwinia chrysanthemi* (3937) to the 5' end of the *pul* cluster of *Klebsiella oxytoca*.



Taken from Condemine *et al.* (1992)

The organisation of the *out* genes, and the equivalent *pul* genes, is shown beneath the restriction map of the 7.5 Kb *Cla*I fragment. See text for further details and discussion.

altered secretion, <30% of Pel accumulates within the cell (Condemine *et al.*, 1992). OutS does not have a putative lipoprotein signal peptide as for PulS and could be cytoplasmic (Condemine *et al.*, 1992). *out*T replaces *pul*A in the equivalent position of the *Ech* cluster. Condemine *et al.* (1992) suggest that OutT or OutB could play a role in the specificity of secretion described earlier.

Using the sequence data to date, a number of homologues to the predicted Out proteins of *Ecc* SCRI193 and *Ech* (EC16) have been identified (see figure 1.13). These may give clues as to the functional roles of the Out proteins (see next section). No OutS has been identified for *Ecc*.

It is interesting to note that 5' to *out*C, in *Ech*, there is a putative KdgR box and 5' to *out*C in *Ecc* SCRI193 there is a putative exo-Peh gene (Lindenberg and Collmer, 1992; Wharam, 1992). In *Ech* (EC16) *out*O regulation is thought to be independent of *out*C-M (Lindenberg and Collmer, 1992). Lindenberg and Collmer (1992) also showed, using *out*::Tn*5-gusA1* fusions, that the *out*C-M operon of *Ech* (EC16) and the *Ecc*71 *out* cluster were induced, weakly, by polygalacturonic acid. They went on to demonstrate that the *Ech* (EC16) *out* cluster was regulated by growth phase, strong induction was found in late logarithmic phase. Further work is needed to reveal the operator/promoter regions in each of these strains. In *Ech* 3937 KdgR has been shown to regulate *out*T, C, D and E (Condemine *et al.*, 1992). *out*S and *out*B are not regulated by KdgR and are constitutively expressed, suggesting that they may be involved with the secretion of other proteins (Condemine *et al.*, 1992).

The homologues depicted in figure 1.13 have been termed "traffic wardens" because they control the flow of protein traffic across membranes (Salmond and Reeves, 1993). In order to attempt to define roles for each of the proteins in the *out* cluster of *Ecc* a brief discussion on the functions of some of the homologues will now be performed.

Figure 1.13 Homologous membrane traffic wardens.

Bacterium	**Homologous proteins (relative to the Pul proteins)**	**Reference(s)**
K. oxytoca	Pul	D'Enfert *et al.*, (1989) D'Enfert and Pugsley (1989) Reyss and Pugsley (1990) Pugsley and Reyss (1990)
Ecc	Out	Reeves *et al.*, (1993) Murata *et al.*, (1990)
Ech	Out	He *et al.*, (1991(a)) Lindenberg and Collmer (1992) Condemine *et al.*, (1992) Ji *et al.*, (1989)
X. campestris	Xps	Dums *et al.*, (1991) Hu *et al.*, (1992)
P. aeruginosa	Xcp	Bally, *et al.*, (1991, 1992)
P. aeruginosa	Pil	Nunn *et al.*, (1990) Nunn and Lory, (1992)
B. subtilis	Com	Dubnau (1991)
Filamentous phage	pIV	Brissete and Russel (1990)
Y. enterolitica	YscC	Michiels *et al.*, (1990, 1991)
P. solanacearum	HrpA	Gough *et al.*, (1992)
H. influenzae	OrfE	Tomb *et al.*, (1991)
P. aeruginosa	PilT	Whitchurch *et al.*, (1991)
A. tumefaciens	VirB11	Christie *et al.*, (1991)

K. oxytoca	(Pul)	S	B	A	C	D	E	F	G	H	I	J	K	L	M	N	O	
Ecc	(Out)				C	D	E	F	G	H	I	J	K	L	M	N	O	
Ech	(Out)	S	B		C	D	E	F	G	H	I	J	K	L	M		O	
X. campestris	(Xps)						E	F	G	H	I	J						
P. aeruginosa	(Xcp)				P	Q	R	S	T	U	V	X	Y	Z			A	
P. aeruginosa	(Pil)						B	C									D	
B. subtilis	(Com)						G1	G2	G3			G4					C	
Filamentous phage	(pIV)					IV												
Y. enterolitica	(Ysc)					C												
P. solanacearum	(Hrp)					A												
X. campestris	(Xps)					D												
H. influenzae	(ORF)					E												
P. aeruginosa	(PilT)						T											
A. tumefaciens	(VirB)						11											

1.5.4.3 The Out proteins - possible roles?

OutO in *Ecc* has been shown to share homology with PulO, XcpA, PilD and ComC (see figure 1.13). PilD is an endopeptidase of *Pseudomonas aeruginosa* that cleaves the type IV leader peptides of type IV prepilin subunits (Nunn and Lory, 1991). TcpJ is also a type IV prepilin peptidase of *Vibrio cholerae* that has also been shown to share high homology to PilD (Kaufman *et al.*, 1991). This type IV prepilin peptidase cleaves specific 6 or 7 basic amino acid residues that have an N methylated phenylalanine at position +1 relative to the cleavage site (Strom and Lory, 1992). This highly conserved NmePhe cleavage site is found in PulG, H, I, J, OutG, H, I, J, and XcpT, U, V and W. In *K oxytoca* it was demonstrated that PulO can process PulG (Pugsley and Dupuy, 1992), it has also been shown that XcpA of *P. aeruginosa* secretion gene cluster can process XcpU (Bally *et al.*, 1992). Studies in this laboratory have also shown that OutO can process OutG (P Douglas, pers. comm.). It was thought that because of the very specific nature of the cleavage of type IV prepilin homologues, the prepilin peptidase may be able to function in heterologous hosts. Mutations in *xcp*A were complemented by the expression of *pul*O (Bally *et al.*, 1992) and PulO can cleave gonococcal prepilin (Dupuy *et al.*, 1992). Other heterologous systems using XcpA or ComC were unable to process prePulG (Pugsley and Dupuy, 1992) suggesting that cleavage may depend on other factors. Because of these results it has been suggested that proteins with NMePhe signal sequences are processed by prepilin like peptidase and may form a pseudopilus like structure between the two membranes. This might serve as a platform for the assembly of other secretion components (Possot *et al.*, 1992; Pugsley, 1993). In this respect PulE, F, OutE, F, XpsE, F, XcpR, S and ComG1, G2 show homology to PilB and C. PilB and C are required for type IV pilus assembly in *P. aeruginosa* (Possot *et al.*, 1992; Nunn *et al.*, 1990).

PulE is particularly interesting because of the homology to a wide range of proteins (see figure 1.13). The possible role of this protein is discussed in section 5.5.3. and has been predicted to act as an energiser for the secretion process by hydrolysing ATP. PulE has also been predicted to be a kinase to phosphorylate other proteins in the system, and activate them, or as a platform for the assembly of a pseudopilus structure

with the type IV pilin like proteins, PulG-J (Possot *et al.*, 1992; Pugsley, 1993).

PulD has been shown to be the only outer membrane protein required for secretion (Pugsley, 1993). Pugsley *et al.* (1991(b)) showed that pullulanase adopts a highly ordered structure prior to secretion which would possibly require a pore like structure. At this stage it is unknown how proteins are translocated across the outer membrane. PulD however is homologous to a number of proteins involved in the molecular trafficking of enzymes, filamentous phages or DNA, across the outer membrane. It is also worth noting that homologies are also found with the human pathogenic *Yersinia* outer membrane proteins (YOP's). This mechanism of secretion does not involve the cleavage of a typical N-terminal signal nor in the recognition of a C-terminal domain (Michaels *et al.*, 1990). This constitutes a novel mechanism of secretion that is Sec independent but does require a large cluster of genes (Michiels *et al.*, 1991). A number of genes have been implicated in the hypersensitive response (HR) reaction of a non-compatible host plant and pathogenic bacterium (for a review see; Willis *et al.*, 1991). The HR reaction is typified by rapid necrosis of infected tissue thus preventing the spread of the bacterial infection. These genes have been termed *hrp* (*h*ypersensitive *r*esponse and *p*athogenicity) and mutations within these genes cause a lack of the HR response in non-host plants, reduced ability to cause disease of host plants and reduced ability to colonize. In *Pseudomonas solanacearum* some of the Hrp proteins are homologous with *Yersinia* Ysc proteins (Gough *et al.*, 1992). There therefore could be some connection between the two types of secretion pathways. Also the outer membrane protein PulD has been shown to share homology with HrpA of *P. solanacearum* and YscC of *Yersinia* (Gough et al., 1992). The *hrp* gene cluster has been implicated in the targeting of bacterial molecules such as Harpin (Wei *et al.*, 1992) to the external milieu where they may illicit the plant response.

1.5.4.4 A periplasmic intermediate in secretion?

At the start of this project little was known about whether there was a true periplasmic intermediate involved in the secretory process. There had been no unequivocal demonstration that the only translocation route in the wild type cell was via the

periplasm, although, there now appears to be considerable circumstantial evidence for it (He *et al.*, 1991(b); Murata *et al.*, 1990; Possot *et al.*, 1992). It could be that mutations in the secretory apparatus cause the artefactural re-routing of normally extracellular enzymes due to the breakdown of the secretion machinery in null mutants for secretion.

Poquet *et al.* (1993) have demonstrated that by uncoupling the expression of *pul*A and B from expression of *pul*C-O and S, in *E. coli*, this resulted in the periplasmic accumulation of pullulanase. Pullulanase was subsequently fully secreted upon the expression of *pul*C-O and S. At least in one case there has been some evidence to suggest that periplasmic intermediates do exist in secretion systems of this sort.

1.5.5 Project aims

In order to address the problem of whether there is a true periplasmic intermediate involved in the secretory process of *Ecc* SCRI193, I decided to attempt to isolate conditional secretory mutants, using localised mutagenesis of the *out* cluster. The initial work concentrated upon linking a selectable marker, Tn*5* or Tn*10*, next to the *out* cluster. Localised mutagenesis could then be adapted to *Ecc* using the *Erwinia* generalised transducing phage øKP. Any conditional secretion mutants isolated by this technology, could be analysed by genetic and molecular approaches. The conditional secretion mutants could then be used in pulse chase experiments to analyse the secretion process.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial strains, bacteriophages and plasmids

The bacterial strains, bacteriophages and plasmids used in this study are listed in tables 2.1, 2.2 and 2.3 respectively.

2.2 Media

Growth media, solutions and assay media were prepared in double distilled water and are listed in table 2.4. All media and solutions were sterilised by autoclaving at 121°C for 20 minutes. Solidification of NB and LB was achieved by the addition of 1.5% (w/v) Bacto agar. An 0.5% (w/v) top LB agar lawn was used for *Erwinia* phage work and an 0.3% top LB agar for the preparation of *Erwinia* phage lysates, both supplemented with $MgSO_4$ to a final concentration of 10^{-2}M. DDA and soft DDA contained 1% and 0.6% (w/v) Bacto agar respectively and were used for the propagation of phage Lambda. Antibiotics were prepared as 100x final concentration stocks and used at the concentrations indicated in table 2.5.

2.2.1 Developing and preparing enzyme detection media

2.2.1.1 Cellulase detection plates

The detection medium for cellulase has been modified from Gilkes *et al.* (1984) by J Hinton (pers. comm.). 4g of carboxymethyl cellulose (Sigma, medium viscocity) was added to 372.4ml of H_2O and 6.4g of Bacto agar. The mixture was shaken vigorously and autoclaved for 30 minutes and then cooled to approximately 60°C. The other constituents were then added, pre-warmed to 60°C, in appropriate amounts, before pouring the plates.

The detection plates were developed by flooding the plate with 0.2% (w/v) congo red (Sigma), for 20 minutes, then bleaching with 1M NaCl for 15 minutes and staining with 1M HCL for 5 minutes. The Cel^+ colonies showed a pink/red translucent halo upon a dark blue background.

Table 2.1 Bacterial strains

Strain	Characteristics	Plasmid phenotype	Source	Reference
E. coli K12				
LE392	F⁻,*hsd*R514($r_k^-m_k^+$), *sup*E44,*sup*F58,*lac*Y1 or *lac*(IZY)6,*gal*K2, *gal*T22,*met*B1,*trp*R55,	-	G.Salmond	de Bruijn & Lupski, 1984
HB101	F⁻,*hsd*S20(r_B^-,m_B^-), *rec*A13,*ara*-14,*pro*A2, *lac*Y1,*gal*K2,*rps*L20, *xyl*-5,*mtl*-1,*sup*E44,	-	G.Salmond	Boyer *et al.*, 1969
DH1	F⁻,*rec*A1,*end*A1,*gyr*A96, *thi*-1,*hsd*R17($r_k^-m_k^+$), *sup*E44,*rel*A1,	-	G.Salmond	Hanahan, 1983
DW75	DW74 (pLVC79, R64 drd 11)	-	G.Salmond	Salmond *et al.*, 1986
Erwinia carotovora subspecies *carotovora*				
SCRI193	Wild-type isolated from potato	-	J. Hinton	Pérombelon & Boucher 1978
HC131	SCRI193(pHCP2) by transfomation	Ap^r $LamB^+$	J.Hinton	Salmond *et al.*, 1986
RJP190	HC131 Out⁻ (EMS)	Ap^r $LamB^+$	P.Reeves	Pers. comm.
RJP208	HC131 Out⁻ (EMS)	Ap^r $LamB^+$	P.Reeves	Pers. comm.
RJP159	HC131 Out⁻ (EMS)	Ap^r $LamB^+$	P.Reeves	Pers. comm.
RJP120	HC131 Out⁻ (EMS)	Ap^r $LamB^+$	P.Reeves	Pers. comm.
RJP211	HC131 Out⁻ (EMS)	Ap^r $LamB^+$	P.Reeves	Pers. comm.
RJP220	HC131 Out⁻ (EMS)	Ap^r $LamB^+$	P.Reeves	Pers. comm.
RJP233	HC131 Out⁻ (EMS)	Ap^r $LamB^+$	P.Reeves	Pers. comm.

Table 2.1 cont.

Strain	Characteristics	Plasmid phenotype	Source	Reference
RJP249	HC131 Out$^-$ (EMS)	Ap^r LamB$^+$	P.Reeves	Pers. comm.
RJP251	HC131 Out$^-$ (EMS)	Ap^r LamB$^+$	P.Reeves	Pers. comm
RJP253	HC131 Out$^-$ (EMS)	Ap^r LamB$^+$	P.Reeves	Pers. comm.
AC4000	HC131 out::Tn*10*	Ap^r LamB$^+$	lab stock	Unpublished
JNH1	HC131, Out$^+$, Tn*5*,	Ap^r LamB$^+$	This study	
JNH2	HC131, Out$^+$, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1000	HC131, Out$^-$, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1005	HC131, Out$^-$, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1006	HC131, Out$^-$, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1007	HC131, Out$^-$, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1008	HC131, Out$^-$, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1010	HC131, Out$^-$, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1011	HC131, Out$^-$, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1012	HC131, Out$^-$, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1013	HC131, Out$^-$, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1014	HC131, Out$^-$, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1015	HC131, Out$^-$, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1016	HC131, Out$^-$, Tn*5*,	Ap^r LamB$^+$	This study	

Table 2.1 cont.

Strain	Characteristics	Plasmid phenotype	Source	Reference
HJN1017	HC131, Out$^-$, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1018	HC131, Out$^-$, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1019	HC131, Out$^-$, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1020	HC131, Out$^-$, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1021	HC131, Out$^-$, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1003	HC131, Out^{ts}, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1004	HC131, Out^{ts}, Tn*5*,	Ap^r LamB$^+$	This study	
HJN1009	HC131, Out$^+$, Tn*5*, *his*$^-$	Ap^r LamB$^+$	This study	

Table 2.2 Bacteriophages

Phage	Characteristics	Source	Reference
Coliphage			
λ_{467}	b_{221}, rex::Tn*5* cI_{857}, O_{am29}, P_{am80},	Lab stock	
λ_{840}	TET(high hopper)	Lab stock	
λcI_{857}	Thermoinducible	P. Reeves	Vollenweider *et al.*, 1980
***Ecc* SCRI193 bacteriophages**			
øKP	*Ecc* generalised transducing phage	I. Toth	Toth *et al.* 1993
ø565	SCRI193 bacteriophage	I. Toth	Pers. comm.
ø575	SCRI193 bacteriophage	I. Toth	Pers. comm.
øD-2	SCRI193 bacteriophage	I. Toth	Pers. comm.

Table 2.3 Plasmids/cosmids

Plasmid/ Cosmid	Characteristics	Plasmid phenotype	Source	Reference
pBR322	Cloning vector	Ap^r, Tc^r,	Amersham Int.	Bolivar *et al.*, 1979
pHIL159/3	pBR322::*outL*$^+$	Ap^r, Tc^s	P. Reeves	Pers. comm.
pNH1004/1	pBR322::*outO*$^+$	Ap^r, Tc^s,	This study	
pNH1004/2	pBR322::*outO*$^+$	Ap^r, Tc^s,	This study	
pHCP2	pBR322::*lamB*$^+$	Ap^r, Tc^s,	Lab stock	Clement *et al.*, 1982
p*Trc*99A	Prokaryotic expression vector	Ap^r	Pharmacia	Amman *et al.*, 1988
pNH31/A	p*Trc*99A::*pelD*$^+$	Ap^r,	This study	
pNH31/B	p*Trc*99A::*pelD*$^+$	Ap^r,	This study	
pNH260	pBR322::*outL*^{ts}	Ap^r,	This study	
pNH700	pBR322::*outL*$^-$	Ap^r,	This study	
pNH200	pBR322::*outL*$^-$	Ap^r,	This study	
pSW1	pIC-19R::*outC*$^+$	Ap^r,	S. Wharam	Pers. comm.
pSW2	pBR322::*outX*$^+$	Ap^r, Tc^r	S. Wharam	Pers. comm.
p3.7R1	pBR322::*outD*$^+$, *outE*$^+$, *outF*$^+$	Ap^r, Tc^r	P. Reeves	Pers. comm.
pJS6161	pUC19::*PelD*$^+$,	Ap^r,	R. Barallon	Pers. comm.
cHIL122	pHC79::*out*$^+$	Ap^r, Tc^r	P. Reeves	Pers. comm.
cHIL220	pHC79::*out*$^+$	Ap^r, Tc^r	P. Reeves	Pers. comm.
cHIL253	pHC79::*out*$^+$	Ap^r, Tc^r	P. Reeves	Pers. comm.
cHIL251/4	pSF6::*out*$^+$	Spc^r,	P. Reeves	Pers. comm.

Table 2.4 Media

Medium	Constituents per litre[a]
NB	13g Oxoid nutrient broth
NBA	13g Oxoid nutrient broth 16g Bacto agar
LB	10g Bacto tryptone 5g Bacto yeast extract 5g NaCl [pH 7.2]
LBA	10g Bacto tryptone 5g Bacto yeast extract 5g NaCl 16g Bacto agar
LBSE	10g Bacto tryptone 5g Bacto yeast extract 58.5g NaCl 0.37g EDTA [pH 7.2]
SOC	20g Bacto tryptone 5g Bacto yeast extract (10ml 1M NaCl) (2.5ml 1M KCL) (10ml 1M $MgSO_4$, 1M $MgCl_2$ filter sterilised) (20ml 1M glucose) [pH 6.9 - 7.0]
DDA	20g Bacto tryptone 8g NaCl (10ml 1M $MgSO_4$) 9g Bacto agar for plates or 2.5g Bacto agar for soft agar

Table 2.4 cont.

Medium	Constituents per litre[a]
Phage buffer	10 mM Tris.HCL
	10 mM $MgSO_4$
	0.01% (w/v) gelatin
	[pH 7.4]
Minimal medium	(20ml 50x phosphate)
	(10ml 10% $(NH_4)SO_4$)
	(10ml 1% $MgSO_4$)
	(10ml 20% Sucrose)
50x Phosphate	350g K_2HPO_4
	100g KH_2PO_4
	[pH 6.9 - 7.1]
Enzyme detection media	
Cellulase (Cel)	10g carboxymethyl cellulose, Sigma
	16g Bacto agar
	(25ml 20% (w/v) Bacto yeast extract)
	(4ml 50% (w/v) glycerol)
	(20ml 50x phosphate)
	(10ml 10% (w/v) $(NH_4)SO_4$)
Protease (Prt)	13g Oxoid nutrient broth
	30g Oxoid gelatin
	16g Bacto agar
Pectate lyase (Pel)	16g Bacto agar
	(5ml 20% (w/v) Bacto yeast extract)
	(10ml 10% (w/v) $(NH_4)SO_4$)
	(1ml 1M $MgSO_4.7H_2O$)
	(9ml 50% (w/v) glycerol)
	(125ml 2% (w/v) Polygalacturonic acid)
	(100ml Pel phosphate buffer)
Pel phosphate buffer	15g Na_2HPO_4, anhydrous
	0.7g $NaH_2PO_4.H_2O$
	[pH 8.0]

Table 2.4 cont.

Medium	Constituents per litre[a]
Pel induction medium (PM)	(5ml 20% (w/v) Bacto yeast extract) (10ml 10% (w/v) $(NH_4)SO_4$) (1ml 1M $MgSO_4.7H_2O$) (9ml 50% (w/v) glycerol) (125ml 2% (w/v) Polygalacturonic acid) (100ml Pel phosphate buffer)
Pel minimal broth (PMB)	As for PM except add 20ml 50x phosphate in place of Pel phosphate buffer
Freezing medium x2	126g K_2HPO_4, anhydrous 0.9g sodium acetate 0.18g $MgSO_4.7H_2O$ 1.8g $(NH_4)SO_4$ 3.6g KH_2PO_4, anhydrous 88g glycerol

[a] - Constituents in brackets indicate seperate autoclaving.

Table 2.5 Antibiotics

Antibiotic	Abbreviation	Final concentration
Sodium ampicillin	Ap	50μg/ml
Chloramphenicol	Cm	50μg/ml
Spectinomycin	Spc	50μg/ml
Tetracycline	Tc	10μg/ml
Kanamycin sulphate	Km	50μg/ml

Prepared as 100x concentration stocks. Tetracycline and chloramphenicol were dissolved in 50% (v/v) ethanol and stored at -20°C, all other antibiotics were dissolved in 100ml sterile double distilled Elga water and stored at 4°C.

2.2.1.2. Protease detection media

The protease assay was as described by Thurn and Chatterjee (1985). Gelatin was dissolved (30g/litre), prior to autoclaving by heating to 60°C.

Detection plates were developed by flooding the plates with 4M ammonium sulphate. Prt^+ colonies give a clear halo on a white opaque background.

2.2.1.3. Pectate lyase detection media

The detection medium was made as described previously by Andro *et al.* (1984). The detection plates were developed by flooding the plates with 7.5% copper acetate (Sigma) for 30 minutes. Pel^+ colonies showed a pale white double halo on a light blue background.

2.2.1.4 Reagents

Enzymes for molecular biology were obtained from Stratgene, Bethesda Research Laboratories (BRL), Boehringer Mannheim, Amersham International, Pharmacia, New England Bio-Labs and Promega. Reagents for media were as stated in the text. All agar was purchased from "Bacto". ^{35}S-methionine and dNTP's were obtained from Amersham International.

2.3 Growth of bacteria

All bacterial strains were grown on Luria broth agar (LBA) containing appropriate antibiotics (table 2.5), at 30°C for *Ecc* or 37°C for *E. coli*. Liquid cultures were grown in 5ml or 10ml volumes in universal (25ml) screw cap bottles and were shaken in an orbital shaker (250rpm). Larger cultures were grown in conical flasks at $1/10^{th}$ total flask volume.

2.4 Strain maintenance

All *E. coli* and *Ecc* strains were maintained on LBA or NBA plates at 4°C for up to 3 months. Long term storage was by freezing liquid cultures, grown under appropriate selective conditions, at -70°C in 2x freezing solution.

2.5 Preparation of high titre bacteriophage lysates

Phage Lambda suicide vectors were propagated on the *E. coli* suppressing strain LE392. LE392 was grown in LB, 10mM $MgSO_4$, over-night and aliquoted into 5ml screw capped bottles in 200μl amounts. Serial dilutions, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-10}, of the λ stock phage were made in phage buffer and 10μl of each added to the 200μl aliquots. The phage were left to adsorb for 10 minutes prior to the addition of 3ml of warm (45°C) soft DDA (10mM $MgSO_4$) and poured onto a dry bottom DDA (10mM $MgSO_4$) plate. The plates were then incubated at 37°C over-night, without inversion. The next day 2 plaques were isolated, using a Pasteur pipette, and resuspended in 1ml phage buffer by vortexing for 30 seconds. The suspension was then pelleted for 1 minute using a high speed microfuge. 10μl, 50μl and 100μl of the lysate supernatant was then added to 200μl aliquots of an LE392 over-night culture (as above) and left to adsorb for 10 minutes at room temperature. 3ml of soft DDA (10mM $MgSO_4$) agar was added and the mixture poured onto a wet bottom DDA (10mM $MgSO_4$) plate. The plates were then incubated at 37°C over-night without inversion. The next day those plates that showed confluent lysis, as compared to a control phage free lawn, were harvested, using a bent Pasteur pipette, into a 25ml universal. 2ml of phage buffer was then used to wash the bottom DDA plate and the "wash" added to the harvested soft agar. This mixture was then vortexed for 30 minutes in the presence of 500μl chloroform. The slurry was then centrifuged (MSE chilspin, 15 minutes, 4°C) to pellet the debris. The supernatant (lysate) was then transferred to a sterile 25ml universal and stored over a few drops of chloroform.

For *Ecc* bacteriophage propagation the host strain HC131 (LamB$^+$) was used. Serial dilutions of the stock phage, 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} were made and 10μl of each added to 200μl of the appropriate host, grown over-night. After 10 minutes adsorption 3ml of 0.3% soft LBA (10mM $MgSO_4$) was added and the mixture poured onto a wet bottom LBA plate. The plates were then incubated at 26°C over-night without inversion. The next day the dilution showing near to confluent lysis was harvested as described above and the lysate stored over a few drops of chloroform at 4 °C. These procedures yielded lysates of up to 10^{11} pfu per ml.

2.6 Transductions

øKP lysates were irradiated by short wave ultra-violet, 7.25μWcm^{-2} x 100 for 60 seconds, prior to transduction. Transductions were then performed essentially as described by Toth *et al.*, (1993). A 5ml over-night bacterial culture of recipient *Ecc* cells was sub-cultured (1/25) and grown in LB, supplemented with appropriate antibiotics, at 30°C with shaking (250rpm) until A_{600} = 0.7. 100-400 μl of ultra-violet irradiated lysate was added to this bacterial culture of recipient *Ecc* cells and incubated at 26°C, for 30 minutes, to adsorb the phage. The culture was then centrifuged (MSE, 4500 rpm, 5 minutes) to pellet the cells and resuspended in 5ml of phage buffer by vortexing. The cells were again pelleted by centrifugation (MSE, 4500rpm, 5 minutes) and the pellet resuspended in 0.5ml LB by vortexing. The cells were then divided onto 3 LBA plates, with appropriate selection, to select for transposants, and spread using an "ethanol flamed" glass rod before incubation at 30°C for 24-48 hours.

Cosmid packaging and cosmid transductions were performed as described by Reeves (1992).

2.7 Transposon trapping

Tn*5* insertions were obtained in *Ecc* (HC131, LamB$^+$) using the λ_{467} transposon delivery system previously described, Salmond *et al.*, (1986).

2.8 Localised mutagenesis

This method was adapted for *Ecc* from Hong and Ames, (1971). Essentially 1.0ml of a high titre øKP.JNH1 lysate ($>10^{10}$ pfu/ml) was added to :

2.0ml phosphate EDTA buffer

3.0ml sterile distilled water

4.0ml hydroxylamine solution

Phosphate EDTA buffer

6.0ml of 1M K_2HPO_4 was added to 43.9ml 1M KH_2PO_4 and the pH adjusted to [pH 6.0]. An equal volume of 10mM EDTA was added and the solution autoclaved.

Hydroxylamine solution

560μl of 4M NaOH was added to 0.35g hydroxylamine and the volume made up to 5.0ml with distilled water.

The lysate was exposed to mutagen, in an oakridge tube (Nalgene), for 21.5 hours (see section 3.5.1) and incubated static at 37°C and the mutagenised phage pelleted for 2.5 hours at 17K and 23°C using a Beckman J2-21 centrifuge and JA-17 rotor. The supernatant was carefully discarded and the oakridge tube blotted dry on a piece of absorbant tissue. The pellet was resuspended over-night in 1.0ml of phage buffer (table 2.4) at 4°C. The mutagenised lysate was then used to infect wild type *Ecc* (section 2.6, omitting the UV irradiation step) and transductants (Km^r) screened for mutant phenotypes (section 2.9).

2.9 Screening for Out$^-$, conditional Out^{ts} and auxotrophic phenotypes

Colonies derived from mutagenesis procedures were replicated onto LBA with appropriate antibiotic selection and incubated at 30°C for 36 hours. These colonies were then screened for extracellular enzyme secretion on enzyme detection media (Pel, Cel and Prt) at both 26°C and 33°C by transferring them using an replicating plate. The detection plates were developed as described in section 2.2.1. Auxotrophs were screened for at 26°C and 33°C on minimal media and any auxotrophy defined by the pool plate method of Holliday (1956).

2.10 Fractionation of *Ecc* spp into supernatants, periplasms and cytoplasms

This method for generating sphaeroplasts was adapted from Osborn and Minson (1974).

5ml bacterial cultures of *Ecc* spp cells were grown over-night, in PMB (see table 2.4), with appropriate antibiotic and 100μl LB, to stationary phase (A_{600} = 3.0-4.0). The supernatants were harvested after centrifugation (MSE, chilspin) at 5000rpm for 10 minutes. The pellet was immediately resuspended in 5ml chilled (4°C) sucrose solution (10mM Tris.HCL, [pH7.8]; 0.75M sucrose) and placed on ice. 250μl of lysozyme solution (2mg/ml lysozyme in 10mM Tris.HCL [pH 7.8]) was quickly added and incubated on ice for 5 minutes. This suspension was transferred to a 50ml beaker, with a magnetic stirrer, and kept at 4°C. Chilled (4°C) EDTA solution (2ml 15mM EDTA [pH 8.0]; 8ml H_2O) was slowly added via a 10ml pipette with a rubber tube connected to the end, over 10 minutes. The rate of addition was controlled by an adjustable clip. After 10 minutes the extent of sphaeroplasting (and thus release of periplasmic fraction) was assessed by looking at a 10μl aliquot of suspended cells under phase contrast microscopy, at high magnification. If this was <75 % then the cell suspension was incubated at 37°C for 10 minutes, to increase the percentage of sphaeroplasts. The cell suspension was then centrifuged (MSE, chilspin) at 5000 rpm for 10 minutes and the periplasmic fraction removed. The remaining sphaeroplasts

were resuspended in 5ml of PMB and sonicated for 3x30 seconds, at an amplitude of 6.0, with a 0.7 inch probe and at 4°C with a 30 second cooling between sonications. Cell debris was removed by centrifugation (MSE, chilspin) at 5000 rpm for 10 minutes and the cytoplasmic fraction removed. Each fraction was stored at -20°C until needed.

2.11 Quantitative spectrophotometric enzyme assays

Samples for quantitative analysis were obtained as described in section 2.10. Enzyme free controls, which in all cases was PMB, were used throughout.

2.11.1 Pectate lyase

Pectate lyase activity was monitored by the breakdown of polygalacturonic acid (Sigma) to unsaturated digalacturonate by measuring the change in absorbance at a wavelength of 235nm and at 37°C (Chatterjee *et al.* 1985). The method described here was obtained from Hinton (pers. comm.).

To a 1ml quartz cuvette the following reagents were added:

876μl reaction mix (pre-heated to 37°C)

22.5μl supernatant/sonicate/periplasm

The mixture was inverted using Nescofilm and placed in a temperature controlled heated cuvette block (37°C) of a Philips PU 8720 scanning spectrophotometer set at 235nm. The data was expressed in the following units ΔA_{235}/min/ml.

Reaction mix	**Reaction buffer**
3.45ml reaction buffer	76.8ml H_2O
3.2ml 0.575% PGA	23.0ml 1M Tris.HCl [pH 8.5]
1.13ml H_2O	78μl 1M CaCl/100ml H_2O

2.11.2 Cellulase

2.11.2.1 Ostazin Brilliant Red assay (OBR-Cellulose)

Essentially this method was developed from Biely *et al.* (1985) by F. Ellard (pers. comm.). Cellulase activity was assayed using the substrate OBR-Cellulose (Sigma) that is broken down by cellulase to release a red pigment and was measured at a wavelength of 550nm after precipitation of intact substrate.

The reaction mix was as follows:

140μl OBR-Cellulose (20.5 mg/ml)
70μl supernatant/sonicate/periplasm
140μl H_2O
7μl 50x phosphate

Samples were incubated at 30°C for 80 minutes and the reaction stopped with 3 volumes ethanol/acetone (2/1, v/v). The samples were then incubated at room temperature for 5-30 minutes and centrifuged in a "high speed" microfuge for 10 minutes at 4°C. The supernatant was removed and measured spectrophotometrically at 550nm against a water blank. The units of activity were expressed as ΔA_{550}/min/ml.

2.11.3 Protease

Ji *et al.* (1987) have described this assay previously. The assay is based on the breakdown of the substrate azocasein (Sigma) by protease to release the "azo" dye, a yellow/orange pigment.

The following reagents were added to 2ml Eppendorf tubes:

750μl 2% (w/v) azocasein (Sigma)
150μl 1M Tris.HCl [pH 8.0]
600μl H_2O

For supernatant and periplasmic fractions 99.75μl

For sonicate fraction 199.5μl

Samples were incubated at 30°C and 750μl aliquots were removed at t=0 and t=4 hours and added to microfuge tubes containing 375μl of 14% perchloric acid, to stop the reaction. After all the samples were collected they were centrifuged in a "high speed" microfuge for 3 minutes. Immediately 750μl of supernatant was transferred to microfuge tubes containing 75μl 10M NaOH. The tubes were inverted to mix and the sample absorbances read at 436nm with water as a reference. The units of activity were expressed as ΔA_{436}/hr/ml.

2.11.4 ß-lactamase assay (BLA)

This assay was performed as described by O'Callaghan *et al.* (1972).

The reaction mixtures were as follows:

1) 100μl sonicate
   700μl 0.1M phosphate buffer [pH 7.0]
2) 5μl periplasmic axtract
   795μl 0.1M phosphate buffer [pH 7.0]
3) 100μl supernatant
   700μl 0.1M phosphate buffer [pH 7.0]

To each reaction mix was added 20μl chromogenic substrate Nitrocephin (4 mg/ml, in DMSO) and the solution inverted. The rate of change of absorbance was followed at 500nm using a Philips PU 8720 scanning spectrophotometer. The units were expressed as ΔA_{500}/min/ml.

2.11.5 ß-galactosidase

This assay was based on Miller (1972).

The reaction mixes, in 1.5ml microfuge tubes, were as follows:

450μl Z buffer

50μl sonicate or 250μl supernatant/periplasm

Z buffer: (per litre)

8.52g Na_2HPO_4 anhydrous

6.24g $NaH_2PO_4.2H_2O$

0.75g KCl

0.25g $MgSO_4.7H_2O$

2.7ml ß-mercaptoethanol

[pH 7.0] not autoclaved

100μl of fresh ONPG (Sigma, 4mg/ml), prepared with Z buffer was added to each reaction. Each reaction was then incubated at 37°C until a faint yellow colour appeared. 250μl of 1M Na_2CO_3 was then added to stop the reaction and the time noted. The rate of change of absorbance was calculated using a Philips PU 8720 scanning spectrophotometer set at 420nm. The units of activity were expressed as ΔA_{420}/min/ml.

2.12 DNA preparation

2.12.1 Preparation of plasmid DNA from a small volume of cells (mini-prep)

This method is based on Maniatis *et al.* (1982) (R. Barralon, pers. comm.). 1.5ml of *E. coli* or *Ecc* bacterial cell culture grown over-night at 37°C or 30°C respectively, was pelleted in 1.5ml microcentrifuge tubes at the high speed setting for 2 minutes. The supernatant was discarded and the pellet resuspended in 100μl of lysis solution

(25mM Tris.HCl, [pH 8.0]; 50mM glucose; 10mM EDTA; lysozyme 2mg/ml) by vortexing and incubated on ice for 5 minutes. 200μl of freshly prepared SDS solution (0.2M NaOH, 1% SDS) was added and the tubes inverted 3 or 4 times to mix. The tubes were then placed on ice for 5 minutes. 150μl of sodium acetate solution (5M acetate/3M Na; made by adding 60ml 5M sodium acetate, 11.5ml glacial acetic acid and 28.5ml H_2O) was added and the tube was vortexed for 10 seconds, inverted, before being placed on ice for 5 minutes. The tube was then centrifuged for 5 minutes at high speed to pellet any non lysed cells, membrane bound chromosomal DNA or other cell debris. The supernatant was transferred to a fresh microfuge tube with a Pasteur pipette and an equal volume of phenol/chloroform (100g phenol, 100ml chloroform, 4ml isoamyalcohol, 0.1g 8,hydroxyquinoline) was added and the tube vortexed. The tube was then centrifuged at high speed, in a microfuge, for 2 minutes and the upper aqueous layer (avoiding the interface) transferred to a fresh microfuge tube containing an equal volume of chloroform:isoamylalcohol (24:1). The tube was vortexed, centrifuged for 2 minutes at high speed and the upper aqueous layer transferred to a fresh tube. To this aqueous layer was added 1/10th volume of sodium acetate solution (as above) and 3 volumes of ethanol or 1 volume of isopropanol. The tube was then placed at -70°C for 1/2 hour, to precipitate the DNA, followed by centrifugation at high speed for 10 minutes to pellet the DNA. The supernatant was removed with a yellow tip and Gilson pipette and the pellet washed with 70% (v/v) ethanol. This was removed, as above, and the pellet dried under a vacuum or in a 50°C oven, for 15 minutes . The pellet was then resuspended in 50μl of TE buffer and stored at 4°C or -20°C.

The phenol/chloroform step was repeated up to 3 times if higher purity DNA was required.

2.12.1.1 "Magic mini-prep"

Magic mini-preps of plasmid DNA were performed using the "Magic mini-prep" kit supplied by Promega. Procedures were followed according to the manufacturers instructions.

2.12.2 Large scale plasmid DNA preparations (Maxi-preps)

This method was used to prepare high quality plasmid DNA for sequencing. The method is based on Maniatis *et al.* (1982) (C Thorpe, pers. comm.).

200ml of LB, in 2 litre flasks containing appropriate antibiotics, was used to culture *E coli* cells at 37°C, with shaking at 250rpm. The cultures were grown over-night under these conditions or chloramphenicol was added (0.17mg/ml) at an absorbance of A_{600} = 0.7-0.9, to amplify the DNA, before incubating for 15-18 hours.

Cells were pelleted by centrifugation using an Beckman J2-21 centrifuge and JA-10 rotor, for 20 minutes at 8K and 4°C. The cells were resuspended in 20ml TES (50mM Tris.HCl [pH 8.0], 5mM EDTA, 50mM NaCl) and transferred to 40ml (sterile) oakridge tubes. The suspension was then centrifuged using an JA-17 rotor, for 5 minutes at 10K and 4°C. The supernatant was discarded and the pellet resuspended by vortexing in 10ml STE (25% sucrose, 50mM Tris.HCl [pH 8.0], 5mM EDTA). The suspension was then frozen at -20°C and stored until needed. The frozen sample was thawed on ice and 1ml of lysozyme solution added (10mg/ml lysozyme, 0.25M Tris.HCl [pH 8.0]) and the tube inverted several times to mix. The tube was left on ice for 10 minutes to enable lysis to occur. Once lysis was seen to occur, the solution became viscous, 2.5ml of 0.5M EDTA [pH 8.0] was added and the suspension left on ice for a further 10 minutes. 16ml of triton lysis mix (0.1% (v/v) Triton X-100, 50mM Tris.HCl [pH 8.5], 50mM EDTA) was added quickly by using a 20ml pipette and a pipette pump and then mixed by vigorous shaking. After 20 minutes incubation on ice the suspension was centrifuged for 30 minutes at 19K using an JA-17 rotor. The

supernatant was then decanted through muslin into a 25ml cylinder containing 28.5g CsCl. This volume was made up to exactly 38ml with TES (see above). The CsCl was completely dissolved by swirling the cylinder and occasional incubation in a 60°C water bath. The solution was then transferred to an oakridge tube and 2ml of ethidium bromide (5mg/ml) was added and then centrifuged at 15K for 20 minutes using an JA-17 rotor. The supernatant was then decanted, through a 20ml syringe and a 16 gauge needle, into heat-sealable Beckman Vti-50 tubes. The tube was then balanced using a 71.25% (v/v) solution of CsCl, heat sealed and centrifuged at 45K for 16 hours and at 22°C using a Vti-50 rotor. The plasmid band was removed as described by Maniatis *et al* (1982). Ethidium bromide was removed by extracting the sample approximately 5 times with salt saturated isopropanol. The plasmid DNA was precipitated by adding 2 volumes of water and 3 volumes of isopropanol, mixing and incubating at -20°C for 24 hours before centrifuging at 15K for 20 minutes and at 4°C using an JA-17 rotor. The supernatant was discarded and the pellet washed in 70% (v/v) ethanol and re-centrifuged for 10 minutes at 15K and at 4°C. The supernatant was discarded and the pellet dried under vacuum. The pellet was resuspended in 100μl TE or H_2O and kept at 4°C or -20°C.

2.12.3 Chromosomal DNA preparation

This method was based on Maniatis *et al*, (1982) by P. Reeves, (pers. comm.).

A 100ml bacterial cell culture was grown over-night in LB at 30°C with shaking at 250rpm. The cell suspension was pelleted in an oakridge tube at 4K for 5 minutes and at 23°C using a Beckman J2-21 centrifuge and JA-17 rotor. The pellet was washed in 5ml of 1M NaCl and frozen at -70°C, for 30 minutes. The pellet was thawed on ice and resuspended in 16ml of TE (50mM Tris.HCl, 50mM EDTA, [pH 8.0]) buffer. 2ml of lysozyme (2mg/ml, made in 250mM Tris.HCl [pH8.0]) was added and incubated on ice for 5-10 minutes until the suspension went thick and viscous. Proteinase K was added to a final concentration of 200μg/ml along with 500μl 10%

(w/v) SDS and the tube shaken vigorously, the suspension had a cloudy appearance. The tube was then incubated at 65°C for 10 minutes until the suspension went clear. 3ml 5M sodium perchlorate was added and the suspension shaken and incubated at 65°C for 20 minutes. The volume was increased to 40ml by adding TE (see above) and mixed by inversion. The suspension was then phenol/chloroform extracted twice (see section 2.12.1) and then chloroform/isoamyalcohol extracted once (see section 2.12.1) using a "shorn-off", blue, Gilson tip, to avoid shearing the DNA. The DNA solution was added to a 100ml glass (washed in ethanol) beaker and two volumes of ethanol added, by gently pouring down the side of the beaker, to precipitate the DNA. An ethanol rinsed glass rod was used to spool the DNA and then left to air dry. The DNA was then dissolved in 5ml TE (see above), with 5μl chloroform, and stored at 4°C.

2.13 Restriction endonuclease digestion of plasmid DNA

Restriction endonuclease digests were normally carried out in 20μl volumes. The digestion of DNA was performed according to the manufacturers instructions (BRL, Pharmacia, Boehringer Mannheim, Amersham International). All digestions were performed for at least 1 hour and at 37°C, unless stated otherwise by the restriction endonuclease suppliers. "Double" digests, using more than one restriction endonuclease, were performed together in the same tube unless the restriction buffers were incompatible. In this case a two step reaction using firstly the lower concentration restriction buffer and then altering the salt concentration by the addition of 1M NaCl. The second digestion could then be performed using the second restriction endonuclease. All digests were analysed by agarose gel electrophoresis.

2.14 Agarose gel electrophoresis

Agarose gels (0.7%) were made by melting the agarose in TBE buffer (1 litre: 10.8g Tris, 5.5g Boric acid, 0.93g EDTA) and ethidium bromide (final concentration 0.5μg/ml), cooling to 50°C and pouring the gel into a gel mould (Bio-Rad sub cells or

mini sub cells). When these gels were solidified, they were submerged in TBE buffer containing ethidium bromide (0.5μg/ml final concentration). Samples for electrophoresis were prepared by adding 0.25 volumes of loading buffer type 1V (0.25% (w/v) bromophenol blue, 40% (w/v) sucrose) and the samples loaded into wells. The electrophoresis was carried out at 100 V for the appropriate time to give good separation and resolution of DNA bands. Higher resolution could be achieved by running the gels for 16 hours, at 5 V for mini sub cells or 25 V for sub cells. After electrophoresis DNA bands could be visualised under long wave UV and photographed using Polaroid positive / negative 665 film.

2.15 Isolation of DNA fragments from agarose gels

2.15.1 Low melting point agarose gel electrophoresis

This method was adapted from Maniatis *et al.* (1982) by V. Mulholland (pers. comm.). Samples were electrophoresed at 80 V through a 1% low melting point agarose gel (low melting point agarose, BRL) as described above (2.14). The gel was solidified at 4°C. The band of interest was identified by long wave UV and removed completely, using a scalpel blade and placed into an microfuge tube. The agarose was melted at 65°C, 100μl of H_2O (to dilute the DNA) and an equal volume of phenol/chloroform was added, vortexed and incubated at room temperature for 10 minutes. The tube was centrifuged at high speed for 5 minutes and the supernatant removed, this process was repeated 3x and the DNA precipitated, washed in 70% (v/v) ethanol and dried under vacuum as described in section 2.12. The pellet was then resuspended in 20μl H_2O.

2.15.2 Direct isolation ("trough-elution") of DNA fragments from agarose gels

This method has been described elsewhere, P. Reeves (1992).

2.16 De-phosphorylation of double stranded DNA (linear)

Re-circularisation of endonuclease restricted vector DNA was prevented by de-phosphorylation using calf intestinal alkaline phosphatase (Boehringer Mannheim) according to the suppliers instructions. The sample was then phenol/chloroform extracted 3x, ethanol precipitated and dried under vacuum (as above, 2.12). The pellet was then re-suspended in an appropriate volume of TE buffer.

2.17 Cloning into plasmid vectors

Plasmid vectors were digested with appropriate restriction endonucleases (see section 2.13) and de-phosphatased (see section 2.16) if required. All restriction endonucleases were removed by phenol/chloroform extraction (see section 2.12).

2.17.1 "Sticky-end" ligations

Usually, a fragment to vector ratio of 4:1 was used, but in practice a range of ratio's was normally made to ensure cloning of the insert in the vector. The vector and insert were mixed and diluted with 30μl 1x ligation buffer (66mM Tris.HCl [pH 7.6], 6.6mM $MgCl_2$, 10mM DTT, 0.4mM ATP). 1μl of T_4 DNA ligase (GIBCO-BRL) was added to initiate ligation and the mixture incubated at room temperature for 4 hours and stored at -20°C until needed. Higher efficiency ligation could be achieved by incubating at 15°C for 16 hours.

2.17.2 Blunt ended ligation

All blunt ended ligations were carried out in 10μl volumes, using 10x ligation buffer (as above) or commercial 5x ligation buffer (GIBCO-BRL), applying the same conditions as above and the reactions incubated at 15°C for 16 hours.

2.18 "End-repair" of linear double stranded DNA

Restriction endonuclease digested DNA that has yielded "sticky ends" or PCR amplified DNA (see section 2.22) that has yielded "ragged" ends, can be made flush

(blunt) by the following reactions :

1) Restriction endonuclease digested DNA :

8μl DNA fragment

1μl dNTP's (2.5mM stocks)

2μl NTB (10x stock)

10U DNA polymerase (Klenow fragment)

10U T4 DNA polymerase

H_2O added to make the final volume 20μl

2) PCR amplified DNA :

20μl Genecleaned* (Geneclean II kit, BIO-101) PCR amplified DNA

2μl dNTP's (2.5mM stocks)

3μl NTB

10U DNA polymerase (Klenow fragment)

10U T4 DNA polymerase

H_2O added to make the final volume 30μl

* The Geneclean II kit (BIO-101) was used according to the suppliers instructions.

NTB 10x : 0.5M Tris.HCl [pH 7.8], 50mM $MgCl_2$, 10mM DTT.

Each reaction was incubated at room temperature for 45 minutes, phenol/chloroform extracted, once, and ethanol precipitated (see section 2.12) before ligation.

2.19 Transformation of *E coli*

This method was adapted from Maniatis *et al.* (1982) by R. Barallon (pers. comm).

2.19.1 Small scale preparation of competent cells, using $CaCl_2$

A 10ml over-night bacterial culture of *E coli* cells, grown in LB and at 37°C, was used to inoculate 10ml of sterile LB at a ratio of 25:1. The sub-culture was grown at 37°C, with shaking at 250rpm, until an A_{600} = 0.5-0.6 was reached. The cells were centrifuged at 4500 rpm for 5 minutes and the supernatant discarded. The pellet was re-suspended in 10ml of 10mM $CaCl_2$ by vortexing and the cells pelleted as above. This step was then repeated. The cells were pelleted, the supernatant discarded and the cells resuspended in 0.5ml 75mM $CaCl_2$/10% (v/v) glycerol and incubated on ice for 30 minutes. The cells were then used in transformations or frozen at -20°C until needed.

2.19.2 Transformation

To 100μl of competent cells in a microfuge tube was added either 1μl DNA or a ligation reaction and the cells "heat shocked" at 42°C, for 2 minutes. 1ml of LB was added and the cells incubated at 37°C, with shaking at 250rpm, for 1 hour. The cells were pelleted in a high speed centrifuge for 1 minute and the supernatant discarded to leave a 0.1ml residual amount of LB. The cells were resuspended by vortexing and spread onto a LBA plate, using an ethanol-flamed glass rod, containing the appropriate antibiotic(s) for selection of transformants and incubated over-night at 37°C.

2.20 Electroporation of *Ecc*

The most efficient way of transforming *Ecc* was by electrotransformation (Solioz and Bienz, 1990). This procedure was performed using a Bio-Rad pulse controller and bacterial electrotransformation was done according to the manufacturers instructions. Preparation of electroporation competent cells was as detailed in the suppliers instructions except that the volumes were scaled down 100x and cells were not frozen but were used fresh. Electrotransformations were performed using 1μl DNA to 40μl recipient cells or 5μl ligation reaction to 40μl recipient cells. *E coli* electrotransformation was performed under the same conditions, if needed.

2.21 Plasmid transfer

2.21.1 Conjugal transfer

Colonies from both donor and recipient strains were mixed together on an LBA plate, using a sterile wire loop, and incubated at 30°C over-night. The cells were then streaked onto selective media, agar plates containing antibiotics, to select for transconjugants that contained the transferred plasmid, and counterselection for the donor strain. The colonies that appeared were further purified by streaking the bacteria, onto MM containing sucrose. This removed any donor auxotrophic *E coli*..

2.21.2 Transfer of plasmids using *Ecc* generalised transducing phage

The generalised transducing phage, øKP (Toth *et al.*, 1993), when propagated on HC131 ($LamB^+$), was used to transfer the plasmid pHCP2 to recipient *Ecc* via transduction (see section 2.6).

2.22 Polymerase chain reaction (PCR)

PCR was used to amplify DNA for cloning any specific gene(s) that would otherwise be difficult to clone from the chromosome (Erlich eds. (1989)). The procedures used here have been adapted from R. M^{c}Gowan (pers. comm.).

To a siliconised 500μl tube (Sigma), was added, in order:

1μl chromosomal DNA

2μl H_2O

80μl reaction buffer mix*

5μl each primer

6μl 100mM $MgCl_2$

*** Reaction buffer mix :**

55μl 10x polymerase buffer

11μl each dNTP (10mM stocks)

334μl H_2O

The tube was then placed in an Hybaid Combi Thermal Reactor, TR2, or Omnigene (Hybaid) and one cycle of denaturation at 95°C and annealing of primers to template DNA at 40°C was performed. To this mix was added 1μl of *Pyrococcus furiosus* (*Pfu*) polymerase (Stratagene) using a "dedicated" 1μl Gilson, which was always kept in a sealed plastic box to prevent cross-contamination with foreign DNA. The contents were mixed by gently "flicking" the tube and then centrifuged at high speed to collect all the reaction mix. The mixture was then overlaid with 50μl paraffin oil and placed into an PCR machine for one cycle of polymerase extension at 75°C. The reaction then proceeded as follows, for 30 cycles:

DNA denaturation, 95°C, 1 minute
annealing of primers to template DNA, 40°C, 2 minutes
polymerase extension, 75°C, 2 minutes

PCR amplification was checked by agarose gel (0.7%) electrophoresis (see section 2.14) of a 15μl sample of PCR reaction.

2.23 Plasmid sequencing

2.23.1 Introduction

Plasmid sequencing of cloned, PCR amplified, double stranded DNA fragments was performed using the following method (V Mulholland, pers. comm.) based on Mierendorf and Pfeffer, (1987). Plasmid DNA was prepared for sequencing by large scale plasmid DNA preparations described in section 2.12.2.

2.23.2 Preparation of template DNA

To 3μg of plasmid DNA was added 1/4 volume of 1M NaOH, 1mM Na_2EDTA and incubated at 37°C for 30 minutes, to denature the plasmid DNA. 1/10 volume of 3M sodium acetate [pH 4.8] was added to neutralise the NaOH and 3 volumes of ethanol added to precipitate the denatured DNA at -70°C for 15 minutes. The DNA was recovered by centrifuging at high speed for 10 minutes, the supernatant carefully removed and the pellet washed in 70% (v/v) ethanol to liberate any remaining salt. The sample was centrifuged at high speed for 1 minute, the supernatant discarded and the pellet dried under vacuum. The pellet was resuspended in 7μl H2O, 1μl primer (see section 2.23.3) and 2μl 5x reaction buffer (200mM Tris.HCl [pH 7.5], 100mM $MgCl_2$, 250mM NaCl), resuspended by vortexing and incubated at 37°C for 15-30 minutes. This procedure enabled the annealing of primer to template DNA.

2.23.3 Sequencing reactions using "Sequenase"

All sequencing reactions were performed as detailed in the Sequenase V2.0 manufacturers (United States Biochemical Corporation) instructions. Multiple sequencing reactions (up to 12, maximum) were performed using micro-titre dishes. 5 minute incubation periods for termination and labelling reaction were strictly adhered to in order to prevent deterioration of the quality of sequence; caused by extended time taken to initiate termination or labelling reactions. Mixing was done by centrifuging the micro-titre dishes for 10 seconds at high speed. Completed reactions were stored at -20°C until needed. Reactions were heated to 80°C for 20-30 minutes prior to loading a sequencing gel (see section 2.23.4).

2.23.4 Sequencing gels

Gradient gels, 1-5%, were poured in 49cm x 20cm x 0.5cm glass plates that were cleaned with detergent and washed twice with ethanol. The back plate was siliconised using "Repelcote" (BDH), allowed to air dry and then cleaned with ethanol, as above.

The plates were then assembled according to the manufacturers instructions. The gradient gels were poured using the following mixtures, in two beakers :

1) 7ml 5x TBE acrylamide urea gel mix
   14μl 25% (w/v) ammonium persulphate
   14μl TEMED

2) 45ml 1x TBE acrylamide urea gel mix
   80μl 25% (w/v) ammonium persulphate
   80μl TEMED

Approximately 8ml of the 1x acrylamide mix was taken up in a 25ml pipette, using a pipette pump, followed by all the 5x acrylamide mix, introducing an air bubble to mix the contents. This solution was pipetted in between the two glass plates at an angle of 45° to the horizontal. The remaining 1x acrylamide mix was pipetted in between the glass plates, in the same fashion, tilting the receptacle, first one way then the other, until it was full. A well forming comb was inserted at the top of the receptacle and clamped into place using clips. Once the gel was set, typically after 1 hour, the comb was removed and the wells washed with 1x TBE buffer. The gel was placed into a gel tank and the upper and lower reservoirs filled with 1x TBE buffer. Samples were then loaded using a 20μl Gilson pipette and electrophoresed at 37W for 3 hours or until the leading dye front reached the bottom of the gel. The gel was then fixed in 10% (v/v) acetic acid for 15 minutes and dried under a vacuum at 80°C in a gel drier (Bio-Rad) for 30 minutes. The gel was then auto-radiographed for 24-48 hours.

2.24 Protein analysis

2.24.1 Concentration of culture supernatants

1 litre of LB containing PGA (0.5% w/v) and appropriate antibiotic(s), was inoculated with 10-15ml of an over-night bacterial cell culture and grown to an A_{600} = 0.5-0.7,

at 30°C, with shaking in an orbital shaker at 150rpm. The cells were centrifuged at 5K for 10 minutes using a Beckman J2-21 centrifuge and JA-10 rotor. The supernatant was collected and concentrated 30 or 40x using an Amicon Hollow Fiber Concentrator, model CH4, with a 10K "cut-off" filter, following the manufacturers instructions. The sample was further concentrated using an Amicon ultrafiltration cell (model 8010) to a final volume of 1-6ml.

2.24.2 Total protein estimation

The total protein content of an individual sample was estimated using an "Protein Assay" kit (Bio-Rad), which was performed according to the manufacturers instructions. The protein standard used in all assay's was BSA (Sigma), 1mg/ml stock solution.

2.24.3 Protein analysis

2.24.3.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

2.24.3.1.1 Protein analysis using the "Bio-Rad, Protean 11" gel apparatus

The following method of protein analysis is based on Silhavy *et al.*, (1984). Gels were poured using a "Bio-Rad, Protean 11" gel rig apparatus. The apparatus was constructed according to the manufacturers instructions. A 12% "lower" acrylamide gel mix was poured between the glass plates, using a 25ml pipette and pipette pump. A gap of 5cm was left at the top portion of the plates and H_2O saturated butanol was added as a thin layer, over the top of the gel, using a Pasteur pipette, in order to facilitate polymerisation (1-2 hours) under anaerobic conditions. Once polymerisation was complete the H_2O saturated butanol was removed by washing with H_2O and the stacking gel poured, as above, until the glass plates were filled. A well-formimg comb was inserted immediately between the plates and the gel left to polymerise (1/2-1 hour). Once polymerised the comb was carefully removed and the wells washed with running buffer, using a Pasteur pipette. The gel was then transferred to the gel rig

apparatus and the samples loaded through the upper buffer chamber. Samples (20-80 μl) were treated by boiling in loading buffer (final concentration 1x) to denature the protein prior to loading, as above. Molecular weight markers were obtained from Pharmacia (low molecular weight determination) and treated the same as test samples, or as prestained SDS-PAGE standards (low range), from Bio-Rad. Samples were electrophoresed into the stacking gel at 33mA for 20 minutes. Gels were then electrophoresed at 10-15 mA over-night.

12% lower gel	**3% stacking gel**
10.0ml 4x lower gel buffer	2.5ml 4x upper gel buffer
16.0ml Acrylamide stock	1.0ml Acrylamide stock
13.8ml H_2O	6.4ml H_2O
0.2ml APS* (10% (w/v),	0.2ml APS* (10% (w/v),
prepared freshly)	prepared freshly)

(*APS - ammonium persulphate)

4x lower gel buffer (1.5M Tris.HCl, [pH 8.8], 0.4% SDS)

181.7g Tris base

40ml 10% (w/v) SDS

H2O to 1 litre

The pH was adjusted to 8.8 with HCl, 1.0ml of TEMED was added and the solution stored at 4°C.

4x stacking gel buffer (0.5M Tris.HCl, [pH 6.8], 0.4% SDS)

60.6g Tris base

40.0ml 10% (w/v) SDS

H_2O to 1 litre

The pH was adjusted to 6.8 with HCl, 2.0ml of TEMED was added and the solution stored at 4°C.

Acrylamide stock

300g acrylamide

8g bis-acrylamide

H_2O to 1 litre

The solution was filtered through Watman filter paper, grade 1, and then stored at 4°C.

4x running buffer stock

60g Tris base

288g glycine

H_2O to 5 litres.

0.1% Bromophenol blue (BpB)

10mg bromophenol blue

10.0ml H_2O

2x sample buffer (SB)

12.5ml 4x stacking gel buffer

20.0ml glycerol

H_2O to 60.0ml

2x loading buffer

0.5ml ß-mercaptoethanol

0.25ml 0.1% (w/v) BpB

4.0ml 10% (w/v) SDS

5.3ml 2x SB

2.24.3.1.2 Protein analysis using the "Phast System" (Pharmacia)

All procedures were followed according to the Phast System user's manual, Pharmacia. Homogeneous, 12%, SDS-PAGE gels were used and samples treated and loaded as described in the users manual.

2.24.3.2 Analytical isoelectric focusing (IEF)

This method was followed as described, R Barralon, (pers. comm.)

The gel was poured between two 18cm x 16cm x 0.5cm glass gel plates, previously washed with ethanol followed by acetone and wiped dry with a tissue, separated by a 1mm thick three-sided spacer and clamped together by 6 large bulldog clips.

The gel consisted of :

4.65ml 29.1% (w/v) acrylamide

4.05ml 0.9% (w/v) N:N'-methylene-bisacrylamide (Kodak)

11.4ml 50% (w/v) glycerol

1.35ml 40% (w/v) ampholytes (Resolyte, Electran)

4.13ml H_2O

(18μl TEMED)

(1.0ml APS (10mg/ml))

Solutions in brackets were added last.

The gel was polymerised over-night, so as to obtain a dry gel for easy sample application. One glass plate was removed using a spatula to lever the plate. Liquid paraffin was applied (approximately 0.5-1.0ml) to a pre-cooled (6°C) IEF gel rig, avoiding bubbles to enable uniform cooling and the gel placed, plate side down, onto the paraffin. Pre-soaked electrode wicks (Bio-Rad 170-4204), 1M NaOH for the cathode and 0.3M citric acid [pH 6.0-10.0] for the anode, were laid onto the respective edges of the gel. The IEF gel was then pre-electrophoresed for 45 minutes at 12W constant power. 10μl samples were loaded onto the gel using filter paper applicators (cut up wicks) placed 1 inch below the top (anode) of the gel. These wicks may be removed 10 minutes into the run. 5-10μl of a marker, cytochrome C (10mg/ml, Sigma), which has a pI=10.35, was loaded beside the test samples. The

samples were electrophoresed at 12W constant power for 85 minutes or until the marker formed a tight, focused, band. The electrode-wicks were then removed, carefully, and the gel overlaid with a pectate lyase overlay (see section 2.24.3.2.1) and the sandwich incubated at 37°C for approximately 70 minutes.

2.24.3.2.1 Pectate lyase (pel) overlay

The gel overlay was prepared using the equipment described in section 2.24.3.2, so that a replica of the IEF gel could be made using the following components:

30ml pel reaction buffer (see section 2.11.1)
30ml PGA (5.75mg/ml)
0.6g agarose

The apparatus was heated to 45°C and boiling agarose was poured into the gel mould using a 25ml pipette and pipette pump. The gel was allowed to set and the gel removed from the glass plates by levering them apart with a spatula, being careful to keep the gel attached to one plate only. The gel was then gently pealed off the remaining plate and overlaid onto the IEF gel. The sandwich was then wrapped in "cling film" and incubated at 37°C for 70 minutes. The over-lay gel was removed and stained in 1% (w/v) CTAB for 20 minutes. Pel activity could be detected by a clear zone upon a white, opaque, background.

2.24.4 Staining of SDS-PAGE gels

2.24.4.1 Silver staining

The following method of silver staining SDS-PAGE gels was adapted from Johannson and Skoog, (1987), by R. Barallon (pers. comm.).

SDS-PAGE gels were fixed in glacial acetic acid:methanol:H_2O (1:4:5) for 30 minutes in a plastic box and washed with distiled H_2O for 5 minutes, with gentle rocking. 25% glutaraldehyde : H_2O (1:1) mix was added and the gel gently rocked for 15 minutes before washing twice in distiled H_2O, for 2x 10 minutes. The gel was then rinsed in 20% (v/v) ethanol for 15 minutes and then stained for 15 minutes in solution A. The gel was then rinsed twice in distilled water, for 5 minutes and developed in solution B for 2-5 minutes. The reaction was then stopped in glycerol:glacial acetic acid:H_2O (11:10:89). Using this method protein levels could be detected down to 10ng.

Solution A

2ml of 20% (w/v) silver nitrate was added to 2ml of 25% (v/v) ammonia, in a 500ml beaker and mixed by swirling. The volume was increased to 10ml with 4% (w/v) sodium hydroxide and further diluted to 200ml with 20% (v/v) ethanol.

Solution B

200μl of formaldehyde and 50μl of 2.3M citric acid were added to 200ml of 20% (v/v) ethanol and mixed by inversion.

For silver staining SDS-PAGE gels using the "Phast System" a Phast-Gel Silver Kit (Pharmacia) was used in accordance with the manufacturers instructions. This method used the modified high sensitivity protocol of Heukeshoven and Dernick, (1988). Sensitivity of protein detection was achieved to 0.05-0.1 ng protein.

2.24.4.2 Coomassie blue

Essentially, coomassie blue (0.05% (w/v)) in a solution of 10% (v/v) glacial acetic acid and 30% (v/v) methanol was used to stain the gel for 15 minutes. De-staining was achieved by washing the gel in the above solution, minus coomassie blue, over-night.

2.24.5 ^{35}S-methionine labelling of protein

This method was adapted from the T7 RNA polymerase expression system of Tabor and Richardson, (1985).

A 5ml or 10ml bacterial cell culture was grown over-night at 30°C, at 250rpm using an orbital shaker, in LB containing 0.5% (w/v) PGA and appropriate antibiotics. This was sub cultured, 1:40, into 5ml or 10ml of fresh media in a 250ml sterilised conical flask and cultured in a New Brunswick, temperature controlled, Aquatherm water bath at 30°C, with shaking at 250rpm, until an A_{600} = 0.4-0.6. 1ml of this culture was removed and centrifuged in a mirofuge at high speed. The pellet was washed once in 5ml of PMB. The washed pellet was resuspended in 5ml of PMB supplemented with 18 amino acids (minus cysteine and methionine), final concentration 0.02% (v/v), and the suspension transferred to a sterile 50ml conical flask. The cells were then cultured at 30°C, using a temperature controlled water bath set at 250rpm, for 60 minutes. 10 or 100μCi ^{35}S-methionine was added for each ml of culture and three 100μl aliquots were removed at 0, 1, 2, 5, 15, (30) and 60 minute intervals. To one aliquot, in a microfuge tube, was added "cold" L-methionine (2mg/ml stock solution) to a final concentration of 200μgml^{-1} in order to stop labelling of protein (the other two aliquots were used as described in section 2.24.5.1). The sample was centrifuged at high speed for 1.5 minutes and the supernatant stored at -20°C until needed for SDS-PAGE analysis (see section 2.24.3.1.1). After the SDS-PAGE gel had been electrophoresed it was dried under vacuum at 60°C using a gel drier (Bio-Rad) for 2 hours and autoradiographed for 1-7 days using ß-max hyperfilm (Amersham International). Radioactive "Rainbow" molecular weight markers (Amersham International) were used to determine the molecular weights of any proteins detected by autoradiography.

2.24.5.1 Incorporation of ^{35}S-methionine into *Ecc* extracellular secreted protein

This method was adapted from Porter, (1984). In order to follow the change in optical density of the cells a 100μl aliquot was added to 900μl PMB with amino acid

supplements as described in section 2.24.5. The A_{600} was recorded for each time point.

The third aliquot of 100μl was added to 2ml ice cold TCA and "cold" L-methionine, final concentration 200μgml^{-1}, in a microfuge tube. The tube was mixed by inversion and incubated on ice for 30 minutes to precipitate proteins and bacterial cells. After 30 minutes the sample was passed through a 0.45μm nitrocellulose filter, by vacuum, and washed; twice with ice cold 5% (w/v) TCA, once with ethanol and once with ethanol:ether (1:1, (v/v)). The filter was placed in a scintillation vial and dried at 60°C, for 10 minutes. 3ml of triton-toluene PPO scintillation fluid was added and the vial placed in an Packard Tri-carb Scintillation counter to record the counts per minute (cpm) of the sample.

2.24.6 Expression of cloned gene(s) using IPTG

An 5ml over-night bacterial cell culture was grown in LB, with appropriate antibiotic, at 30°C or 37°C (*E coli* or *Ecc* respectively) with shaking at 250rpm. The over-night culture was sub-cultured (1:40) into 10ml LB with appropriate antibiotic and grown at 30°C or 37°C, with shaking at 250rpm, to an A_{600} = 0.5. IPTG was then added to a final concentration of 0.3M and the cultures incubated for a further 2 hours. The cells were then centrifuged at 4500 rpm for 10 minutes and the supernatant removed and concentrated, 20x, using an Amicon ultrafiltration cell (model 8010). The sample was then stored at -20°C until needed. The intra-cellular fraction was obtained by resuspending the pelleted cells in 5ml of LB, by vortexing, and consequent sonication for 3x 30 seconds, at an amplitude of 6.0, with a 0.7 inch probe and at 4°C with 30 seconds cooling in between sonications. The cell debris was removed by centrifugation at 4500rpm for 10 minutes and the sample stored at -20°C.

CHAPTER 3

LOCALISED MUTAGENESIS OF THE *Erwinia carotovora* (STRAIN SCRI193) *out* GENE CLUSTER

3.1 Introduction

In the search for Out$^-$ secretion mutants (Pel$^-$, Cel$^-$, Prt$^+$) of *Ecc* strain SCRI193, many reports have shown (see section 1.5.4.2) that a number of Out$^-$ mutants have been isolated. These Out$^-$ mutants have been generated randomly, by exposure to chemical(s) such as EMS, or by transposon inactivation of specific gene(s). These secretory (Out$^-$) mutants have been isolated at a relatively low frequency of 0.2% (Reeves, 1992), using EMS, or 0.1% using transposon mutagenesis (Murata *et al.*, 1990). No chemical mutagenesis studies yielded any conditional secretion mutants, probably due to the low chance of mutating the specific secretion gene cluster (*out* cluster), see section 1.5.4.2. In order to increase the chance of introducing lesions into the *out* gene cluster, and thus enhance the likelihood of isolating conditional secretory (Out^{ts}) mutants, this study employed the use of localised mutagenesis (Hong and Ames, 1971) of the *out* cluster.

3.1.1 Hydroxylamine, a chemical mutagen

There are many ways in which to induce mutational damage to DNA, these include a variety of agents such as : UV light, ionizing radiation and chemicals. The damage caused to DNA can be in the form of ; A) breaks, base dimers and cross-links to the structure of DNA, or, B) missing, modified or incorrect base changes (Zubay, 1986). Chemically one of the most specific *in vitro* mutagens is hydroxylamine (NH_2OH). The mode of action of hydroxylamine is a preferential reaction with cysteine, with which it forms a specific adduct and can then base pair with adenine. This ultimately results in the replacement of an cysteine-guanine base pair with an adenine-thymine base pair (a transitional event).

There are disadvantages with using chemical mutagens. Firstly, they can induce multiple lesions in the gene(s) of interest unless conditions for mutagenesis have been monitored, to produce exposure times that will yield low, but detectable, numbers of mutants. Secondly, chemical mutagens tend to be highly toxic and carcinogenic.

In this study hydroxylamine was used in localised mutagenesis of the *out* cluster. In order to do this, a selectable marker was first linked to the *out* gene cluster by transposon trapping.

3.2 Transposon trapping

λ_{467} was used to infect HC131 and approximately 5000 Km^r transductants were isolated and pooled to form a heterologous culture. The generalised transducing phage, øKP, was propagated on the heterologous culture to make a øKP heterologous lysate. This lysate was then used to transduce known Out^- secretion mutants defective in *out* genes mapping to the 5' (RJP190 and RJP120) and 3' (RJP159 and RJP249) end of the *out* cluster. This was performed in order to attempt to isolate a transductant carrying a transposon(s) linked to either end of the *out* cluster. 1169 transductants (Km^r) were screened on enzyme detection media for restoration of the Out^+ phenotype. Two transductants were isolated, JNH1 (from RJP190) and JNH2 (from RJP159), that exhibited the Out^+Km^r phenotype, inferring linkage to the *out* cluster.

λ_{840} was also used as described above in order to obtain Tn*10* linkage to the 5' (RJP211 and RJP233) and 3' (RJP253) end of the *out* cluster. 600 Tc^r resistant colonies were screened, unsuccessfully, for restoration of Out^+ phenotype.

Screening transductants on enzyme detection media proved to be difficult, due to pseudopositive halos, probably caused by superinfection by øKP and lysis of the transductant cells. A further factor that may also have contributed to false positives could be due to the øKP lysate titre being very high, $>10^{11}$ pfu/ml. In order to attempt to overcome this problem, short-wave UV irradiation of øKP lysates was performed prior to infection (see section 3.3).

3.3 UV killing curve of the generalised transducing phage ϕKP

3.3.1 Introduction

Before irradiating lysates with short wave UV light, a killing curve was constructed to estimate the optimum length of time for exposure. At the same time transductional frequency was monitored, at each irradiation time interval, to establish if an increase in transductant numbers could be attained by irradiating the lysate, which has been shown previously with the P1 phage of *E coli*. (Masters *et al.*, 1984).

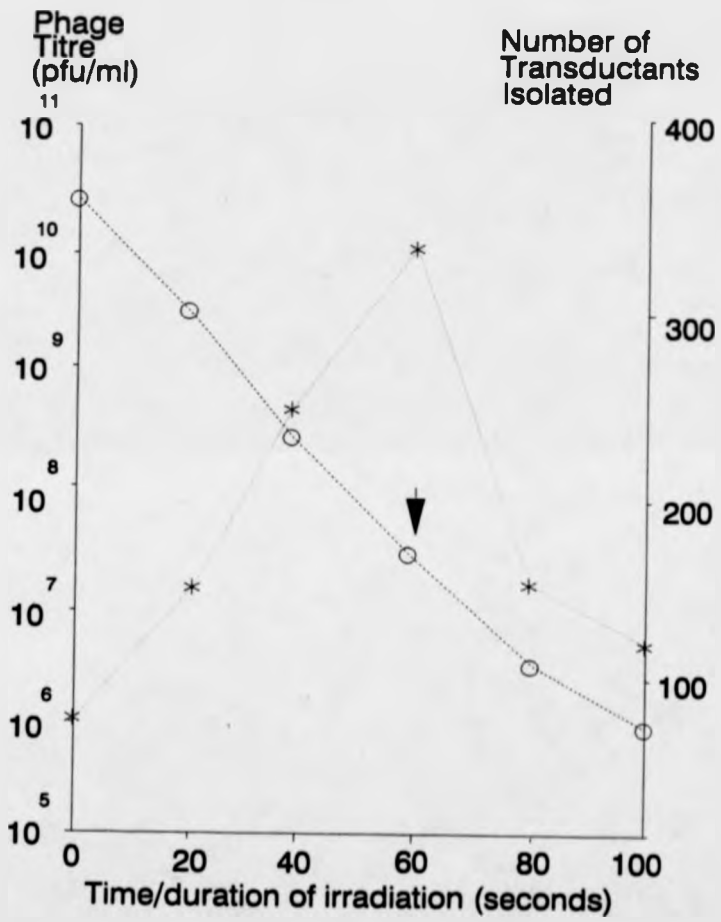
3.3.2 Killing curve

A øKP.JNH1 lysate was diluted 1:10 in phage buffer and irradiated under short-wave UV at 7.25μWcm^{-2}. 200μl aliquots were removed at 20 second intervals, to a maximum of 100 seconds, for transduction (see section 2.6) using HC131 as a host and for øKP titration. The results are illustrated in figure 3.1. From the graph, a 3 log drop in phage titre was shown to correspond to 60 seconds exposure to UV, which also yielded the maximum number of transductants (indicated by the arrow). The experiment was repeated to confirm the results. The 60 second time of exposure to short-wave UV was used in all subsequent øKP transductions.

3.4 Mapping of the transposon, Tn*5*, to the *out* cluster by co-transduction

In order to position the transposon to one end, 5' or 3', of the *out* cluster, øKP lysates were to be made on each of the strains JNH1 and JNH2 (see section 2.5). Only JNH1 was found to be øKP sensitive. Linkage of Tn*5* in JNH2 was not pursued further. The JNH1 lysate was used to re-transduce (section 2.6) known Out$^-$ mutants to Km^r. In this way, by screening the Km^r transductants for the percentage that were now Out$^+$, this gave a relative position of the known Out$^-$ mutation to the Tn*5* insertion. This enabled the construction of a putative linkage map of Tn*5* to known *out* mutations (figure 3.2), the crude relative positions of which were already known (Reeves, 1992).

Figure 3.1 UV killing curve of ϕKP.JNH1

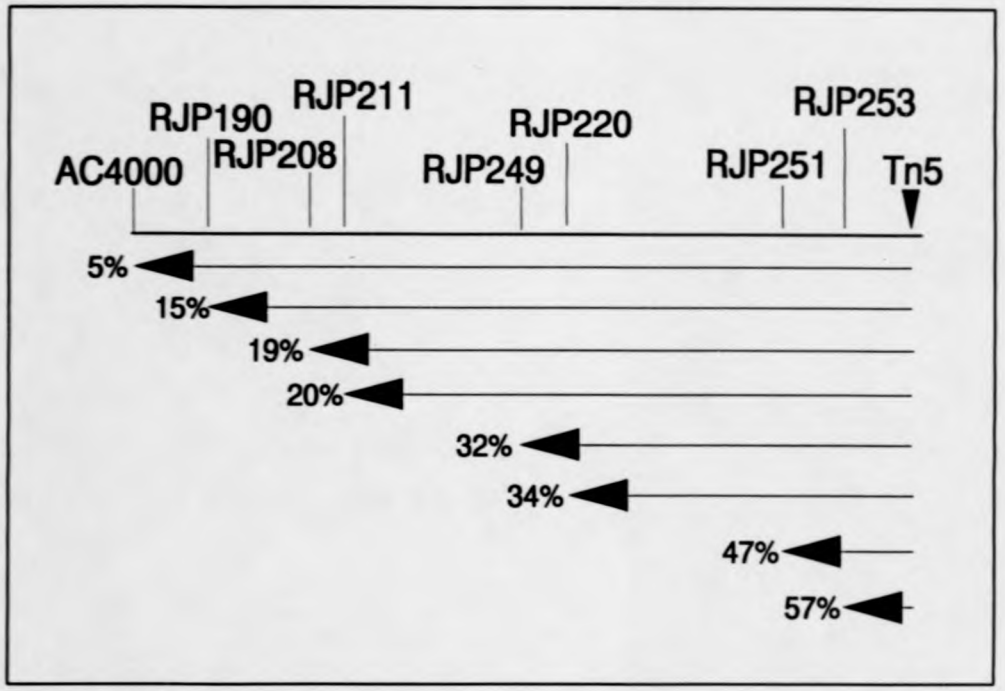


Key

○ - ϕKP.JNH1 lysate titre

* - Number of kanamycin resistant transductants

Figure 3.2 Linkage map of Tn5 to known out mutations



Key - Arrows represent % co-transduction frequency of Out+Km^r to each known out mutation shown above.

3.5 Localised mutagenesis of the *Ecc* spp *out* cluster, using ϕKP.JNH1

3.5.1 Hydroxylamine killing curve of ϕKP.JNH1

In order to derive the time of exposure for a 3 log drop in phage titre, estimated to produce mutants with single mutations, 1.0ml of øKP.JNH1 was mutagenised (see section 2.8) with hydroxylamine. 100μl aliquots, diluted 100x in LBSE (table 2.4), were taken every 6 hours, to a maximum of 48 hours, and used to titrate the phage. A mutagen "free" sample, water added instead of hydroxylamine, was used as a control for the experiment. The killing curve is illustrated in figure 3.3. The time taken for a 3 log drop in phage titre is indicated by the arrow, 21.5 hours. The experiment was repeated to confirm the results.

3.5.2 Localised mutagenesis

Localised mutagenesis (see section 2.8) was performed using 21.5 hours exposure of øKP.JNH1 to hydroxylamine, as derived from the killing curve (section 3.5.1). This lysate was then used to transduce HC131, and transductants, (Km^r), screened for mutant phenotypes on enzyme detection media (section 2.9). The mutagenised phage viability was checked by titrating samples taken during the mutagenesis procedure (A; t=0, B; t=21.5 hours, C; pellet supernatant and D; re-suspended mutagenised phage). A theoretical recovery percentage can be obtained by using the following equation :

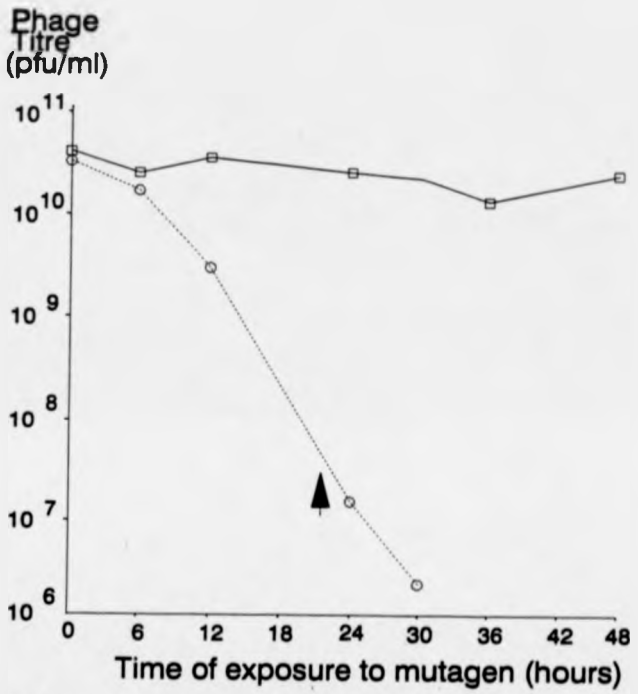
$$\frac{(B-C)}{B} \times 100$$

An actual percentage of mutagenised phage was obtained by using the following equation :

$$\frac{D}{B} \times 100$$

In all mutagenesis experiments the percentage of viable phage recovery was >70%. This reduction in phage titre could be due to the harsh centrifuging needed to pellet the

Figure 3.3 Killing curve of ΦKP.JNH1 using hydroxylamine



Key:

□ - Control sample titre (water added instead of mutagen)
○ - ΦKP.JNH1 lysate titre

phage or some degree of phage killing, due to the mutagen.

3.5.3 Putative Out$^-$ mutants generated by localised mutagenesis of the *Ecc out* cluster

Table 3.1 shows the range of mutant phenotypes isolated by screening the Km^r transductants (section 3.5.2) on detection media (section 2.9). The "window" for screening mutants at 26°C and 33°C was determined by monitoring wild type *Ecc* and Out$^-$ (RJP251) on enzyme detection media at increasing (>30°C) and decreasing (<30°C) temperatures. It was found that above 33°C the Out$^-$ phenotype was unreliable, small halos were detected, which was thought to be due to lysis. Thus the temperature of 33°C was chosen to avoid problems in the screening procedure. The lower limit was set at 26°C because below this temperature the wild type halos were seen to diminish slightly.

Figure 3.4 shows examples of two Out$^-$ mutants, one of which is conditional, isolated during localised mutagenesis. At position 1 is the wild type control (HC131) which shows the expected halos on enzyme detection media at both temperatures (26°C and 33°C). Position 2 is an example of an Out$^-$ putative secretion mutant (HJN1000) that has very reduced halos for pectate lyase (Pel) and cellulase (Cel), at both temperatures, but wild type halos for protease (Prt), detection plates. At position 3 is an example of a putative conditional, Out^{ts}, secretion mutant (HJN1004), that exhibits a wild type phenotype at 26°C but much reduced, Out$^-$, halos at 33°C.

3.6 Discussion

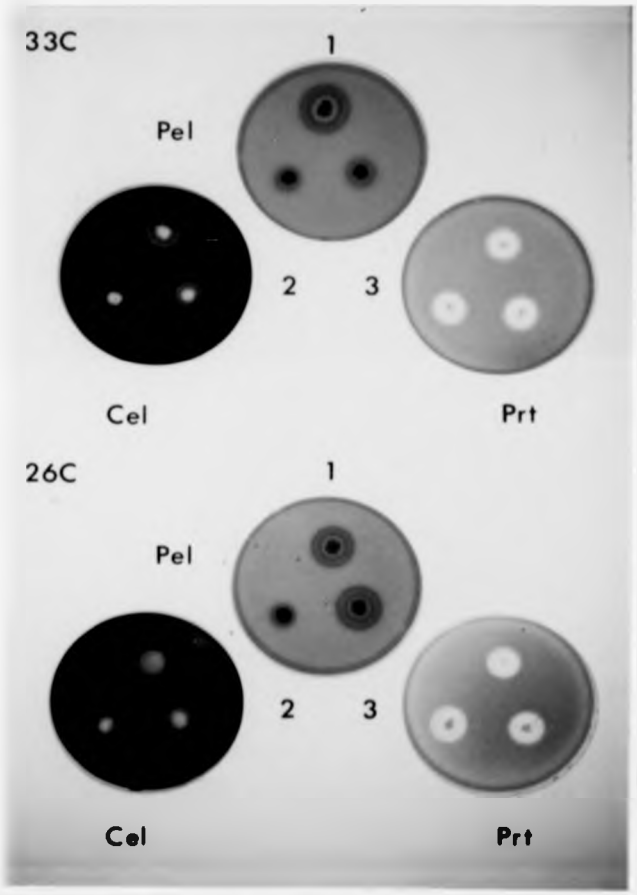
It has been shown, figure 3.1, that by irradiating øKP lysates, to a maximum after 60 seconds, prior to infection of host bacterial cells, actually increases the numbers of transductants. This phenomenon has been discussed by Margolin, (1987), who showed that UV (short-wave) irradiation of coliphage P22 results in an increased number of complete transductions and a decreased number of abortive transductions. The 60

Table 3.1 Putative mutants isolated by localised mutagenesis of the *Ecc* spp. *out* cluster.

Strain	Total	Frequency[a]	Phenotype
HJN1000			Out$^-$ (Pel-,Cel-,Prt+)
HJN1005			Out$^-$ (Pel-,Cel-,Prt+)
HJN1006			Out$^-$ (Pel-,Cel-,Prt+)
HJN1007			Out$^-$ (Pel-,Cel-,Prt+)
HJN1008			Out$^-$ (Pel-,Cel-,Prt+)
HJN1010			Out$^-$ (Pel-,Cel-,Prt+)
HJN1011			Out$^-$ (Pel-,Cel-,Prt+)
HJN1012			Out$^-$ (Pel-,Cel-,Prt+)
HJN1014			Out$^-$ (Pel-,Cel-,Prt+)
HJN1015			Out$^-$ (Pel-,Cel-,Prt+)
HJN1016			Out$^-$ (Pel-,Cel-,Prt+)
HJN1017			Out$^-$ (Pel-,Cel-,Prt+)
HJN1018			Out$^-$ (Pel-,Cel-,Prt+)
HJN1019			Out$^-$ (Pel-,Cel-,Prt+)
HJN1020			Out$^-$ (Pel-,Cel-,Prt+)
	(15)	(0.6%)	
HJN1003			Out^{ts} (Out$^-$, 33°C
HJN1004			Out^{ts} Out$^+$, 26°C)
HJN1013			Out^{ts}
HJN1021			Out^{ts}
	(4)	(0.2%)	
HJN1001			Auxotrophic
HJN1002			Auxotrophic
HJN1009			Auxotrophic
	(3)	(0.1%)	
HJN1022			Reduced growth rate
HJN1023			Reduced growth rate
	(2)	(0.1%)	

a - Percentage of the number of transductants screened (2368)

Figure 3.4 Examples of two putative secretion mutants (Out$^-$, Out^{ts}) on enzyme detection media.



Key:

1. Wild type control (HC131)
2. Putative Out$^-$ secretion mutant (HJN1000)
3. Putative Out^{ts} secretion mutant (HJN1004)

Pel - Pectate lyase detection media
Cel - Cellulase detection media
Prt - Protease detection media

second exposure time also corresponds to a 3 log drop in phage titre and proved to be successful in "cleaning up" a difficult screening problem (see section 3.2), by reducing the number of phage present, so abating lysis due to superinfection.

The transposon Tn*5* has been successfully linked to the *out* cluster and positioned, to the 3' end of the *out* cluster, by co-transduction linkage data, figure 3.2. The relative positions of the known Out$^-$ mutants to the transposon, are consistent with their relative positions by cosmid complementation as defined by Reeves, (1992). Cotransduction mapping of the transposon is not always totally satisfactory, due to the fact that not all markers are transduced with the same frequency (Masters, 1985). A better way of physically mapping the transposon would have been by three-point crosses, by ordering two mutations with respect to a third known marker, or by deletion mapping. For this study, however, only crude linkage data was required in order to ascertain if the transposon was truly linked to the *out* cluster and to which end, 3' or 5', it was linked. Fine mapping of mutations in Out$^-$ mutants generated in this study will be discussed in later chapters.

Localised mutagenesis has been successfully adapted for *Ecc* and has clearly shown that Out$^-$ mutants can be generated at fairly high frequencies, see table 3.1, which are much higher than those reported elsewhere, using random mutagenesis, see section 3.1. The ease of recovery of *out* mutations in the *out* cluster increases the chance of procuring conditional, *out*^{ts}, mutants, which in this study were isolated at a frequency of 0.2%. Interestingly, three auxotrophs were isolated, which, if linked to the *out* cluster, could be used as an selectable auxotrophic markers. Two strains, HJN1022 and HJN1023, appeared to exhibit a reduced growth rate on detection media. Further analysis of this phenotype will be examined in chapter 4.

The putative mutants isolated in this study were characterised, as discussed in following chapters, to prove that they were true secretion mutants, by localising

intracellular accumulation of extracellular enzymes. Further to this, molecular genetic characterisation was used to map particular lesions to specific gene(s).

CHAPTER 4

CHARACTERISATION OF Out⁻ SECRETION MUTANTS GENERATED BY LOCALISED MUTAGENESIS OF THE *out* GENE CLUSTER

4.1 Introduction

Localised mutagenesis (section 3.5.2) of the *out* cluster, using hydroxylamine, has generated a wide variety of mutants (section 3.5.3). The majority of mutants produced an Out$^-$ (Pel$^-$, Cel$^-$, Prt$^+$) phenotype. This phenotype has been shown by previous workers (Andro *et al.*, 1984; Ji *et al.*, 1987; Thurn and Chaterjee, 1985; Gibson *et al.*, 1988; Reeves *et al.*, 1992; Murata *et al.*, 1990; He *et al.*, 1991(a)) in *Ecc* and *Ech*, to be the result of a disruption in the secretion process, due to a mutation in one or more of the Out proteins, that are essential for the secretion of Pel and Cel. Beside isolating putative Out$^-$ and Out^{ts} mutants this study also revealed 3 putative auxotrophs (HJN1001, HJN1002 and HJN1009) and two possible growth rate mutants (HJN1001, HJN1002).

This chapter is concerned with characterising the putative mutants isolated and ascertaining whether or not the Out$^-$ and Out^{ts} mutants are truly secretion mutants. If they are secretion mutants, then by growing up bacterial cultures of each of the mutants in induction medium, the cells could then be lysed and fractionated to produce extracellular and intracellular fractions. These fractions could then be analysed, by spectrophotometry, to assay for cellular or extracellular location. This should indicate that the extracellular enzymes are synthesized but have somehow been blocked in the secretion pathway.

To confirm that the mutations that have been generated, are indeed due to localised mutagenesis of the *out* cluster, each of the Out$^-$ or Out^{ts} mutants were positioned on the linkage map (section 3.4) with respect to the transposon, Tn*5*. This was done by making øKP lysates on each of the mutant strains and transducing into the wild type HC131, selecting for Km^r Out$^-$.

4.2 Are the Out$^-$ or Out^{ts} mutations, generated by localised mutagenesis, linked to the transposon Tn*5* ?

øKP lysates were made (section 2.5) on each of the Out$^-$ or Out^{ts} putative secretion mutants, the titres were all $> 10^8$pfu/ml. These lysates were used to transduce (section 2.6) the wild type HC131, and the percentage co-transduction frequency of Km^r Out$^-$ (figure 4.1) used to position the mutations, relative to the transposon, Tn*5*. The higher the frequency of co-transduction the closer the mutation was to the transposon, Tn*5*. Conversely, the lower the frequency, the further away the mutation was from Tn*5*. The results are shown in figure 4.2 and the mutations are positioned in comparison to the original linkage map of known Out$^-$ mutants (figure 3.2). This data assumes that each mutation lies to the left of the transposon Tn*5*, within the *out* cluster, but the mutation(s) could lie to the right of the transposon Tn*5*, outside the *out* cluster. Mapping the mutations to the *out* cluster is discussed in chapter 5.

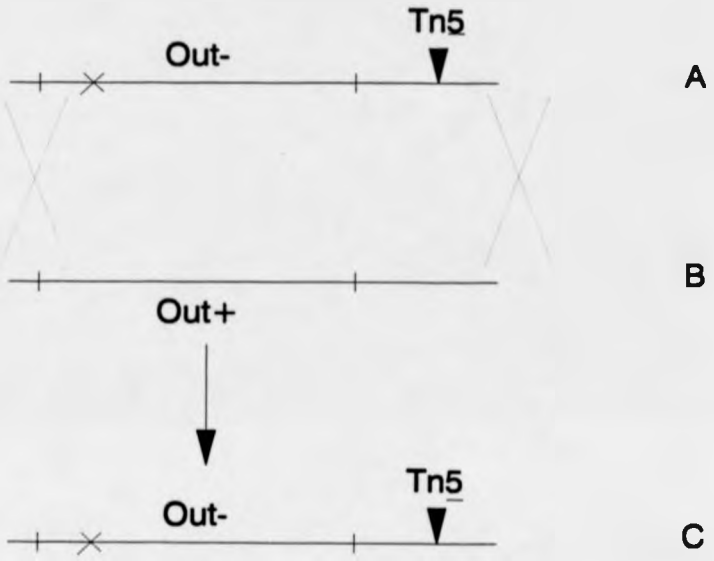
4.2.1 Discussion

All the mutations generated in this study were mapped relative to the transposon Tn*5*. This method of mapping mutations has already been discussed (section 3.6) as only a "crude" estimate of the physical position of the lesion causing the mutant phenotype. Further "fine" mapping of the lesion(s) to specific area(s) or specific gene(s), within the *out* cluster, is described in chapter 5.

4.3 Are the Out$^-$ or Out^{ts} mutants "true" secretion mutants ?

The putative Out mutant strains were fractionated according to the procedures outlined in section 2.10. All strains were cultured at 30°C except for the conditional Out^{ts} mutants, which were also cultured at 26°C and 33°C. Quantitative spectrophotometric analysis was performed, on each fraction, as described in section 2.11.

Figure 4.1 Mapping of secretion mutants generated by localised mutagenesis.



Key :

A - Donor, Out- or Out(ts) secretion mutant

B - Recipient, wild type Ecc (HC131)

C - Transductant

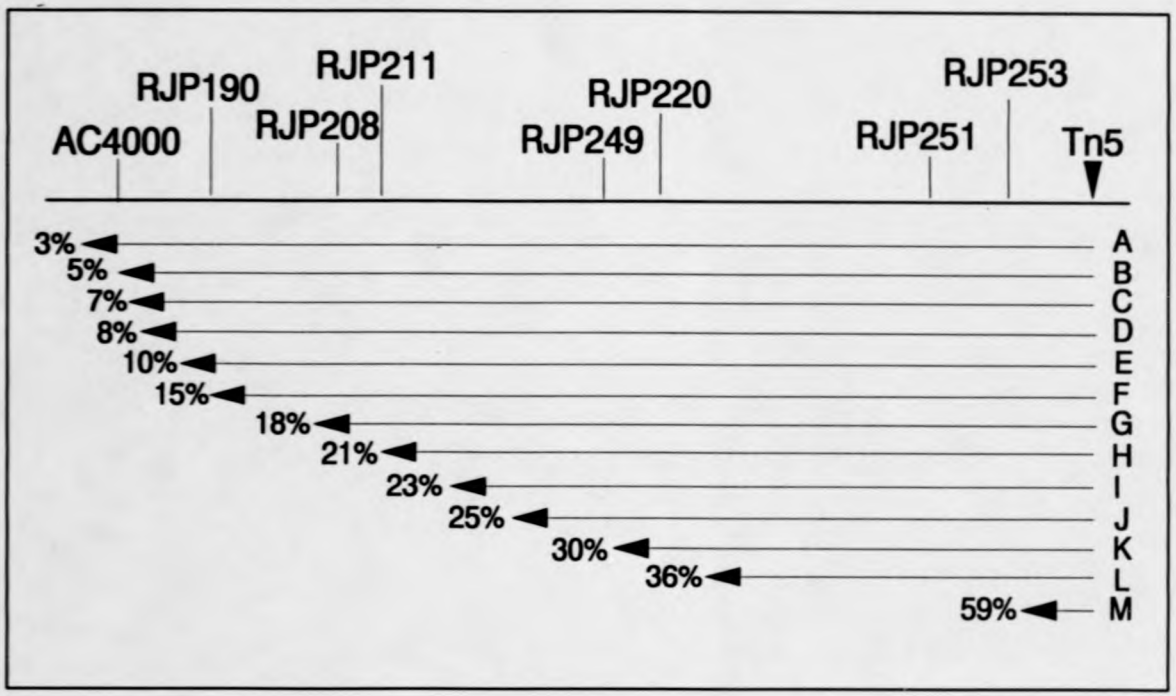
- Homologous recombination
(recombination events may also occur between any of these points)

Figure 4.2
The percentage co-transduction frequency of Out$^-$ Km^r for each secretion mutant, is shown by the arrows.

Key :

A - HJN1003
B - HJN1000
C - HJN1007, HJN1014
D - HJN1018
E - HJN1016, HJN1017
F - HJN1006
G - HJN1020
H - HJN1012
I - HJN1010, HJN1015
J - HJN1004, HJN1005, HJN1011
K - HJN1013
L - HJN1019, HJN1021
M - HJN1008

Figure 4.2 Linkage map of Out- secretion mutants generated by localised mutagenesis.



4.3.1 Results

The results of spectrophotometric analysis are displayed in tables 4.1 and 4.2. In all cases the periplasmic and cytoplasmic markers, ß-lactamase and ß-galactosidase, were assayed, to ensure that the fractionation procedures were successful. The wild type strain HC131 and the known Out$^-$ mutant RJP220 were used as controls.

Table 4.1 shows that control enzyme markers have been localised to the appropriate fractions (>64% of Bla in the periplasm and >88% of ß-galactosidase in the cytoplasm). Each of the putative Out$^-$ secretion mutants isolated in this study show periplasmic accumulation of Pel and Cel at 30°C, similar to that of the control Out$^-$ secretion mutant RJP220. For the putative conditional (Out^{ts}) secretion mutant, HJN1003, there was some extracellular secretion of Pel and Cel, but this was not to wild type levels. In each case Prt was secreted to the external medium to wild type levels.

The results shown in table 4.2 indicate that from the 4 putative conditional (Out^{ts}) secretion mutants, only 2 produced the desired phenotype (HJN1003 and HJN1004). For these two mutants Pel and Cel accumulated periplasmically at the restrictive temperature (33°C), but exhibited an Out$^+$ phenotype (wild type) at the permissive temperature (26°C). The two putative Out^{ts} mutants (HJN1013 and HJN1021) that did not show this conditional phenotype, were shown to be Out$^-$ secretion mutants.

4.3.2 Discussion

These results confirm that the putative Out$^-$ secretion mutants, generated by localised mutagenesis, are "true" secretion mutants. This indicates that Pel and Cel are still synthesized but cannot be secreted. The fact that Prt secretion into the external medium is not affected in any of these mutants supports the hypothesis of an independent pathway of secretion (section 1.5.4).

Table 4.1 Extracellular enzyme assays of Out⁻ secretion mutants, fractionated after culturing at 30°C

	Enzyme											
	Pel				Cel				Prt			
	Percentage enzyme activity											
Strain	S	P	C	%wt	S	P	C	%wt	S	P	C	%wt
HC131	90	8	2	100	94	0	6	100	99	1	0	100
RJP220	1	72	27	63	2	74	24	144	98	1	1	79
HJN1000	1	41	58	316	1	44	55	197	91	4	5	18
HJN1003	35	53	12	111	40	43	17	367	98	1	1	113
HJN1004	7	69	24	197	8	69	23	458	94	3	3	33
HJN1005	1	72	27	167	0	83	17	400	92	4	4	19
HJN1006	1	61	38	132	3	51	46	21	95	2	3	26
HJN1007	1	65	34	164	0	80	20	411	86	7	7	12
HJN1008	5	60	35	188	5	65	30	669	93	3	4	22
HJN1010	1	63	36	174	0	77	23	134	100	0	0	7
HJN1011	1	57	42	163	0	73	27	137	100	0	0	13
HJN1012	1	56	43	194	0	84	16	14	100	0	0	34
HJN1013	3	57	40	154	4	67	29	29	100	0	0	14
HJN1014	1	74	25	175	0	73	27	78	100	0	0	11
HJN1015	1	62	37	191	1	87	12	127	100	0	0	8
HJN1016	1	61	38	185	0	76	24	72	100	0	0	60
HJN1017	1	69	30	174	0	89	11	117	100	0	0	54
HJN1018	1	69	30	155	5	80	15	362	100	0	0	16
HJN1019	1	54	45	122	3	78	19	422	100	0	0	39
HJN1020	1	77	22	142	8	81	11	390	100	0	0	33
HJN1021	5	74	21	143	7	78	15	295	100	0	0	54

Enzyme							
Bla				β-gal			
Percentage enzyme activity							
S	P	C	%wt	S	P	C	%wt
4	93	3	100	0	1	99	100
5	89	6	131	0	8	92	24
3	64	3	124	1	5	94	71
4	90	6	164	0	10	90	71
1	86	13	154	1	8	91	41
4	87	9	171	0	4	96	65
7	78	15	155	0	4	96	54
7	77	16	162	1	11	88	40
4	77	19	127	1	6	93	56
6	80	14	165	1	5	94	48
6	84	10	189	2	2	96	41
3	75	22	143	0	7	93	63
6	80	14	200	1	3	96	45
3	88	9	112	0	8	92	48
4	84	12	183	1	5	94	51
4	80	16	182	1	5	94	54
4	87	9	164	0	4	96	56
3	90	7	155	1	6	93	43
5	84	11	144	1	3	96	62
1	95	4	214	1	3	96	50
2	93	5	213	1	6	93	50

Key :

S - Supernatant fraction
P - Periplasmic fraction
C - Cytoplasmic fraction
%wt - Percentage of wild type level

Table 4.2 Extracellular enzyme assays of conditional Out^{ts} secretion mutants, fractionated after culturing at 26°C and 33°C

	Enzyme											
	Pel				Cel				Prt			
	Percentage enzyme activity											
Strain	S	P	C	%wt	S	P	C	%wt	S	P	C	%wt
26°C												
HC131	92	4	4	100	81	2	17	100	100	0	0	100
RJP220	1	82	17	82	0	90	10	89	99	0	1	114
HJN1003	82	15	3	79	66	29	5	119	100	0	0	114
HJN1004	73	23	4	88	76	10	14	89	100	0	0	100
HJN1013	8	73	19	58	29	58	13	58	100	0	0	60
HJN1021	10	60	30	99	2	70	28	220	100	0	0	124
33°C												
HC131	85	12	3	100	80	5	15	100	100	0	0	100
RJP220	1	57	42	87	9	68	23	138	100	0	0	265
HJN1003	6	72	22	99	0	83	17	116	100	0	0	15
HJN1004	2	85	13	51	4	85	11	209	100	0	0	350
HJN1013	2	63	35	75	0	76	24	130	100	0	0	39
HJN1021	1	73	26	113	0	100	0	110	100	0	0	80

Enzyme							
Bla				ß-gal			
Percentage enzyme activity							
S	P	C	%wt	S	P	C	%wt
4	86	10	100	1	7	92	100
2	95	3	91	1	3	96	54
2	85	13	103	3	2	95	72
1	95	4	96	1	3	96	39
5	88	7	135	1	5	94	89
6	79	15	210	1	4	95	78
6	83	11	100	0	5	95	100
10	78	12	149	0	4	96	46
4	90	6	286	1	5	94	68
4	92	4	210	1	5	94	55
11	75	14	325	1	5	94	57
4	90	6	350	1	6	93	60

Key :

S - Supernatant fraction
P - Periplasmic fraction
C - Cytoplasmic fraction
%wt - Percentage wild type level

This pleiotropic effect of mutation(s) in a cluster of genes that are essential for the secretion of extracellular enzymes, has been demonstrated before (Andro *et al.*, 1984; Ji *et al.*, 1987; Thurn and Chaterjee, 1985; Gibson *et al.*, 1988; Reeves *et al.*, 1993; Murata *et al.*, 1990; He *et al.*, 1991(a)) for *Ecc* and *Ech*. The general assumption is that the secretion of these extracellular enzymes (Pel and Cel) is by two independent steps (see figure 1.5). The accumulation of these naturally extracellular enzymes in the periplasm may not be a "true" indication that these enzymes normally reside in the periplasm prior to their secretion into the extracellular medium. Their presence in the periplasm could be an artifactual re-routing of the enzymes due to the disruption in the secretory machinery. In order to test the possibility, this study has generated some conditional (Out^{ts}) secretion mutants. These mutants could be used in pulse-chase experiments (see chapter 6) to determine whether or not the secretion machinery could be thermally renatured, and the periplasmic accumulated enzymes (Pel and Cel) secreted into the extracellular medium.

4.4 Assay for auxotrophic mutants

The three putative auxotrophic mutants (table 3.1) were screened for auxotrophy using the pool plate method of Holliday (1956). From the pool plate assay it was found that only HJN1009 was auxotrophic. The auxotrophy was defined as a histidine requirement. This was confirmed by growing the strain on minimal media with and without histidine. The medium containing histidine supported growth whilst the media lacking histidine did not.

The reversion frequency of the auxotroph, HJN1009, was calculated to be 3.2×10^{-8}. The linkage (by cotransduction) of the His^- auxotrophy, to the transposon Tn*5* (section 4.2), was 41%. This figure was obtained by screening Km^r transductants for the percentage that were His^- on minimal media. This would indicate that the auxotrophy is linked to the transposon, but does not infer linkage to the proximal side of Tn*5* or the other side.

The two prototrophs, HJN1001 and HJN1002, were probably reduced growth rate mutants because their original isolation as putative auxotrophs on minimal medium could simply be due to their poor growth on this medium.

4.4.1 Reduced growth rate mutants ?

Two putative reduced growth rate mutants (table 3.1) were isolated during the screening process (section 2.9). These strains (HJN1022 and HJN1023) along with the two confirmed prototrophs (HJN1001 and HJN1002) , see section 4.4, were investigated for a reduced growth rate phenotype.

An over-night culture of each mutant was sub-cultured, 1/25, into 5ml of LB or minimal sucrose broth (see table 2.4) and the optical density (A_{600}) recorded over 5 hours. The results are illustrated in table 4.3. From the results it is clear that none of the putative mutant strains show a reduced growth rate phenotype in these liquid media, compared to the wild type HC131 control.

4.4.2 Discussion

The auxotrophic mutation in HJN1009 has shown to be linked to the transposon Tn*5*, by cotransduction. In order to ascertain whether the lesion causing the auxotrophy is linked to the *out* cluster and to which side of Tn*5* a further experiment is necessary. This involves using a øKP lysate, propagated on a known Out$^-$ secretion mutant (eg. RJP251), to transduce the auxotrophic (His$^-$) strain HJN1009, selecting for His$^+$. If the lesion is to the left of the transposon then there will be a high percentage of transductants that are Km^s. Conversely, if a high percentage of transductants are Km^r then the lesion could be to the right of the transposon.

The putative mutants, HJN1001 and HJN1002, did not exhibit an auxotrophic or reduced growth rate phenotype. The two putative reduced growth rate mutants, HJN1022 and HJN1023, were shown to have normal growth rates. None of these

Table 4.3 Doubling times (minutes) of reduced growth rate mutants of Ecc spp.

Strain	LB	MSB
HC131	69	72
HJN1001	68	63
HJN1002	78	59
HJN1022	72	72
HJN1023	68	71

Key: LB - Luria Broth, MSB - Minimal Sucrose Broth.
Doubling times expressed in minutes.

putative mutants were pursued further.

4.5 Phage sensitivity in Out$^-$ mutants of *Ecc*.

4.5.1 Introduction

Four bacteriophages , øKP, øD-2, ø565 and ø575 have been shown to infect *Ecc* (Toth, 1992). Only one of these, øKP, was shown to be a generalised transducing phage. Reeves, (1992), demonstrated that an EMS generated Out$^-$ secretion mutant (RJP190) was resistant to øD-2 and ø565, at low titre, but remained sensitive to infection by øKP and ø575. High titre lysates of øD-2 and ø565 exhibited normal infectivity. This pleiotropic phenotype (ø^r, Out$^-$) is unusual in that it has not been reported elsewhere. It infers that there might be a link between the secretion of extracellular enzymes and phage infection.

The majority of Out proteins in *Ecc* are predicted to be inner membrane associated (Reeves *et al.*, 1993). This has also been shown to be the case in other bacterial systems, such as the secretion of pullulanase by the *Pul* cluster in *Klebsiella oxytoca*. The lesion that causes the pleiotropic phenotype in RJP190 has not yet been mapped to a specific gene(s). It has been mapped, crudely, by cosmid complementation (Reeves, 1993) to the 5' end of the *out* cluster and thus could be in *out*D that encodes an putative outer membrane protein (P. Douglas, pers. comm.). If this is correct then this mutant could be extremely interesting in that the lesion could be affecting the correct orientation of another protein, a receptor protein, necessary for phage adsorption. Alternatively, the protein, OutD, could play a part in both phage adsorption and secretion.

4.5.2 *Ecc* bacteriophage sensitivity testing of Out$^-$ and Out^{ts} mutants

Bacterial top agar lawns of each mutant strain (section 2.5) were spotted (10μl) with appropriate bacteriophage (øKP, øD-2, ø565) and incubated over-night at 30°C. This

procedure was also performed at 26°C and 33°C for the Out^{ts} mutants.

4.5.3 Results/Discussion

All of the strains were sensitive to infection by the bacteriophage tested and so further analysis was not pursued. Ideally the bacteriophage ø575 should have been tested for sensitivity on all the strains, but unfortunately, at the time of this experiment, was unavailable.

4.6 General conclusions

All the secretion mutants, Out^{-} and Out^{ts}, generated through localised mutagenesis of the *out* cluster, have been mapped relative to the transposon Tn*5* (section 4.2). These mutant strains have subsequently been analysed, by spectrophotometry (section 4.3), to reveal a periplasmic accumulation of Pel and Cel, which is conditional upon temperature in the Out^{ts} strains.

One auxotroph, HJN1009, has been defined to be histidine requiring, and has shown linkage to the transposon Tn*5* (section 4.4).

In characterising all the putative mutants isolated by localised mutagenesis of the *out* cluster (see chapter 3), those mutants illustrated in table 4.4 have now been defined as "true" secretion or auxotrophic mutants.

These mutants have been investigated further, using molecular genetic techniques, to map a specific lesion(s) to an actual gene(s). The particular lesion(s), once mapped to a specific gene, could then be identified at the nucleotide level, using the molecular techniques discussed in chapter 5.

Table 4.4 Secretion mutants isolated by localised mutagenesis of the *Ecc out* cluster.

Strain	Total	Frequency[a]	Phenotype
HJN1000			Out^- (Pel-,Cel-,Prt+)
HJN1005			Out^- (Pel-,Cel-,Prt+)
HJN1006			Out^- (Pel-,Cel-,Prt+)
HJN1007			Out^- (Pel-,Cel-,Prt+)
HJN1008			Out^- (Pel-,Cel-,Prt+)
HJN1010			Out^- (Pel-,Cel-,Prt+)
HJN1011			Out^- (Pel-,Cel-,Prt+)
HJN1012			Out^- (Pel-,Cel-,Prt+)
HJN1013			Out^- (Pel-,Cel-,Prt+)
HJN1014			Out^- (Pel-,Cel-,Prt+)
HJN1015			Out^- (Pel-,Cel-,Prt+)
HJN1016			Out^- (Pel-,Cel-,Prt+)
HJN1017			Out^- (Pel-,Cel-,Prt+)
HJN1018			Out^- (Pel-,Cel-,Prt+)
HJN1019			Out^- (Pel-,Cel-,Prt+)
HJN1020			Out^- (Pel-,Cel-,Prt+)
HJN1021			Out^- (Pel-,Cel-,Prt+)
	(17)	(0.85%)	
HJN1003			Out^{ts} (Out^-, 33°C
HJN1004			Out^{ts} Out^+, 26°C)
	(2)	(0.10%)	
HJN1009			Auxotrophic
	(1)	(0.05%)	

a- Percentage of the number of transductants screened (2368).

CHAPTER 5

MOLECULAR / GENETIC CHARACTERISATION OF Out$^-$ AND Out^{ts} SECRETION MUTANTS OF *Ecc*, GENERATED BY LOCALISED MUTAGENESIS OF THE *out* GENE CLUSTER

5.1 Introduction

The work, described in earlier chapters, showed that the lesion(s) in each Out$^-$ or Out^{ts} secretion mutant was linked by cotransduction to the transposon Tn*5* (section 4.2). This "crude" method of mapping mutations within the *out* cluster is satisfactory for initial proof that the lesions are within the desired area of the *Ecc* chromosome. In order to finely map each mutation to a specific gene(s) within the *out* cluster, a number of cosmids that contained defined inserts of *out* cluster DNA, were available for complementation studies (Reeves, 1992). These complementation studies were facilitated by the incorporation of the plasmid pHCP2 (LamB$^+$) into each of the mutant strains. The expression of *lam*B enabled infection of *Ecc* via phage lambda adsorption to the membrane associated LamB protein. Once injected, lambda DNA acts as a suicide delivery vector (see Salmond *et al.*, 1986).

The *out* cluster (*out*C-*out*O) has now been sequenced (Reeves *et al.*, 1993). This information was used to enable further "fine" mapping of lesions responsible for the Out$^-$ or Out^{ts} phenotype, to specific gene(s), by sub-cloning, PCR amplification and cloning.

5.2 Incorporation of pHCP2 into Out$^-$ and Out^{ts} strains

pHCP2 was successfully incorporated into each Out$^-$ or Out^{ts} mutant strain via conjugal transfer (section 2.21.1), øKP transduction (section 2.21.2) or electroporation (section 2.20).

5.3 Cosmid complementation

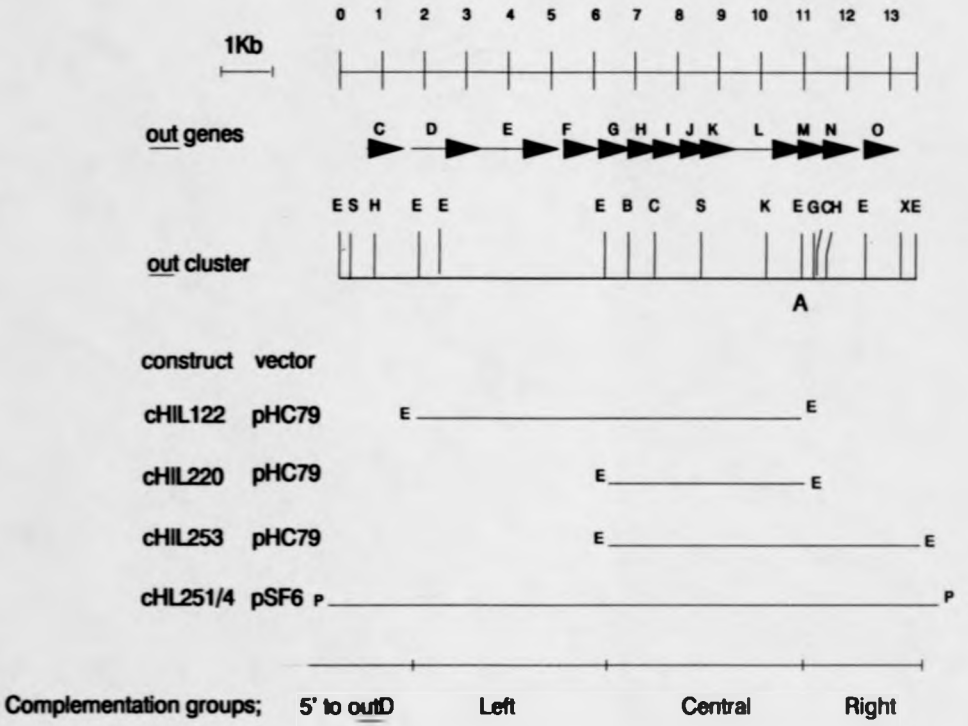
Four Out$^+$ cosmids (cHIL220, cHIL253, cHIL122 and cHIL251/4) were available for complementation studies (Reeves, 1992; Reeves *et al*, 1993). The *out* cluster DNA that each cosmid contains is illustrated in figure 5.1.

Each cosmid was transferred into the mutant strains by transduction (section 2.6),

Figure 5.1

The *out* cluster DNA is represented by the thick black line. The relative position of each gene within the *out* cluster is represented by the arrows. Cosmids cHIL122, cHIL220 and cHIL253 have *Eco*R1 genomic *Ecc* DNA fragments cloned into pHC79. *Eco*R1 fragments are represented by thin black lines. cHIL251/4 has a large, unspecified, (>30 Kb) *Pst*1 fragment of genomic *Ecc* DNA (P. Reeves, pers. comm.), cloned into pSF6. The *Pst*1 fragment is represented by a thin black line. Abbreviations : E, *Eco*R1; H, *Hin*d111; S, *Sal*1; B, *Bam*H1; C, *Cla*1; K, *Kpn*1; G, *Bgl*11; X, *Xho*1.

Figure 5.1 Genetic map of cosmids covering the out cluster



using high titre cosmid lysates ($>10^{10}$ pfu/ml), or by electroporation in the case of cHIL251/4. Transductants, containing the appropriate cosmid and conferring the relevant antibiotic resistance, were then screened on enzyme detection media for the restoration of the Out^+ phenotype, at 30°C. For the Out^{ts} mutants the assay was done at both 26°C and 33°C. The results of the complementation studies are shown in table 5.1.

5.3.1 Results / Discussion

The cosmids, cHIL220, cHIL122 and cHIL253 have been used to position a number of mutations to the left, middle or right of the *out* cluster, as shown in table 5.1. These data, along with the cotransductional linkage data of each mutation with respect to the transposon Tn*5* (see section 4.2), indicates that the transposon lies to the right of the *out* cluster.

Some mutations were not complemented by any of these cosmids, suggesting that either: a) these mutations mapped outside the known *out* cluster, possibly in some regulatory or other essential gene, or b) the mutations mapped to the *out* cluster, but were upstream or downstream, in gene(s) that have not yet been defined. The fact that all of the mutations were shown to be linked by cotransduction to the transposon Tn*5* (section 4.2), and that 74% of the mutations were mapped to the *out* cluster by the cosmids cHIL122, cHIL220 and cHIL253 suggested that the mutations were close to, or within, the known *out* cluster. Mutations that were not complemented by the above cosmids, showed cotransductional linkage of between 5-10% (see section 4.2). This suggested that the lesions mapped to the far left (5') of the *out* cluster. In order to attempt to show that the mutations were indeed close to, or within the *out* cluster, a cosmid containing a large insert (>30 Kb) of *Ecc* DNA (cHIL251/4) was used. This cosmid (cHIL251/4) does not have much DNA 3' of *out*O (Reeves, pers. comm.). cHIL251/4 restored the Out^+ phenotype to the previously non complemented mutants (table 5.1). This indicates that the mutations are probably 5' to the *out* gene cluster, by

Table 5.1 Cosmid complementation of Out$^-$ and Out^{ts} mutations.

Mutant strain	Cosmid cHIL122	cHIL220	cHIL253	cHIL251/4	Pos.[a]
HJN1003	+	-	-	ND	Left
HJN1010	+	-	-	ND	Left
HJN1020	+	-	-	ND	Left
HJN1006	+	+	+	ND	Central
HJN1011	+	+	+	ND	Central
HJN1012	+	+	+	ND	Central
HJN1013	+	+	+	ND	Central
HJN1015	+	+	+	ND	Central
HJN1016	+	+	+	ND	Central
HJN1019	+	+	+	ND	Central
HJN1004	-	-	+	ND	Right
HJN1005	-	-	+	ND	Right
HJN1008	-	-	+	ND	Right
HJN1021	-	-	+	ND	Right
HJN1000	-	-	-	+	5'to outD
HJN1007	-	-	-	+	5'to outD
HJN1014	-	-	-	+	5'to outD
HJN1017	-	-	-	+	5'to outD
HJN1018	-	-	-	+	5'to outD

Key :

+ Restoration of phenotype to Out$^+$

- Not complemented (Out$^-$ or Out^{ts})

ND Not done

Pos[a] Relative position in the *out* cluster

cotransductional linkage data and cosmid complementation. Sequencing of the 5' region of the *out* cluster is currently underway, in this laboratory, in order to define gene(s) upstream of *out*C. One such gene, *out*X, has been cloned and sequenced (Wharam S, 1992). This gene is 5' to *out*C and its protein product has been predicted to be homologous to exo-polygalacturonase (Peh-X). In order to ascertain whether this clone (pSW1) could complement the putative 5' mutations (see table 5.1) it was transferred to each strain as described above. It was found that pSW1 did not complement any of these putative 5' mutants. It could be that the gene(s) that has this lesion(s) lies further upstream of the *out* cluster.

Of particular interest in this study is the characterisation of conditional Out^{ts} secretion mutants. In order to attempt to define which gene(s) had been mutated in each case, further fine mapping was necessary.

5.4 Mapping of the lesion responsible for the Out^{ts} phenotype in the conditional secretion mutant HJN1004

5.4.1 Introduction

Cosmid cHIL253 contains a 7.6 Kb insert of *out* DNA (see figure 5.1) which was cloned into the *Eco*R1-digested vector, pHC79 (Reeves, 1992). From the complementation data of HJN1004 (table 5.1) only the cosmid c253 was shown to complement this *out*^{ts} mutation. This would imply that the lesion causing the Out^{ts} phenotype maps 3' to the second common *Eco*R1 restriction site, labelled A in figure 5.1. From the available sequence data of the *out* cluster, it was possible to predict that the lesion maps to one of the following *out* genes: *out*L, *out*M, *out*N or *out*O.

It is known that OutO, or its homologues PulO, XcpA, PilD, TcpJ,and ComC (see figure 1.13), are thought to be type IV prepilin peptidases from their protein sequence similarities to known peptidases. It has now been shown (P. Douglas, pers. comm.)

that this OutO putative peptidase cleaves OutG at a consensus type IV pilin cleavage site (NMePhe), see section 1.5.4.3. Three other proteins, OutH-J, also have a consensus type IV prepilin cleavage site. Prepilin peptidase activity on type IV pilin like proteins has now been shown for OutO homlogues, except ComC (Pugsley and Dupuy, 1992 ; Bally *et al.*, 1992; Nunn and Lory, 1992; Kaufman *et al.*, 1991). It was interesting therefore to ascertain if the *out*^{ts} mutation mapped to *out*O.

5.4.2 Subcloning of *out*O

The cosmid cHIL253 was digested with *Hin*dIII (section 2.13) and a 2 Kb fragment was purified by low melting point agarose gel electrophoresis (section 2.15.1), see figure 5.2. The resulting 2 Kb fragment was ligated (section 2.17) into *Hin*dIII restricted pBR322, and treated with calf intestinal alkaline phosphatase (section 2.16). The ligation reaction was then used to transform (section 2.19.2) or electroporate (section 2.20) the recipient strain DH1. Transformants were selected on LB + Ap media.

5.4.3 Results

12 putative clones were isolated on LB + Ap media. 10 clones were shown, via plasmid DNA preparation (section 2.12), and *Hin*dIII restriction digests, to contain the 2 Kb insert. This 2 Kb insert was shown to be in both orientations, pNH1004/1 and pNH1004/2, see figure 5.3. The subcloned insert contained an intact *out*O with a truncated version of *out*N.

5.4.4 Complementation studies using subclones of *out*O and *out*L

5.4.5 Introduction

The subclone pHIL159/3 (table 2.3) was available for complementation studies. This subclone (see figure 5.4) was constructed by cloning a *Hin*dIII *Sal*I fragment, isolated from the plasmid pHIL159/1, into pBR322 (Reeves, 1992). This construct contained insert DNA of *out*L with truncated forms of *out*K and *out*M.

Figure 5.2

(1) Cosmid cHIL253 was digested with *Hind*111 to release a 2 Kb fragment which was purified by low melting point agarose gel electrophoresis. This fragment was ligated into *Hind*111 digested vector pBR322 (2). See text for details.

Abbreviations : E, *Eco*R1; B, *Bam*H1; C, *Cla*1; S, *Sal*1; K, *Kpn*1; H, *Hind*111; Bg, *Bgl*11; Ap, Ampicillin resistance; Tet, Tetracycline resistance. Dotted lines indicate truncated forms of the gene(s).

Figure 5.2 Subcloning of outO

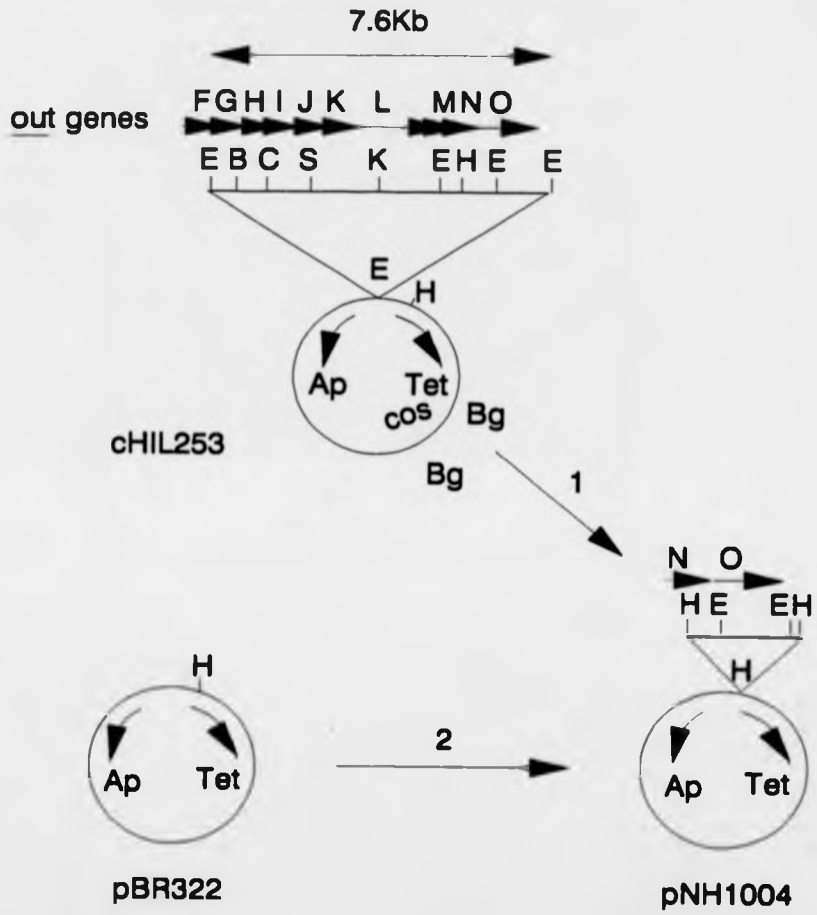
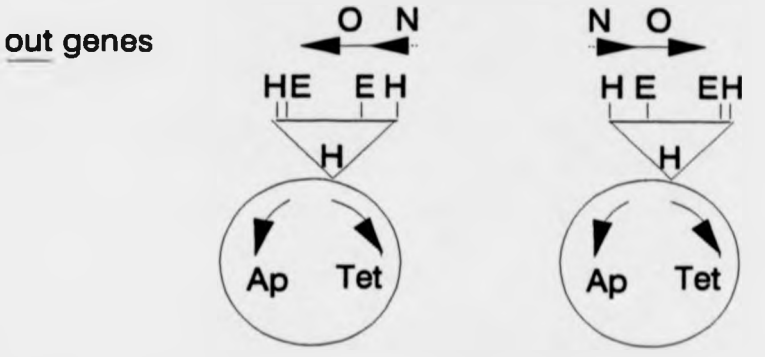
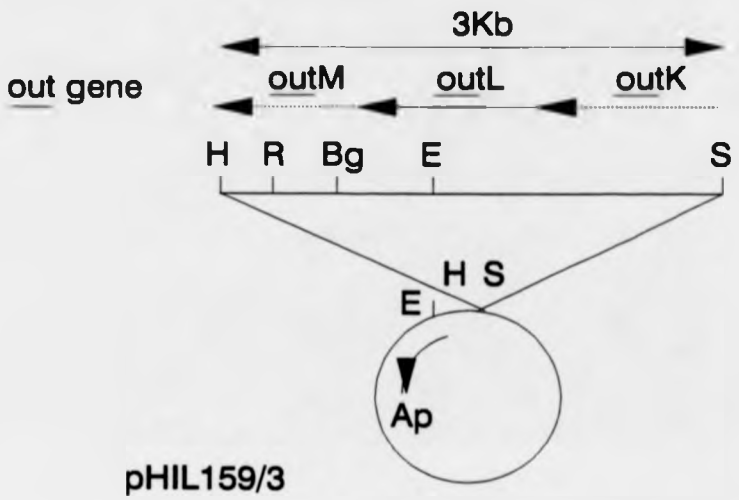


Figure 5.3 Orientations of outO subclones



Key:
Arrows indicate the orientation of out genes.
Abbrieviations; H, Hind111; E, EcoR1;
Ap, ampicillin resistance; Tet, tetracycline resistance.
Arrows with dotted lines indicate a truncated form of the gene.

Figure 5.4 Genetic map of pHIL159/3



Key:
Arrows indicate the orientation of out genes.
Arrows with dotted lines indicate truncated forms of the gene(s).
Abbrieviations; H, Hind111; R, EcoRV; Bg, Bgl11; E, EcoR1; S, Sal1; Ap, ampicillin resistance.

In order to map the lesion(s) to specific gene(s) using the subclones pHIL159\3, pNH1004\1 and pNH1004\2, the mutations that were complemented by the cosmids cHIL253 and cHIL220, were tested for complementation by the subclone pHIL159\3. This showed which mutants had lesions in *out*L. Mutations that were not complemented by pHIL159\3, but were complemented by the cosmid cHIL253, were then tested for complementation by the subclones pNH1004\1 and pNH1004\2. This showed which mutants had lesion(s) in *out*O.

5.4.6 Results

Each of the subclones pHIL159\3, pNH1004\1 and pNH1004\2, were electroporated (section 2.20) into appropriate mutant strains in which the mutations mapped, by cosmid complementation, to the right of the *out* cluster (see table 5.1). As well as doing this for the mutants generated in this study, the secretion mutants (RJP) generated by EMS mutagenesis (Reeves, 1992) that had mutations that mapped to the 3' end of the *out* cluster, were also tested for complementation. Transformants, Ap^r, were screened for restoration of the Out^+ phenotype on enzyme detection media at 30°C or at both 26°C and 33°C for the Out^{ts} mutant HJN1004. The results of the screening are shown in table 5.2.

5.4.7 Discussion

Complementation studies with the plasmid pHIL159\3 has revealed 4 mutants that have lesions in *out*L, see table 5.2. These include the conditional (out^{ts}) secretion mutant HJN1004, 2 null Out^- secretion mutants HJN1008 and HJN1021, and the EMS generated Out^- secretion mutant RJP159, which was used as a control.

From table 5.2, it was shown that two Out^- mutations map to *out*O, HJN1005 and RJP249. HJN1004 and RJP159 were used as negative controls. It appears from the complementation studies, that both orientations, pNH1004\1 and pNH1004\2, restore the Out^+ phenotype. This could be due to other promoters in the vector pBR322

Table 5.2 Complementation of mutations in *out*L and *out*O

Mutant strain	Subclone		
	pNH1004\1	pNH1004\2	pHIL159\3
HJN1004	-	-	+
HJN1005	+	+	-
HJN1006	ND	ND	-
HJN1008	ND	ND	+
HJN1011	ND	ND	-
HJN1012	ND	ND	-
HJN1013	ND	ND	-
HJN1015	ND	ND	-
HJN1016	ND	ND	-
HJN1019	ND	ND	-
HJN1021	ND	ND	+
RJP159	-	-	+
RJP249	+	+	-
RJP250	-	-	-
RJP220	ND	ND	-

Key :

+ Restoration of Out$^+$ phenotype

- not complemented

ND Not done

driving the expression of *out*O. Even if this expression is low, it appears to be sufficient to allow complementation of the lesion(s) in the mutated *out*O strain.

Complementation analysis has mapped the mutation in the conditional secretion mutant (Out^{ts}), HJN1004, to *out*L along with two null (Out^-) mutations in HJN1008 and HJN1021. Two null (Out^-) secretion mutants, HJN1005 and RJP249, have mutations that map to *out*O.

5.5 Mapping of the lesion responsible for the Out^{ts} phenotype in the conditional secretion mutant HJN1003

5.5.1 Introduction

The conditional Out^{ts} secretion mutant HJN1003 was mapped to the 5' end of the *out* cluster by cosmid complementation (section 5.3) and by cotransduction (section 4.2). In order to attempt to map the lesion(s) responsible for the conditional phenotype to a specific gene(s), two plasmids were available for complementation analysis, pSW1 and p3.7R1 (figure 5.5). p3.7R1 contains a 3.7 Kb insert that contains an intact *out*E and a truncated versions of *out*D and *out*F. pSW1 contains a 1.8 Kb insert that has an intact *out*C with truncated forms of *out*X and *out*D. By transferring each of these plasmids into HJN1003, complementation analysis showed whether the lesion(s) responsible for the Out^{ts} phenotype was in the gene *out*C or *out*E. If the complementation analysis proved negative, then this would have indicated that the lesion(s) could have been in *out*D or *out*F.

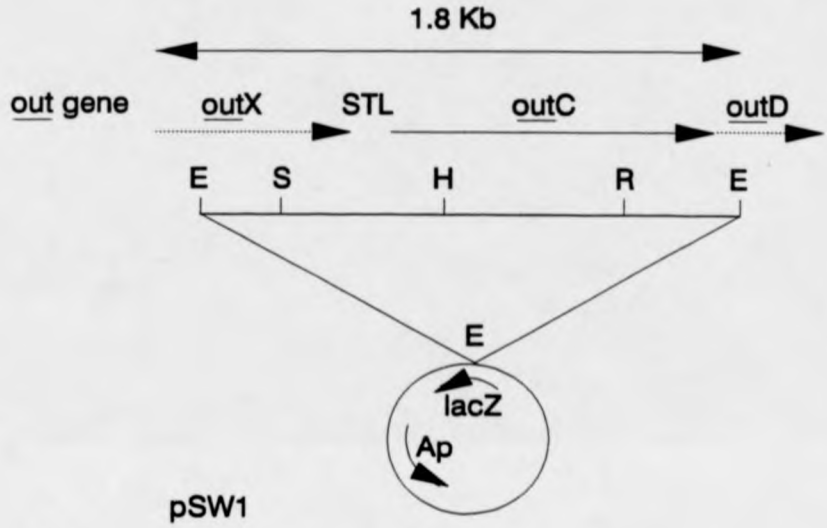
5.5.2 Complementation analysis of HJN1003

Each of the plasmids pSW1 and p3.7R1 was transferred to the Out^{ts} mutant strain HJN1003 by electroporation (section 2.20). Ap^r transformants were screened at 26°C and 33°C, using enzyme detection media, for restoration of the Out^+ phenotype.

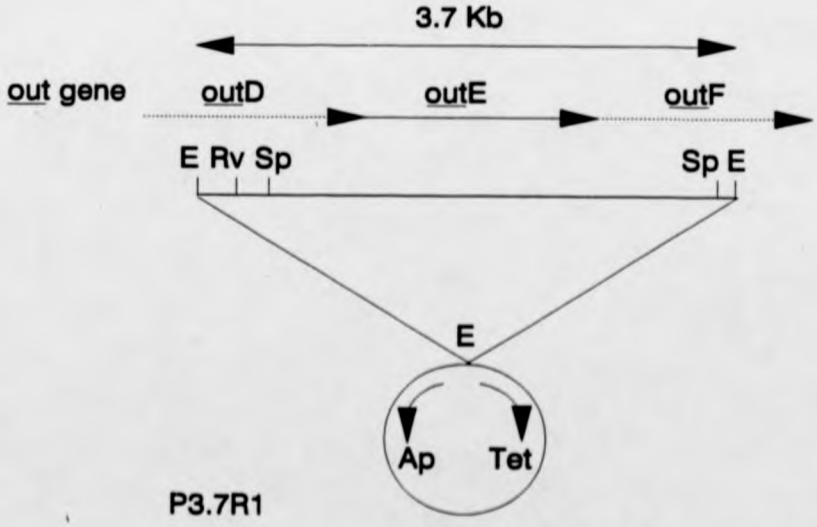
Figure 5.5

Arrows indicate orientation of *out* genes. Arrows with dotted lines indicate truncated forms of the gene. Abbreviations : E, *Eco*R1; S, *Sal*1; H, *Hin*d111; R, *Eco*RV; Sp, *Sph*1; STL, stem loop structure; Ap, ampicillin resistance; Tet, Tetracycline resistance; *lac*Z, ß-galactosidase reporter gene of pIC19-R.

Figure 5.5 Genetic maps of pSW1 and p3.7R1



pSW1



P3.7R1

5.5.3 Discussion

It was found that the plasmid pSW1 complemented the Out^{ts} mutation at 33°C, the restrictive temperature. This indicates that the lesion responsible for the Out^{ts} phenotype lies in *out*E. *out*E has been shown by sequence analysis (Reeves *et al.*, 1993) to contain a putative ATP binding site, as the protein sequence contains both Walker A and Walker B consensus sequences (Walker *et al.*, 1982) which are thought to indicate a nucleotide binding fold. These motifs are also found in nucleotide binding proteins with ATPase or kinase activity (Pugsley, 1992). OutE has been shown to be homologous to a group of closely related proteins, PulE, ExeE, XcpR, XpsE, PilB, TcpG-1 and ComG1 (see figure 1.13). These related proteins are all involved in protein secretion or DNA uptake (ComG1) and have homology to type IV pilin like proteins, or are involved directly in type IV pilus assembly (see Pugsley, 1993). The exact role of OutE in the secretion process is undefined. It has been suggested (Possot *et al.*, 1992; Pugsley, 1993) that its homologue PulE may act as : 1) an energiser for the secretion process by hydrolysis of ATP, or 2) a kinase to phosphorylate other proteins in the secretory machinery and thereby activate them [this was based on the fact that the closely related protein VirB.11 of *Agrobacterium tumefaciens*, has been shown to have ATPase and autokinase activity (Christie *et al.*, 1991)], or 3) a platform for the assembly of a pseudopilus with type IV pilin-like proteins (Pul G-J).

It is therefore of great interest that this study has generated a conditional secretion mutant with a lesion(s) that maps to *out*E. Future work would involve the sequencing of this gene to define the lesion that is responsible for the Out^{ts} phenotype, and biochemical analysis of the binding affinity of the mutated and wild type protein, for ATP.

5.6 PCR amplification and cloning of *outL*

5.6.1 Introduction

In order to define the lesion responsible for the Out^{ts} phenotype of HJN1004, it was necessary to clone the gene (*outL*) in which the lesion(s) was known to map. Once cloned, the gene could then be sequenced to reveal the lesion(s). This could also be done for the other Out^{-} mutants with lesion(s) in *outL* ; HJN1008 and HJN1021.

From the sequence data of the *out* cluster it has been revealed that there are no major restriction sites available at either end, 3' or 5', to *outL*. There was thus no easy way to directly clone this gene from the mutant strain by conventional cloning techniques. Because the sequence of the wild type *Ecc out* cluster was already known (Reeves *et al.*, 1993) it was comparatively easy to use PCR to amplify the gene and to consequently clone the DNA into a suitable vector. Because PCR is known to generate mutations [due to misreading of the DNA template with enzymes such as *Taq* polymerase (Keohavaong and Thilly, 1989)], the polymerase *Pfu*, derived from *Pyrococcus furiosus* (Lundberg *et al.*, 1991), was used. This polymerase has proof reading (exonuclease) ability (3' to 5') and is known to have a 12 times lower error rate than *Taq* polymerase and 5 times lower error rate than Vent polymerase (derived from *Thermococcus litoralis*), Kohler *et al.*, (1991). *Pfu* also has a higher thermostability than Vent and shows less non specific priming and false background extension products. As the gene *outL* is known to be 1.3 Kb, the low error rate of *pfu* should enable the amplification, cloning and subsequent sequencing of this gene with a lower probability of misreading errors introduced by the PCR process.

5.6.2 PCR amplification of *outL*

5.6.2.1. Designing oligonucleotide primers for PCR amplification of *outL*

The design of oligonucleotides (primers) for use in the PCR reactions was based on 3

basic factors (Erlich, 1989). Firstly, each primer contained 20 bases, of which the GC content was approximately 50% and long stretches of polypyrimidines or polypurines were avoided. Secondly, each primer was designed to avoid the incorporation of putative secondary structures that may interfere with annealing to template DNA. Thirdly, primer complementarity was avoided at the 5' and 3' ends to prevent primers annealing to each other.

For ease of cloning the amplified DNA product, *Hin*dIII restriction sites were engineered to the 5' ends of the oligonucleotides. Further to this, three bases were added 5' to the engineered restriction sites to enable restriction endonuclease digestion. The primers designed for the amplification of *out*L are shown in figure 5.6. Primer 9730 was designed so that a putative Shine-Dalgarno ribosome binding site was incorporated into the PCR product, this could be advantageous in the expression of the gene.

5.6.2.2 Optimisation of the PCR reaction with *Pfu* DNA polymerase

The plasmid pHIL159\3 was used as a positive control for the PCR reaction as this plasmid carries the intact wild type *Ecc out*L DNA. As this gene was cloned into a relatively high copy number (30-50) plasmid (pBR322) many copies of *out*L were present which would increase the probability of primer annealing to the wild type DNA template.

PCR amplification (section 2.22) of the wild type *out*L DNA was achieved using reaction buffer 2, provided with the polymerase (Stratagene). The next step was to optimise the PCR amplification using chromosomal preparations of the *out*L secretion mutant strains. Initially there was no amplification products seen by agarose gel electrophoresis (section 2.14). A range of primer and $MgCl_2$ concentrations was used to optimise the amplification of *out*L DNA in plasmid and chromosomal preparations. The results are shown in figure 5.7.

Figure 5.6 PCR amplification primers

Oligonucleotide primers (5' - 3') :

1) NH9730

CCC / AAG CTT / CGG CAA TAT GGA GGA TAT AG
Hind111

2) NH11096R

AAA / AAG CTT / GAC CAT AAG CTG ACG CTC GC
Hind111

Figure 5.7 Optimisation of the PCR amplification of *out*L with *Pfu* polymerase.

PCR amplification reaction mixtures were made as described in section 2.22. A range of primer concentrations were used in PCR reactions with either the plasmid pHIL159/3 (*out*L$^+$) DNA (1.0µl) or with chromosomal DNA (1.0µl) from the wild type strain SCRI193 (tracks 2-11). A range of $MgCl_2$ concentrations were used in similar PCR reactions (using 2.0µl of each PCR primer) to optimise the $MgCl_2$ concentration necessary for PCR amplification with *Pfu* polymerase (tracks 12-19).

Track:
1. λ*pst* markers
2. pHIL159/3 (plasmid DNA) 5µl each PCR primer (see figure 5.6)
3. pHIL159/3 (plasmid DNA) 2.5µl each PCR primer (see figure 5.6)
4. pHIL159/3 (plasmid DNA) 2.0µl each PCR primer (see figure 5.6)
5. pHIL159/3 (plasmid DNA) 1.2µl each PCR primer (see figure 5.6)
6. pHIL159/3 (plasmid DNA) 0.8µl each PCR primer (see figure 5.6)
7. SCRI193 (chromosomal DNA) 5µl each PCR primer (see figure 5.6)
8. SCRI193 (chromosomal DNA) 2.5µl each PCR primer (see figure 5.6)
9. SCRI193 (chromosomal DNA) 2.0µl each PCR primer (see figure 5.6)
10. SCRI193 (chromosomal DNA) 1.2µl each PCR primer (see figure 5.6)
11. SCRI193 (chromosomal DNA) 0.8µl each PCR primer (see figure 5.6)
12. pHIL159/3 (plasmid DNA), 1.52mM $MgCl_2$
13. pHIL159/3 (plasmid DNA), 3.52mM $MgCl_2$
14. pHIL159/3 (plasmid DNA), 5.52mM $MgCl_2$
15. pHIL159/3 (plasmid DNA), 7.52mM $MgCl_2$
16. SCRI193 (chromosomal DNA) 1.52mM $MgCl_2$
17. SCRI193 (chromosomal DNA) 3.52mM $MgCl_2$
18. SCRI193 (chromosomal DNA) 5.52mM $MgCl_2$
19. SCRI193 (chromosomal DNA) 7.52mM $MgCl_2$
20. Control (no template DNA)
21. λ*pst* markers

5.6.2.3 Results

Figure 5.7 shows the optimum primer and DNA concentrations required for the amplification of *out*L, which were; 5μl of each primer and a 7.52 mM concentration of $MgCl_2$. These conditions were used to amplify wild type *Ecc out*L and the mutated forms of *out*L from the secretion mutants HJN1004, HJN1008 and HJN1021. The amplification of each sample, including the control pHIL159\3, are shown in figure 5.8.

5.6.2.4 Discussion

The results have shown the successful amplification of the *out*L alleles using PCR. Because the quantity of amplification product using *Pfu* DNA polymerase is small compared to that found using *Taq* DNA polymerase, it was thought that cloning the amplification product into the plasmid vector pBR322 would be the simplest route for sequence analysis rather than direct PCR sequencing (Pharmacia P-L Biochemicals, 1990).

5.6.3 Cloning of the PCR product, *out*L

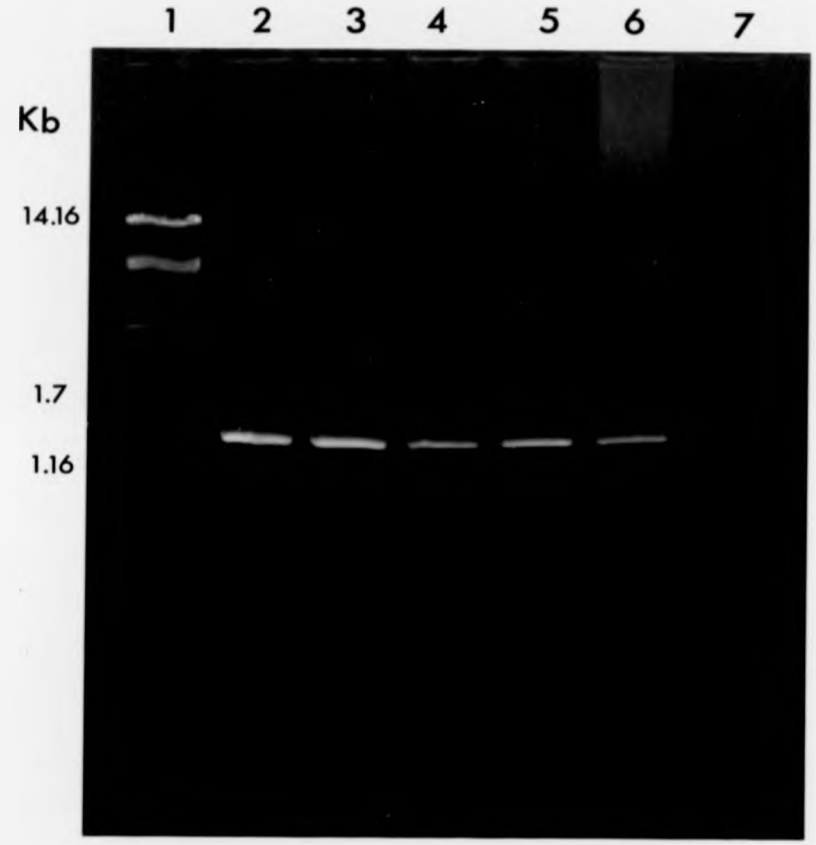
5.6.3.1 Cloning using "sticky ends"

PCR amplified DNA was prepared for cloning by ethanol precipitation and / or phenol extraction (section 2.12.1). The DNA was then restricted (section 2.13), using *Hin*dIII, to produce protruding "sticky ends" for ligation (section 2.17.1) into *Hin*dIII digested vector, pBR322. The ligation reactions were then used to transform (section 2.19.2) or electroporate (section 2.20) DH1, and Ap^r transformants were selected on LB + Ap.

5.6.3.1.2 Results / Discussion

No clones were isolated using this approach. This could be due to; 1) the inability of the restriction endonuclease to cleave the short DNA sequences that flank the restriction

Figure 5.8 PCR amplification of *out*L



PCR amplification was performed as described in section 2.22.

Track:
1. λ*pst* markers
2. SCRI193 (chromosomal DNA)
3. HJN1004 (chromosomal DNA)
4. HJN1008 (chromosomal DNA)
5. HJN1021 (chromosomal DNA)
6. pHIL159/3 (plasmid DNA)
7. Control (no template DNA)

sites of the PCR amplified DNA, or 2) the stability of the restriction endonuclease. Because of these possible reasons it was decided that the simplest route towards cloning the PCR amplified DNA was to use blunt cloning.

5.6.3.2 Blunt cloning PCR amplified DNA

5.6.3.2.1 Introduction

The vector pBR322 (see appendix 1) was digested with *Eco*RV to yield blunt ends. The PCR amplified DNA was prepared for cloning by ethanol precipitation and / or phenol extraction (section 2.12.1). Following this the DNA was "end repaired" (section 2.18) to render the linear double stranded DNA blunt ended. The PCR amplified DNA was then ligated (section 2.17.2) into the *Eco*RV-treated vector. The ligation reactions were used to transform DH1. Ap^r transformants were selected on LBA + Ap. These Ap^r transformants were then screened for Tc^s as any insert cloned into *Eco*RV digested pBR322 will disrupt the Tc^r gene (insertional inactivation). Putative Tc^s clones could have their plasmid DNA prepared and then digested with *Hin*dIII. As a *Hin*dIII site is present at either end of the insert to be cloned (*out*L) and is present only once in pBR322, then by electrophoresing *Hin*dIII digested DNA it was possible to identify clones containing the insert.

5.6.3.2.2 Results

The numbers of Ap^r transformants isolated during the cloning process are shown in table 5.3 , along with numbers of putative Tc^s clones. Of the 17 putative clones, 6 were shown to contain the desired insert DNA. Unfortunately the wild type *Ecc* DNA was not cloned.

In order to check that each clone contained the desired insert DNA *Kpn*I digests were performed. Because there is only a single *Kpn*I site within the insert DNA (*out*L) and there are no *Kpn*I sites within the vector (pBR322) DNA, this proved to be a

Table 5.3 Cloning of PCR products

PCR amplified *Ecc* strain	N^{o} of Ap^{r} transformants	N^{o} of Tc^{s} putative clones	N^{o} of clones
HC131	286	0	0
HJN1004	353	3	1
HJN1008	114	7	2
HJN1021	258	7	3

Key :

N^{o} - number

Ap^{r} - Ampicillin resistant

Tc^{s} - Tetracycline sensitive

reasonable test to confirm that the DNA cloned was *out*L. In all cases *Kpn*1 digested the DNA.

5.6.3.2.3 Discussion

The mutated *out*L gene from each of the Out^{ts} or Out^{-} mutants, HJN1004, HJN1008 and HJN1021, was successfully PCR amplified and cloned into pBR322 (pNH260, pNH700 and pNH200 respectively). This was not the case for wild type *Ecc* HC131. There was no obvious reason for the failure of cloning this PCR product. This was slightly unfortunate, as the reason for cloning the wild type *out*L gene was that by showing that there were no errors in the sequence, this would have indicated that no errors were introduced by the PCR process.

In order to show that the PCR process did not introduce any errors that were detectable phenotypically, the biological activity of the gene product was tested. This was done by transferring the PCR clone of the mutated *out*L gene from HJN1004 (Out^{ts}), into each of the other *out*L (Out^{-}) strains and screening for conditional complementation.

5.6.3.3 Biological activity of the PCR cloned gene product

The PCR clone pNH260 was electroporated into strains of HJN1004, HJN1008 and HJN1021. Ap^{r} transformants were isolated on LB + Ap media and screened for conditional complementation at both 26°C and 33°C, on enzyme detection media. The *out*L wild type clone, pHIL159/3, was used as a positive control. In addition, the PCR clones pNH700 and pNH200 were electroporated into each of the mutant strains HJN1004, HJN1008 and HJN1021. The resulting transformants were screened at 30°C on enzyme detection media for restoration of the Out^{+} phenotype. Those transformants that were isolated using the strain HJN1004 were screened at both 26°C and 33°C.

5.6.3.3.1 Results

The results of the complementation analysis are shown in table 5.4. Partial complementation to Out$^+$, was seen with the null mutations of *out*L, HJN1008 and HJN1021, using the clone pNH2600. This tentatively suggests that the PCR gene product is biologically active, as some complementation to Out$^+$ can be detected. The complementation analysis was performed using enzyme detection media and so is not quantitative. In order to ascertain whether this data is conclusive, quantitative enzyme assays, using spectrophotometry (see section 4.3), of supernatant, periplasmic and cytoplasmic fractions could be performed.

Complementation of the conditional secretion mutant, HJN1004, was seen with the clone pNH200. Complementation to Out$^+$ was seen at the normally restrictive temperature of 33°C. No other combination of clone and (Out$^-$) mutant strain showed complementation. Negative controls used were the PCR cloned mutated gene, transferred into the original, cured, mutant strain. The positive control used was the clone pHIL159/3, transferred into the mutant strain HJN1021.

5.6.3.3.2 Discussion

Putative conditional complementation of each of the null *out*L mutants has been shown using the PCR clone pNH260. This tentatively suggests that there have been no errors introduced into the PCR product that are detrimental to the protein products biological activity. This does not rule out the fact that errors could have been introduced that are "silent" and do not exhibit a detectable phenotype.

The PCR clone pNH200 was shown to complement the conditional secretory mutant, HJN1004, at the restrictive temperature of 33°C. This clone did not complement the original mutant strain from which it was derived, as expected, but also did not complement the other Out$^-$, mutated *out*L strain HJN1008. The original strain HJN1021, from which the PCR clone was derived, was complemented by the subclone

Table 5.4 Complementation analysis using the PCR cloned gene products

Cured strain	PCR clone	Temperature	
		26°C	33°C
HJN1004	pNH260	WT	NC
HJN1008	pNH260	(+)	NC
HJN1021	pNH260	(+)	NC
HJN1004	pNH700	WT	NC
HJN1004	pNH200	WT	WT

Cured strain	PCR clone	Temperature
		30°C
HJN1008	pNH700	NC
HJN1008	pNH200	NC
HJN1021	pNH700	NC
HJN1021	pNH200	NC
HJN1021	p159/3	WT

Key :

WT - Complementation to wild type (Pel^+, Cel^+)

(+) - Partial complementation

NC - No complementation

pHIL159/3, which carries wild type *out*L. These results suggest that a mutation in the *out*L gene of HJN1021, can suppress the mutation responsible for the conditional phenotype of HJN1004. One possible explanation for this phenomenon could be that OutL exists, *in vivo*, as a dimer, and two, different, mutated copies of the same gene product could interact to suppress each others mutation.

A crude way of testing this theory would be to introduce the PCR clone pNH200 into wild type *Ecc*, HC131. By quantifying, by spectrophotometry, the secretion of extracellular enzymes (Pel and Cel) in this strain and comparing it to that of the wild type *Ecc* strain without the PCR clone, it may be possible to show that the mutated gene product reduces the amount of secretion of Pel and Cel. In order to identify the specific lesion(s) in each of the PCR cloned genes that is / are responsible for the particular phenotype of the OutL mutant strains, it was necessary to sequence each clone.

5.6.4 Sequence analysis

5.6.4.1 Introduction

The knowledge gained from the sequence of the *out* cluster (Reeves *et al.*, 1993) enabled a sequencing strategy to be devised (figure 5.9) using oligonucleotide primers designed from the existing sequence. Sequencing of the plasmid clones was performed as described in section 2.23. The aim of the sequencing strategy was to have both strands of DNA sequenced to reveal the specific lesion(s) induced by localised mutagenesis.

5.6.4.2 Results

Where possible, each sequencing reaction (section 2.23.3), using the same oligonucleotide and corresponding termination reaction, for each plasmid clone, was electrophoresed (section 2.23.4) alongside each other. This made identification of

Figure 5.9

The arrows represent oligonucleotide primers for sequencing.

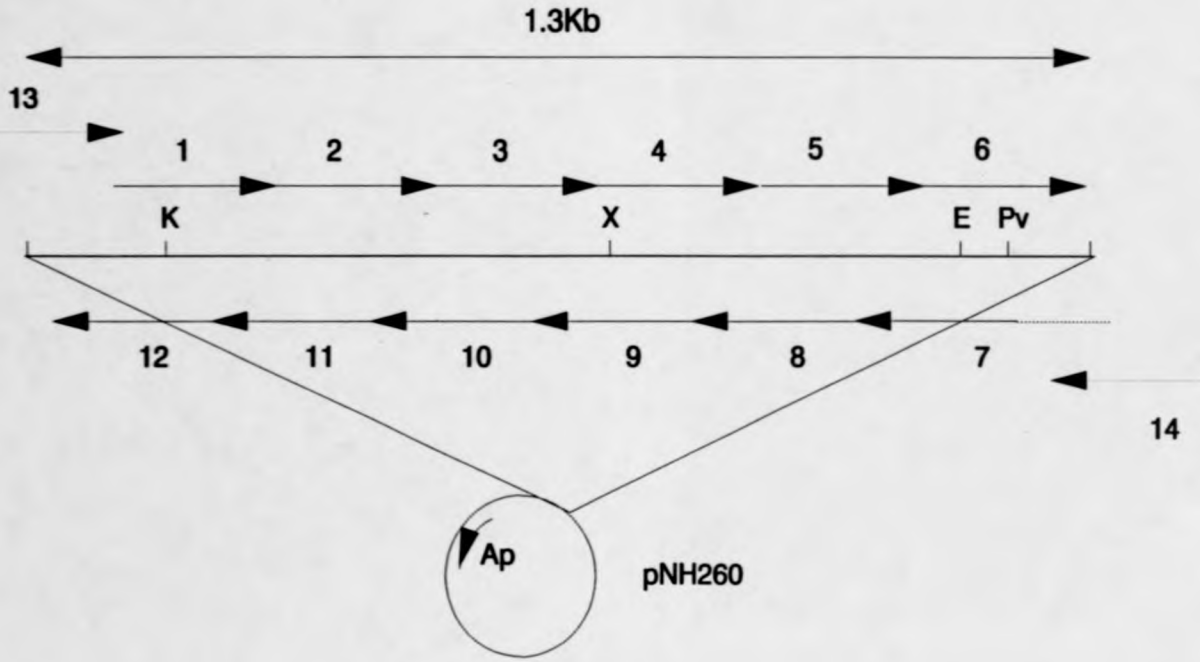
Oligonucleotide primers :

Name	Sequence (5' - 3')
1 - NH9886	AGG TGC GTT CAT CCA AT
2 - NH10089	CAA CTG GCG ACG GAA AT
3 - NH10290	TGA TGA CAT GTG GCT GT
4 - NH10474	AAA CGG ATC TCT TCA CG
5 - NH10672	AAC ATT GGC GGC AGG AA
6 - NH10860	TTC AGT CGC TGT CTT AC
7 - NH11096R	see figure 5.7
8 - NH10854R	GAT CGC GCT ATT CTG TG
9 - NH10661R	TAA AGC TGA TAG TGC GC
10 - NH10452R	CTC TTC CTG CCA TTC GT
11 - NH10235R	ATA ACG TAT CGA CGC TG
12 - NH10009R	AGT GGC TGG TAC CAG AA
13 - NH140	TAG GCA TAG GCT TGG TT
14 - NH240R	ATA TCG CGA TCG TCG TG

Primers 13 and 14 were designed to sequence into the insert from pBR322. See text for details.

Abbreviations : H, *Hin*dIII; K, *Kpn*I; X, *Xho*II; E, *Eco*RI; Pv, *Pvu*I; Ap, ampicillin resistance.

Figure 5.9 Sequencing strategy for PCR clones



specific mutations easier. 85% of each clone has been sequenced, double stranded, using the above techniques. The sequences of each clone have been compared to that of the wild type known sequence (Reeves *et al.*, 1993).

Clone pNH700 has revealed a single mutation in the codon CCT that encodes the amino acid proline (Pro^{159}, figure 5.10), to TCT that encodes the amino acid serine (Ser^{159}, figure 5.10). This mutation has been found in both DNA strands, see figure 5.11.

Clone pNH260 has revealed 2 mutations. One lies within the same codon as described above (CCT) that encodes Pro^{159}, and changes the amino acid to (CTT) leucine (Leu^{159}). The second mutation was found in the codon GAC that encodes for aspartic acid (Asp^{260}, figure 5.10), and changes the codon to AAC which encodes asparagine (Asn^{260}). These mutations have been detected in both DNA strands, see figures 5.11 and 5.12.

5.6.4.3 Discussion

Sequencing has revealed a single mutation in *out*L from the secretion mutant HJN1008. It is this single amino acid change (Pro^{159} to Ser^{159}) that causes the Out^- phenotype. It is extremely interesting to note that in the *out*L gene of HJN1004 there is a mutation within the same codon that changes the amino acid from Pro^{159} to Leu^{159}. Unfortunately it was found that another lesion existed in the sequence of *out*L that changed Asp^{260} to Asn^{260}. In order to distinguish which lesion is responsible (or if both are needed) for the conditional phenotype in HJN1004 it would be necessary to remove one of the mutations, possibly by site directed mutagenesis, and see whether the remaining mutation would still exhibit the conditional phenotype, or is "silent". It is interesting that the lesion causing the conditional phenotype of HJN1004 could be in the same codon as the lesion responsible for the null phenotype of HJN1008. This would imply that this amino acid residue (Pro^{159}) is extremely important in the

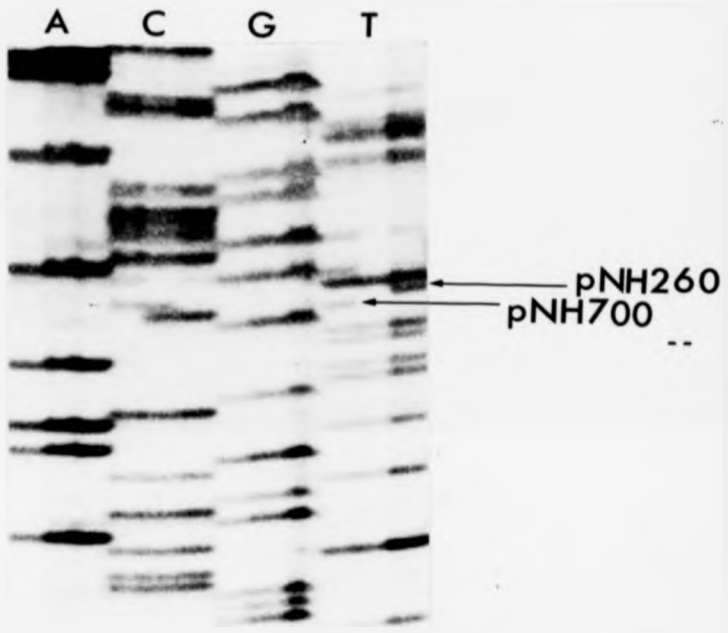
Figure 5.10 Protein analysis of OutL clones

Figure figure 5.10 shows the amino acid sequence of wild type OutL. The amino acid changes that were identified in the clones pNH260 and pNH700, see text for details, are depicted alongside the wild type amino acid sequence. The amino acid residues that are in bold type-face, and underlined, represent the putative hydrophobic domain of the protein.

Key:
A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

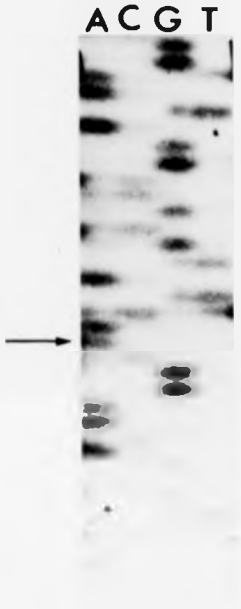
```
          1                                                                 52
Wild type M K I A G K W K R K A A K A P L H R G T V A R H P C L I V R L P V E E Q G E I E W Q V R S S N G E S L L
pNH260
pNH700

          53                                                                104
Wild type S Q G R G S I E Q V R P A L A A Y P S V T F T R V L V P A T D V T F Y A L T L P R Q A R R H V T Q V V P
pNH260    -
pNH700

          105                                                               156
Wild type F M L E D Q L A T E I E K L H F A V L E I H G D D G T V A V V E K N R M Q R W L A Q C D A L G L S V D T
pNH260
pNH700

          157                                                               208
Wild type L L P D A R V L P K H Q D G W S A L Q H D D M W L F R Q P T G H A M A A E S S W C G D L L K A S M P L P
pNH260       L
pNH700       S
          209                                                               260
Wild type A I Y S Y S A A S V G G E L A Q Y E W Q E E G E W K A Q P E T D L F T L A A T A H L P A S V D L R Q G D
pNH260                                                                     N
pNH700

          261                                                               312
Wild type Y A P D K A W Q N T L L P W R G V G I A F A C Y L L L V V A D A G W A H Y Q L Y Q Q A E H W R Q E S V R
pNH260
pNH700

          313                                                               364
Wild type V Y R Q I F P S E T N V V N P R A Q M Q Q H L Q R T A A G G A G K A L L D Q L T P L Q Q L M T Q N S A I
pNH260
pNH700

          365                                                               416
Wild type K I Q S L S Y D G A A G E F R L A L Q G T S Y Q E L E Q F Q Q Q A A A Y Y Q V Q A G E M R Q E N D R V E
pNH260
pNH700

          417          425
Wild type G R L T L R S Q Q
pNH260
pNH700
```

Figure 5.11 Sequencing of mutations in *out*L.



The sequence of the *out*L clones pNH700, pNH200 and pNH260 are shown above. The four tracks A, C, G, and T show the sequence of each of the clones in parallel, for ease of identifying any mutation(s). The mutations within *out*L in pNH700 and pNH260 are indicated by the arrows. These mutations were shown to be present in both DNA strands (data not shown).

Figure 5.12 Sequencing of a second mutation in *out*L cloned from HJN1004.



The second mutation is indicated by the arrow. This mutation was shown to be present in both DNA strands (data not shown).

biological functioning of this protein. It could be a critical site of interaction between this protein and another, involved in the secretion machinery. It can be seen, figure 5.10, that the mutations at position 159 and 260 are in the N-terminal portion of the protein. The predicted hydrophobic membrane spanning domain of the protein is depicted by the shaded portion of figure 5.10.

The topology of the protein is being deduced by other members of the group and, to date, it is suggested that OutL spans the membrane only once (P. Reeves, pers. comm.). The N-terminal and C-terminal portions of the protein are in the cytoplasm and periplasm respectively (P. Reeves, pers. comm.). It could be suggested that the N-terminal portion transduces a signal from a cytoplasmic protein, such as *out*E, which is consequently communicated to the rest of the secretory apparatus, via the C-terminal part of the protein.

Unfortunately the complete sequence for pNH200 was not achieved by the end of this project. The lesion within this cloned gene has yet to be identified. Once this lesion is identified, predictions about the putative dimerisation of this protein *in vivo* can be investigated (see section 5.6.3.3.2).

5.6.4.4 Secondary structure prediction analysis of *out*L and its mutated forms

The secondary structure of wild type OutL along with the mutated forms of this gene that have been sequenced, are depicted as "squiggle plots" in figures 5.13, 5.14 and 5.15. These predictions have been made by the use of "protein structure" and "plotstructure" in the UWGCG programmes (Devereux *et al.*, 1984). It can be seen that the plot of the mutated OutL from HJN1004 (Out^{ts}) differs from the wild type OutL in two regions, labelled A and B in figure 5.14. At position A there is a predicted additional ß-turn region and at position B there is a larger predicted area of hydrophobicity. These two regions, A and B, correspond to the amino acid changes depicted in figure 5.10. The amino acid change at position B also appears to shift the

Figure 5.13 Secondary structure prediction for OutL (wild type)

Key to symbols :

ß-turn region :

ß-sheet :

random coil :

a-helix :

Figure 5.13 shows the predicted secondary structure of OutL using "proteinstructure" and "plotstructure" in the UWGCG programmes (Devereux *et al.*, 1984). Secondary structure was predicted using the algorithms of Chou and Fasman (1978). Hydrophobicity regions were predicted using the algorithms of Kyte and Doolittle (1982), with a window of 20 residues.

Hydrophobicity regions are represented by the red diamonds, whereas hydrophilicity regions are represented by the green ovals.

One major hydrophobicity region is predicted to the C-terminal half of the protein, which is predicted to be the membrane spanning domain of the protein. See text for details and discussion.

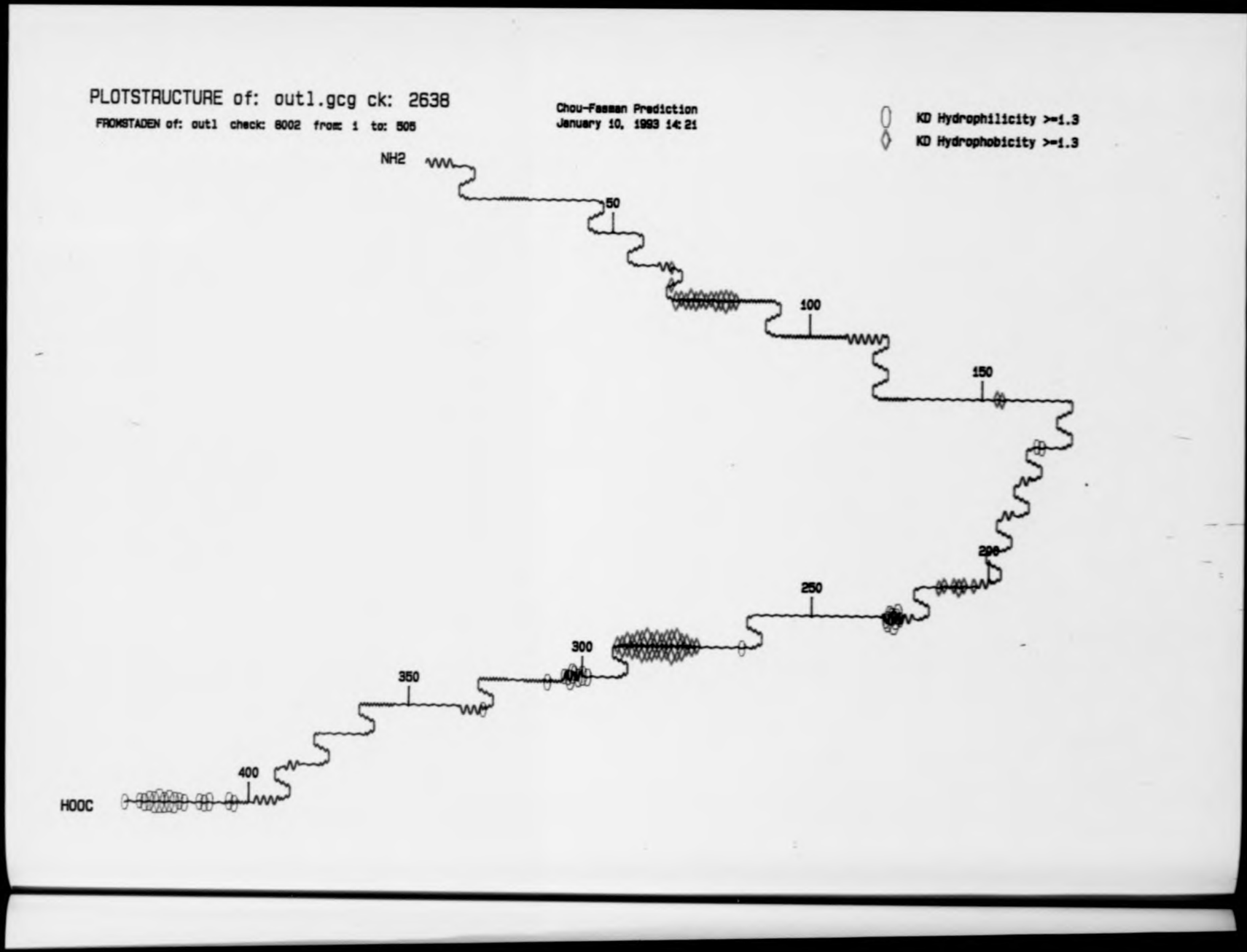

PLOTSTRUCTURE of: outl.gcg ck: 2638
FROMSTADEN of: outl check: 8002 from: 1 to: 505
Chou-Fasman Prediction
January 10, 1993 14:21
KD Hydrophilicity >=1.3
KD Hydrophobicity >=1.3
NH2
50
100
150
200
250
300
350
400
HOOC

Figure 5.14 Secondary structure prediction for OutL (pNH260)

Key to symbols : see figure 5.13

Figure 5.14 shows the predicted secondary structure of OutL using "proteinstructure" and "plotstructure" in the UWGCG programmes (Devereux *et al.*, 1984). Secondary structure was predicted using the algorithms of Chou and Fasman (1978).
Hydrophobicity regions were predicted using the algorithms of Kyte and Doolittle (1982), with a window of 20 residues.
Hydrophobicity regions are represented by the red diamonds, whereas hydrophilicity regions are represented by the green ovals.

See text for details and discussion.

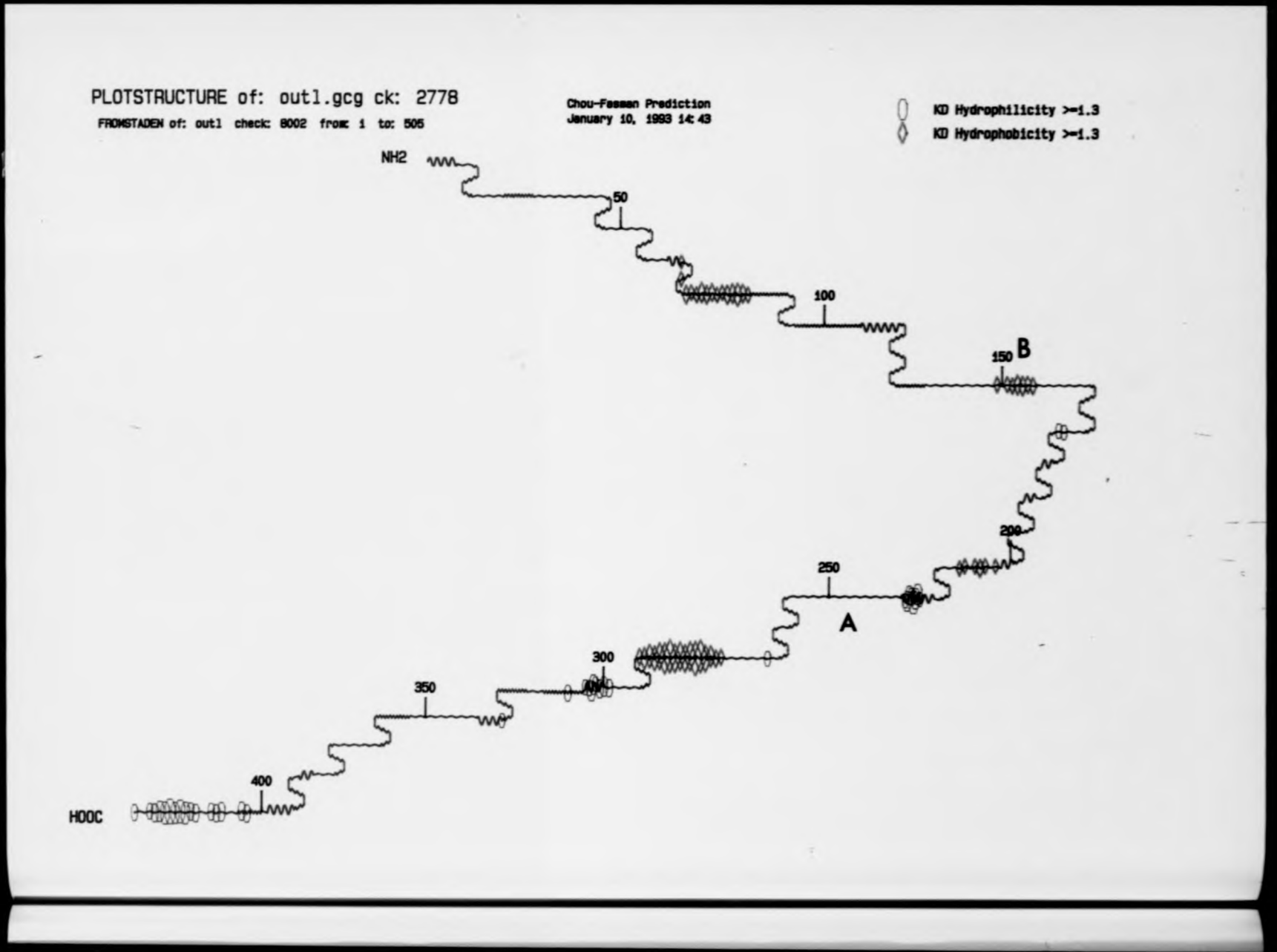
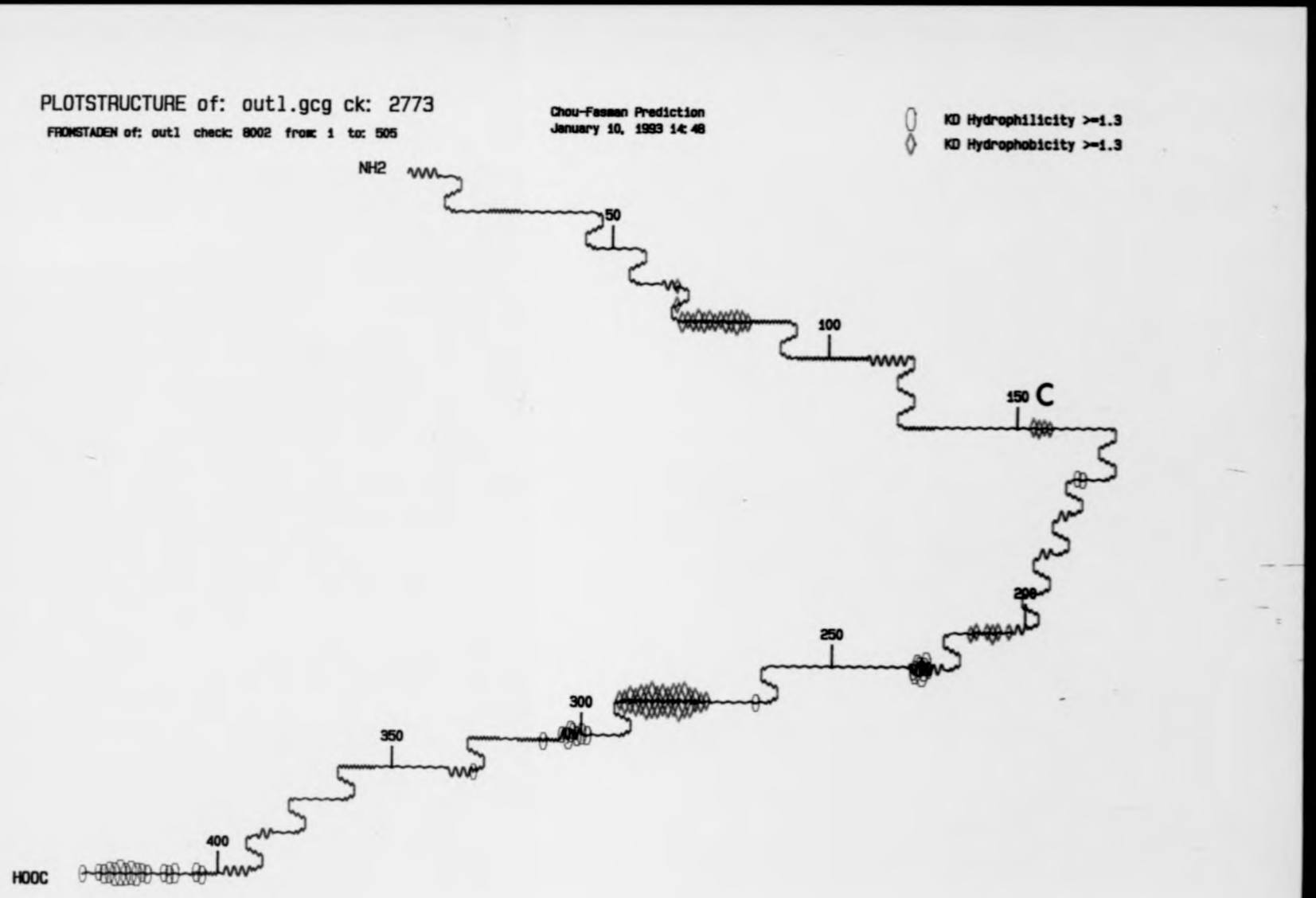

PLOTSTRUCTURE of: outl.gcg ck: 2778
FROMSTADEN of: outl check: 8002 from: 1 to: 505
Chou-Fasman Prediction
January 10, 1993 14:43
KD Hydrophilicity >=1.3
KD Hydrophobicity >=1.3
NH2
50
100
150
B
200
250
A
300
350
400
HOOC

Figure 5.15 Secondary structure prediction for OutL (pNH700)

Key to symbols : see figure 5.13

Figure 5.15 shows the predicted secondary structure of OutL using "proteinstructure" and "plotstructure" in the UWGCG programmes (Devereux *et al.*, 1984). Secondary structure was predicted using the algorithms of Chou and Fasman (1978).
Hydrophobicity regions were predicted using the algorithms of Kyte and Doolittle (1982), with a window of 20 residues.
Hydrophobicity regions are represented by the red diamonds, whereas hydrophilicity regions are represented by the green ovals.

See text for details and discussion.


PLOTSTRUCTURE of: outl.gcg ck: 2773
FROMSTADEN of: outl check: 8002 from: 1 to: 505
Chou-Fasman Prediction
January 10, 1993 14:48
KD Hydrophilicity >=1.3
KD Hydrophobicity >=1.3
NH2
50
100
150
C
200
250
300
350
400
HOOC

alignment of the protein structure compared to that of the wild type, figure 5.13. Exactly how these changes affect the assembly/function of the protein is yet to be determined.

The sequence of the null (Out$^-$) mutation in HJN1008, figure 5.15, shows a small increase in hydrophobicity at position C. This is the only noticeable change from that of the wild type form (figure 5.13).

It is generally known that the thermostability of a protein can be altered by the substitution of a single amino acid residue (Yutani *et al.*, 1977; Imanaka *et al.*, 1986). What amino acid residues control the thermostability of certain proteins ? Imanaka *et al.*, (1992) successfully isolated temperature sensitive penicillinase repressors by changing proline residues at predicted ß-turns. Proline residues are suggested to be strong protein folders forming fairly rigid structures (Levitt, 1978). It could therefore be tentatively suggested that the amino acid substitution of Pro^{159} for Leu^{159} (hydrophobic, aromatic side chain), in the clone pNH260, could be the residue causing the temperature sensitivity of HJN1004. The second amino acid change from Asp^{260} to Asn^{260} changes the residue from a negative charge to non charged, which may lead to a relatively minor change in the proteins secondary structure.

The amino acid change of the Pro^{159} to Ser^{159} (polar) in pNH700 causes a null (Out$^-$) phenotype. The thermostability is not altered but the amino acid substitution is enough to disrupt the secretory machinery.

5.6.4.5 General conclusions

Most of the mutations generated by localised mutagenesis have been mapped to the *out* cluster by cosmid complementation (section 5.3). This data, along with cotransduction data (section 4.2), suggest that the position of the transposon Tn*5* with respect to the *out* cluster is to the right (3'). Those mutants (Out$^-$) that have not been complemented

by any cosmids other that cHII.251/4 (see section 5.3.1) are predicted to be linked to the *out* cluster by cotransduction linkage data. Further "fine" mapping is required using DNA upstream of the *out* cluster.

The two conditional (Out^{ts}) mutants, HJN1003 and HJN1004, have had the lesions responsible for their conditional phenotypes mapped to *out*E and *out*L (see sections 5.4 and 5.5), respectively. For HJN1004, PCR amplification, cloning and sequencing (see section 5.6) has revealed two lesions in the mutated *out*L gene. Further analysis is required to distinguish which mutation is responsible for the Out^{ts} phenotype. Sequencing of the PCR clone pNH700 has identified the lesion responsible for the null (Out-) phenotype of HJN1008. This lesion causes the amino acid substitution of Ser^{159} Pro^{159}.

CHAPTER 6

INVESTIGATION OF THE EXTRACELLULAR PROTEIN SECRETION PATHWAY IN *Ecc* USING CONDITIONAL SECRETORY (*out*^{ts}) MUTANTS.

6.1 Introduction

As discussed, in chapter 1, there is emerging a common two-step mechanism of secretion of extracellular enzymes across the outer membrane of Gram-negative bacteria termed the general secretory pathway, GSP (Pugsley, 1992). The first stage in the secretion of extracellular enzymes, via this pathway, is thought to be analogous to the Sec-dependant export process of *E. coli* (for reviews see: Wickner *et al.*, 1991; Schatz and Beckwith, 1990). Once the enzyme has translocated the inner membrane to the periplasmic space it is then transported across the outer membrane in an, as yet, undefined manner. Pleiotropic mutants defective in extracellular enzyme secretion in a wide range of bacteria (see chapter 1) have been used to demonstrate that a number of accessory proteins are required for translocation across the outer membrane in Gram-negative bacteria. These studies include the use of secretion mutants for Pel, Cel and Peh by *Ecc* and *Echr* in which the normally extracellular protein accumulates periplasmically (Andro *et al.*, 1984; Ji *et al.*, 1987; Thurn and Chatterjee, 1985; Gibson *et al.*, 1988; Reeves *et al.*, 1993; Murata *et al.*, 1990; He *et al.*, 1991(b)) .

The genes encoding the accessory proteins have been shown to be clustered together, in both *Ecc* and *Echr* (Reeves *et al.*, 1993; Lindenberg and Collmer, 1992). In *Ecc* there are 13 *out* genes that are essential for the secretion of Pel, Cel and Peh and have been termed "membrane traffic wardens" due to the fact that they are involved in controlling the flow and location of proteins that are translocated across the outer membrane (Salmond and Reeves, 1993). Similarities between the *out* genes of *Ecc* and other similar (GSP type) clusters of genes encoding accessory protein products, has been described, in detail, in chapter 1.

It is the periplasmic accumulation of normally extracellular enzymes, in secretion mutants (described above), that is the subject of this study. To date there has been no unequivocal evidence that the only translocation step, after export, is via the periplasm. There is considerable circumstantial evidence for it (He *et al.*, 1991(b),

Murata *et al.*, 1990; Possot *et al.*, 1992; Hu *et al.*, 1992) but this does not mean that the enzymes could not have artefactually been re-routed to the periplasm, due to the breakdown of the secretion machinery (see section 1.5.4.4 for a discussion). In attempting to address this question, I have isolated conditional secretory (*out*^{ts}) mutants in order to try to perform "pulse-chase" experiments the results of which might show whether the periplasmicaly accumulated enzymes are able to re-enter the secretory machinery or if they are permanently "shunted" out of the system.

6.2 How many proteins are secreted into the extracellular mileu of *Ecc* ?

In addressing this question, two important points could be answered; 1) how many proteins are secreted by *Ecc* ?, and 2) are there other secreted proteins, other than the expected Pel and Cel, also missing from the culture supernatant in the Out$^-$ or Out^{ts} mutants ?

It has been shown for *Ecc* strain *Ecc* 567 (Ward and DeBoer, 1989) that a large number (>20) of proteins have been detected, by SDS-PAGE analysis, in culture supernatants after 72-96 hours, post-inoculation. This analysis could represent proteins that have been released by the cells during stationary phase or by leakage from dead cells. In order to deduce how many proteins are secreted by *Ecc* strain SCRI193, similar experiments were performed using larger volumes of cells for a much shorter time period (see section 2.24.1), so as to reduce the possibility of leakage from dead cells. In parallel to wild type *Ecc* the Out$^-$ secretion mutant HJN1019 was also analysed, for reasons described above. Once the supernatants were concentrated 30 times or 40 times (see section 2.24.1) samples were analysed by SDS-PAGE using the "Bio-Rad, Protean 11" gel system as described in section 2.24.3.1.1 or by the "Phast System" (Pharmacia) as described in section 2.24.3.1.2. Gels were stained by the silver staining technique described in section 2.24.4.1.

6.2.1 Results/Discussion

Upon repeating the experiment several times it was found that proteins could not be identified to any sufficient resolution. This could be explained by a number of reasons:

1) The amount of protein in the sample was too low to be detected by the silver staining procedure. This was discounted by performing total protein estimations on each sample as described in section 2.24.2. Each loaded sample was found to be within the detection limits (data not shown) of the silver staining technique (see section 2.24.4.1).

2) Smearing was seen on some gels which could indicate that the proteins have been degraded by proteases. These could have been present in the supernatant concentrating apparatus (Amicon).

3) Problems with concentrating some supernatants. It was found that for mutant (Out^-) strains grown in LB/PGA (0.5% w/v) concentration procedures were difficult compared to that of wild type *Ecc*. This could be explained by the fact that wild type *Ecc* secretes the necessary extracellular enzymes (Pel) to break down the PGA substrate. In the secretion (Out^-) mutant strains there is no secretion of these extracellular enzymes and so no break down of PGA. This could therefore cause problems with concentration procedures due to the PGA in the supernatant, and consequently filters were blocked easily.

Because of the problems encountered with concentrating the supernatants of these strains, it was thought that another approach would be to radiolabel the proteins using ^{35}S-methionine.

6.2.2 ^{35}S-methionine labelling of proteins in *Ecc*

The method for ^{35}S-methionine labelling has been described in section 2.24.5. In

order to show that ^{35}S-methionine was being taken up by *Ecc* an incorporation curve was constructed as described in section 2.24.5.1. The results of ^{35}S-methionine incorporation in *Ecc* are shown in figure 6.1. The results suggest that there is maximal incorporation early on (0-2 minutes), post-inoculation with radiolabel. Incorporation is still evident up to 60 minutes post-inoculation, although comparatively reduced. The change in optical density, A_{600}, is 0.1 OD unit (data not shown).

6.2.2.1 Results/Discussion

The results of ^{35}S-methionine radiolabelling using 10 or 100μCi per ml cell culture, see section 2.24.5, was inconclusive. Using 10μCi per ml of cell culture, some protein bands could be detected but the resolution was very poor. By using 100μCi per ml the resolution was not improved by any great amount. The experiments were repeated but without any marked improvement. This was puzzling because it was shown that *Ecc* does incorporate ^{35}S-methionine, see above. It could be that there needs to be more ^{35}S-methionine present for a longer time period for incorporation into the secreted proteins, or that there are not enough methionine residues present in the secreted proteins. For CelV of *Ecc* there are 8 methionine residues present in the transcribed protein (V. Cooper, pers. comm.). This should have enabled at least the identification of CelV by this technique, but this was not shown to be the case.

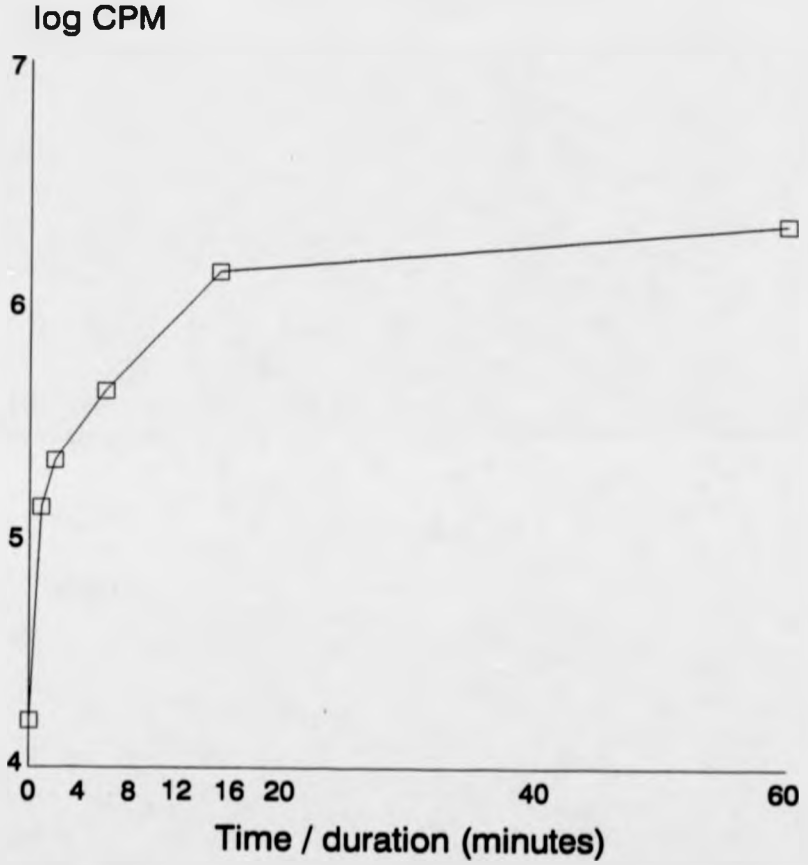
Due to time constraints, this approach was not pursued further.

6.3 "pulse-chase" - an approach to understanding extracellular protein secretion in *Ecc*

6.3.1 Introduction

In this study Out^{ts} secretion mutants of *Ecc* were isolated which were to be used to study the second step in the GSP. In order to be able to use these mutants, it was necessary to be able to identify the extracellular protein in the wild type strain and in

Figure 6.1 Incorporation of 35S-methionine in Ecc



Key :
CPM - Scintillation counts per minute

the Out^{ts} or Out^{-} secretion mutants. In a "pulse-chase" experiment, labelled extracellular protein has to be "chased" with unlabelled methionine, across the outer membrane, and into the extracellular milieu. In the conditional (Out^{ts}) secretion mutants the extracellular protein would be expected to reside in the periplasm at the restrictive temperature (33°C). When the culture is shifted to the permissive temperature (26°C) the question is "what happens to the periplasmically accumulated protein ?". The best way in which to perform this experiment would be to "pulse" bacterial cultures of wild type *Ecc* and the Out^{ts} and Out^{-} mutant strains with radiolabel, at the restrictive temperature then to "chase" with unlabelled methionine, once the temperature has been shifted down to the permissive temperature. By taking samples at various time points and fractionating them (see section 2.10) into supernatant, periplasmic and cytoplasmic fractions, the samples could then be electrophoresed on an SDS-PAGE gel, followed by immunoprecipitation with a monoclonal antibody raised against Pel or Cel.

Antibodies for this experiment were supposed to be available before the end of this project, but due to unforeseen circumstances were not available. Therefore, two alternative approaches were designed using the p*Trc*99A expression vector system and the bacteriophage T7 RNA polymerase / promoter system.

6.3.2 p*Trc*99A expression vector system

This vector system is inducible by IPTG due to the *trc* promoter. This is a *trp/lac* hybrid promoter which is fully repressed by the product of the *lacI*^{q} allele, which is also present in the vector, and there is also the *lacZ* ribosome binding site. The copy number of this vector is high, 30 copies per chromosome (Amann *et al.*, 1988). It was unknown whether the *trp/lac* promoter would work in *Ecc*, but it was demonstrated to be functional in *Pseudomonas putida* (Bagdasarian *et al.*, 1983), and the *tac*P promoter was demonstrated to be functional in four non-*Escherichia coli* bacteria (Fürste *et al.*, 1986).

The principle behind using this vector, was to attempt to clone either Pel or Cel and induce expression by the addition of IPTG. In that way it should have been possible to visualise the protein by concentrating the supernatants as described in section 2.24.6. Once proteins had been detected by SDS-PAGE, the band corresponding to the overexpressed cloned gene product, could be identified by Pel overlays, see section 2.24.3.2.1. Once this had been done "pulse chase" experiments could be performed as described earlier.

6.3.2.1 Subcloning of *pelD*

pelD was cloned previously (R. Barralon, pers. comm.) as a 2.3 Kb *Sac*1-*Sph*1 fragment into pUC19. This clone , pJS6161, was digested with *Sac1-Sph1* (section 2.13) and the resulting 2.3 Kb fragment purified by "trough elution" (section 2.15.2), see figure 6.2. The vector, p*Trc*99A (see appendix 1) was restricted with *Sma*1 to yield blunt ends and treated with calf intestinal alkaline phosphatase (see section 2.16). The 2.3 Kb insert was "end repaired" (see section 2.18) for cloning into the blunt ended vector and ligated as described in section 2.17.2. Ligation reactions were electroporated (section 2.20) into DH1, and transformants selected on LB + Amp.

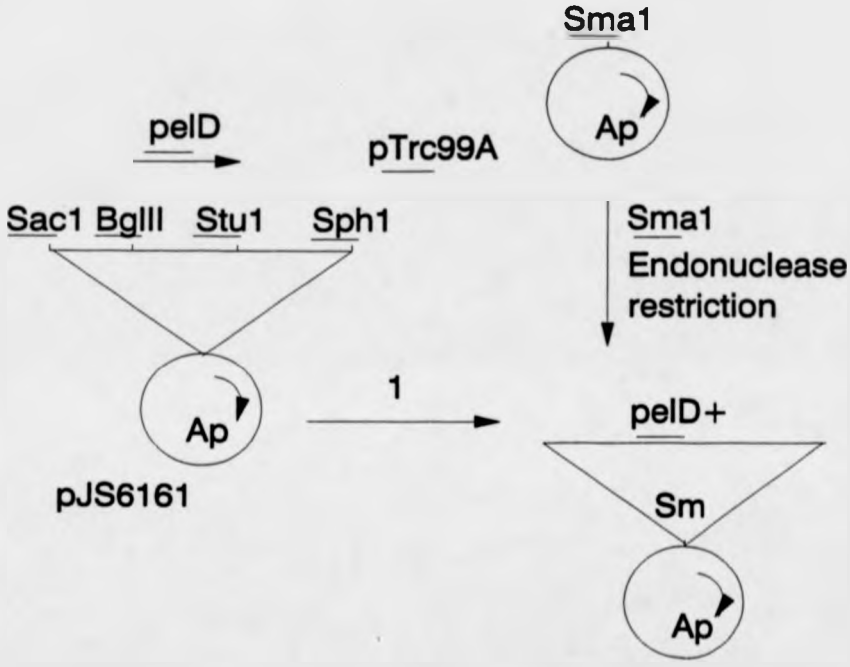
6.3.2.2 Results / Discussion

20 Ap^r transformants were isolated. 4 clones were shown, via plasmid DNA preparation (section 2.12), and *Bgl*11 restriction digests, to contain the 2.3 Kb insert. This 2.3 Kb insert was shown to be in both orientations, see figure 6.3, by double digestion with *Sac*1 and *Bgl*11.

6.3.2.3 Does the subclone, pNH31A, overexpress PelD upon induction with IPTG ?

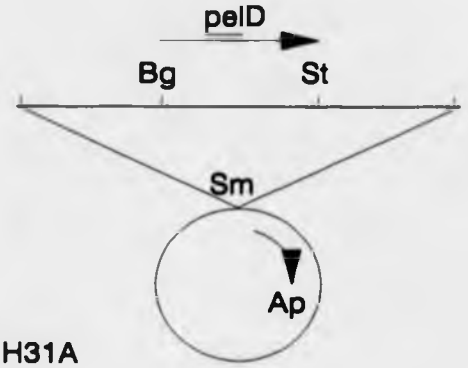
DH1 containing the subclones pNH31A and pNH31B, were cultured and induced by IPTG, as described in section 2.24.6, along with control DH1 cells. 150μl of each supernatant and sonicate sample was added to 0.5 x 0.5 cm wells in Pel detection

Figure 6.2 Sub cloning strategy for pelD.

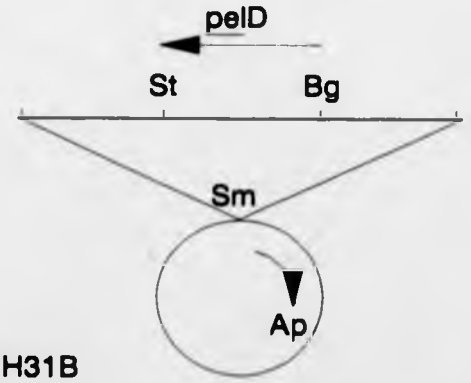


Key:
1 - BglII and Stu1 endonuclease restriction
and 2.3Kb fragment isolation by "trough elution".
Sm - Sma1
Ap - Ampicillin resistance

Figure 6.3 Orientation of subclones pNH31A and pNH31B



pNH31A



pNH31B

Abbreviations :

Bg, Bgl11; St, Stu1; Sm, Sma1; Ap, ampicillin resistance.

media (see table 2.4). Samples were left overnight at 30°C before developing the detection media (see section 2.2.1.3).

No halos were identified for either of the subclones or in the DH1 control cells. It was thought that this could be due to the detection limits of the Pel medium or that the samples were not concentrated enough by the Amicon system.

Each subclone, pNH31A and pNH31B, was transferred by electroporation (section 2.20) to the cured *Ecc* strains; HJN1003, HJN1004, HJN1014 and SCRI193. Induction with IPTG, see above, was carried out with HJN1004, HJN1014 and SCRI193 containing the subclones described above. When the sonicate and supernatant samples were electrophoresed by SDS-PAGE, Pel overlays (section 2.24.3.2.1) were used to distinguish the overexpression of Pel in the relevant samples. No overexpression of Pel was seen in any of the strains indicated above. This was confirmed by performing IEF on each of the samples (section 2.24.3.2). Again, no over-production of Pel was seen. The original SDS-PAGE gel was silver stained (section 2.24.4.1) to reveal proteins in the supernatant (data not shown).

6.3.2.3.1 Discussion

The results described in section 6.3.3.3 indicate that although it appears from restriction enzyme analysis that *pelD* has been successfully cloned into p*Trc*99A, the clone does not overexpress PelD. The concentrating of the supernatants has revealed a number of proteins (data not shown). This is surprising because no extracellular proteins could be detected using the techniques described in section 6.2. This is probably because there was no PGA in the culture medium. No obvious differences could be detected between the extracellular protein secretion of the secretion mutants, HJN1004, HJN1014 and that of the wild type *Ecc* strain SCRI193. This may be because the detection of Pel and Cel is masked by the presence of similar molecular weight proteins, or that they are not present in detectable amounts. No other proteins

were affected in their secretion by the secretion mutants HJN1004 or HJN1014. It could be that the *out* cluster is specific for the secretion of Pel, Cel and (Peh), and that no other secreted extracellular protein is able to enter this pathway.

6.3.3 Bacteriophage T7 RNA polymerase / promoter vector system

This system, for exclusively expressing specific genes using the T7 polymerase promoter as a control, was designed by Tabor and Richardson, 1985. The principle behind this system relies upon the specificity of T7 RNA polymerase for its own promoters. Also T7 RNA polymerase is insensitive to rifampicin, whereas in *Escherichia coli* transcription by RNA polymerase is inhibited by rifampicin. These facts were exploited by constructing a set of cloning vectors with multiple restriction sites downstream of the T7 RNA polymerase / promoter, ø10 (see Tabor, 1990). The clone could then be introduced into an *Escherichia coli* strain containing pGP1-2. pGP1-2 contains the intact T7 RNA polymerase gene under the control of the λ_{PL} promoter that is repressed by the temperature sensitive repressor protein product of cI^{857}. Thus induction of the T7 RNA polymerase gene can be achieved by raising the temperature from 30°C to 42°C. The cloned gene product can be uniquely labelled by growing the cells in minimal medium followed by the addition of rifampicin to dampen down transcription by *Escherichia coli* RNA polymerase. Subsequently ^{35}S-methionine could be added to label the proteins.

This study has generated Out^{ts} secretion mutants, thus by cloning either Pel or Cel into one of these vectors and expressing them by the technique described above, the system would be useless because induction occurs by raising the temperature to 42°C. An alternative method of induction using IPTG uses the M13 phage mGP1-2 (Tabor and Richardson, 1987). This phage contains the T7 RNA polymerase gene under the control of the p_{lac} promoter of *Escherichia coli*. The principle requires that the host cells carry the F factor. When the host cells are infected with mGP1-2 and IPTG is added to induce the p_{lac} promoter, T7 RNA polymerase is produced and can therefore

initiate transcription of the desired cloned gene as described above.

For this technique to work in *Ecc* the strain must carry the F factor. One such plasmid carrying the F factor is available in this laboratory. The M13 phage mGP1-2 is also now available, but arrived too late for use in this study, due to time constraints.

6.4 General discussion

Several attempts have been made during this study to try to perform "pulse chase" experiments with the Out^{ts} secretion mutants. Unfortunately, although various strategies have been devised, radiolabelling and consequent SDS-PAGE analysis has proved difficult. Overexpression of Pel or Cel by using either of the T7 or p*Trc*99A systems, may have caused an overloading effect on the secretion system. The only "clean" way of performing the "pulse chase" experiment is to use monoclonal antibodies, as described in section 6.3.1.

CHAPTER 7

GENERAL CONCLUSIONS

CHAPTER 7

GENERAL CONCLUSIONS

7.1 Introduction

At the start of this project little was known about the second stage of secretion of extracellular enzymes across the outer membrane of Gram negative bacteria. Since that time it has been indicated that there are clusters of several genes that are responsible for the efficient secretion and targeting of extracellular proteins, in many species of bacteria. In particular the secretion of pullulanase by *Klebsiella oxytoca* has been intensely studied and the products of the *pul* gene cluster have been found to share significant homology to various secretory machinery in other bacterial species. In *Erwinia* both *Ech* and *Ecc* have been shown to have similar clusters of genes (*out*) that have a very similar structural organisation to that of the *pul* cluster. Many suggestions have been proposed for the roles that each of the accessory proteins play in the secretion process. These have been described, where appropriate, throughout this thesis. One intriguing question that this study has addressed is whether there is a true periplasmic intermediate in the secretory process ? The results and summary of the major findings of this study are described below.

7.2 Summary of work in this study

The major findings are as follows:

1) The transposon Tn*5* was linked to the *out* cluster and localised mutagenesis of this region (using hydroxylamine) was performed by using the *Erwinia* generalised transducing phage øKP. This is the first time this technology has been used in *Ecc* (chapter 3).

2) Localised mutagenesis generated 17 Out$^-$ secretion mutants (Pel$^-$, Cel$^-$, Prt$^+$), 2 conditional (Out^{ts}) secretion mutants and 1 auxotroph (histidine requiring), see chapter 4.

3) Each secretion mutant (Out$^-$) was shown to accumulate Pel and Cel within the periplasm. This was conditional upon temperature in the Out^{ts} secretion mutants (chapter 4).

4) In each secretion mutant the mutation that was responsible for the Out$^-$ or Out^{ts} phenotype was mapped, using cotransduction frequencies, with respect to the transposon Tn*5* (chapter 4).

5) By using cosmids that contain various inserts of *out* DNA, most of the mutations generated by localised mutagenesis were complemented, by restoration of the Out$^+$ phenotype, and mapped to the *out* cluster (chapter 5).

6) By comparing the complementation data with the cotransduction linkage data it was predicted that the Tn*5* insertion lies 3' to the *out* cluster (chapters 4 and 5).

7) The lesion(s) responsible for the Out^{ts} phenotype of HJN1003 and HJN1004, have been mapped to *out*E and *out*L respectively (chapter 5).

8) PCR cloning and sequence analysis has revealed two lesions in *out*L from the Out^{ts} strain HJN1004 and one lesion in *out*L in the Out$^-$ strain HJN1008 (chapter 5).

9) Secondary structure predictions of the mutated forms of OutL, in HJN1004 and HJN1008, have revealed small changes in the predicted hydrophobicity of both proteins. In the case of HJN1004 there is also predicted to be an additional ß-turn (chapter 5).

10) Attempts to use "pulse chase" to analyse protein secretion proved difficult but future experiments are envisaged with the recent production of antibodies raised against Cel

(chapter 6).

7.3 Concluding remarks

Throughout this project many advances have been made in understanding the second stage of secretion of extracellular enzymes in Gram negative bacteria. The large clusters of genes that encode accessory proteins, "traffic wardens", involved in the recognition and targeting of these enzymes, have been found in many diverse bacteria (see chapter 1). Not only are these systems present in *Erwinia* and *Klebsiella* spp. but it has been shown that a similar system of secretion has evolved, if only in part, in other bacteria. Now that *Ecc* has been shown to have 13 accessory protein genes (*out*) it is intriguing to speculate as to the functions that each protein plays in the overall secretion process. Several putative roles have been suggested for the Pul proteins of *Klebsiella oxytoca*. Because the two systems, *Erwinia* and *Klebsiella*, share significant homology at the protein level, these putative roles may also apply to *Ecc*, see chapter 1 for a discussion.

Understanding how these proteins interact with each other in the secretion process will tell us a lot about the system. For example the OutE protein has been implicated in three roles, firstly, in energising the secretory process by hydrolysing ATP. Secondly, OutE could act as a kinase to phosphorylate other proteins, thereby activating them. Thirdly, OutE could act as a platform for the assembly of type IV pilin like strucutres (see chapter 1 for a discussion). It has been shown from this study that a mutation(s) within this gene exhibits a conditional secretion phenotype (HJN1003). Some of the above putative roles could now be examined using this mutant. Sequencing of the mutation will tell us whether the lesion lies within the putative ATP binding site.

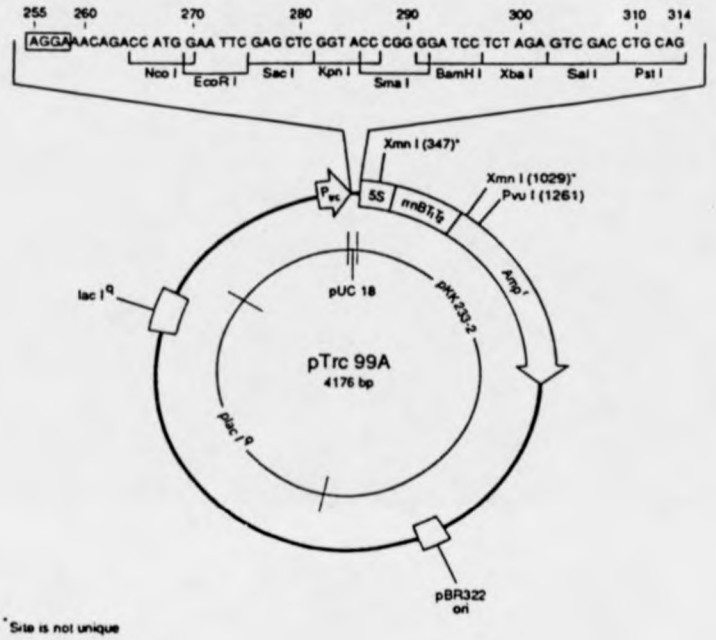
The mutation within the conditional secretion mutant HJN1004 has been mapped to *out*L and has been sequenced to reveal two lesions within the gene. OutL has been predicted to

be an integral inner membrane protein that is thought to span the membrane once (Reeves *et al.*, 1993). The larger N-terminal portion of this protein is cytoplasmic while the C-terminal portion resides in the periplasm. An interesting feature is that for each mutated *out*L that has been so far been sequenced all have revealed mutations within the N-terminal portion of the protein. This means that a lesion on the cytoplasmic portion of the protein causes the breakdown of the secretion machinery. This is interesting because the majority of the Out proteins are predicted to be inner membrane associated. How can a mutation on the cytoplasmic face of the inner membrane cause such a massive disruption of the secretory mechanism across the outer membrane ? Is there some sort of signal transduction happening with OutL ?

It was unfortunate that the presence of a true periplasmic intermediate was not confirmed or refuted by using the conditional secretion mutants isolated in this study. It is now possible, that with the use of antibodies raised against Cel, that this question may be resolved.

The adaptation of localised mutagenesis to *Ecc* has proved successful in isolating conditional secretion mutants along with a new "battery" of secretion (Out$^-$) mutants. In analysing some of these mutants at the molecular level, many more questions as to the role of these proteins in the secretory process have been raised. Research has progressed substantially in the last few years in investigating secretion. The secretion mutants generated in this study have proved to be extremely interesting and will be valuable in future studies on secretion.

Appendix 1.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Nco I
EcoR I
Sac I
Kpn I
Sma I
BamH I
Xba I
Sal I
Pst I
Xmn I (347)*
Xmn I (1029)*
Pvu I (1261)
5S
rrnB T1 T2
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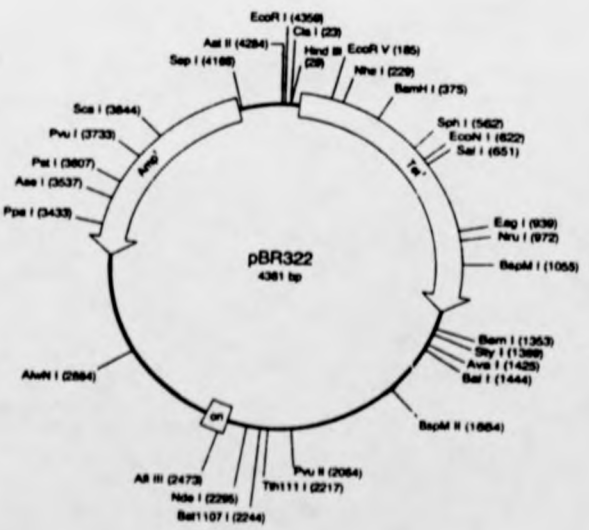
pHK 233-2
pUC 18
lac I q
pTrc 99A
4176 bp
plac I q
pBR322 ori
*Site is not unique


EcoR I (4358)
Cla I (23)
Hind III (29)
EcoR V (185)
Nhe I (229)
BamH I (375)
Sph I (562)
EcoN I (622)
Sal I (651)
Pst I (3807)
Aae I (3537)
Pae I (3433)
Eag I (939)
Nru I (972)
BspM I (1055)
pBR322
4361 bp
Bsm I (1353)
Sty I (1389)
Ava I (1425)
Bal I (1444)
BspM II (1664)
AlwN I (2684)
ori
Pvu II (2084)
Tth111 I (2217)
Bal1107 I (2244)
Nde I (2295)
Afl III (2473)

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