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**THE MOLECULAR AND GENETIC CHARACTERIZATION OF THE
SECRETORY APPARATUS OF
ERWINIA CAROTOVORA SUBSP. *CAROTOVORA***

by

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A thesis presented for the degree of
Doctor of Philosophy

Department of Biological Sciences,
University of Warwick.

September 1992.

To my parents

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SUMMARY.

Erwinia carotovora subsp. *carotovora* (*Ecc*) is a Gram negative phytopathogen, which causes soft-rot of various crops. The major pathogenicity determinants are macerating enzymes: including pectinases (*Pel*) and Cellulases (*Cel*). These are thought to be secreted from the cell by a two-step mechanism, via a periplasmic intermediate. The proteins required for the second step of secretion (transport across the outer membrane) are encoded by a cluster of (*out*) genes. This thesis describes an analysis of the structure of the *out* gene cluster and secretion of *Pel* by *Ecc*.

The synthesis and secretion of *Pel* by the wild-type *Ecc* strain HC131 was characterized, to enable accurate studies to be made of secretion-deficient (*Out*⁻) mutants.

The *out* gene cluster was analysed using restriction mapping and Southern hybridizations. Mutagenesis (using *TnlacZ* and *TnphaA*) was performed, in an attempt to identify a putative regulatory region of the *out* cluster. The expression of individual *out* genes under various conditions was investigated using gene fusions.

DNA sequence analysis of the upstream region of the *out* cluster identified two genes: *outC* and *outX*. These were expressed *in vivo*, and their protein products were visualized using SDS-PAGE.

OutX was found to encode exo-Peh: predicted to be a lipoprotein, bound to the *Ecc* outer membrane. This protein is proposed to have a role in the regulation of *pel* (and possibly *out*) gene expression. A putative promoter region was identified upstream of *outX*, suggesting that the start of the *out* gene cluster had been found.

While this project was in progress, DNA sequence analysis and data bank searches revealed homologies between the *Out* proteins of *Ecc* (including *OutC*), and proteins involved in the trafficking of various macromolecules across the Gram negative cell envelope. It is thought that the *Ecc* *Out* system may represent the major pathway out of Gram negative cells.

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ACKNOWLEDGEMENTS

I would like to thank George Salmond and Michel Perombelon for supervising this project. In particular, I thank George for his reassurance: 'it always worked for me', and Michel for seeing things from a different perspective: 'studying pathogenicity is like trying to find how many angels will fit on the point of a pin'!

I thank all those in Micro II (past and present) who helped make my time in the lab enjoyable. Special thanks to Dave W. and Phil for their advice and encouragement, Vince for his advice and discouragement and Mike for his discouragement and insults: they will not be forgotten! Thanks to Paul R. for not laughing too much at my early attempts to play squash and to Kevin for not laughing at all. I thank Paul D. for showing me the way to Colab (and everywhere else in/around Warwickshire).

Thanks to Jeni Lockett for synthesizing sequencing primers for me. Thanks also to Phil for his help with sequence analysis, and to Vince for advice on everything else to do with computers: his failure to get 'riled' while manning a 24 hr help line/call out service is much appreciated!

I would like to thank my landlady for adding interest to life in Coventry, and Mrs Titman of the accommodation office for her sympathy and humour. I thank Simon and Roni for making escape possible, by lending me their house (and spider collection). Special thanks to Rita for putting up with me while I was writing up: I hope I was not the only reason she decided to leave Coventry!

Thanks to Ed for helping me to escape from the lab and forget about *Erwinia* from time to time, and to my parents for their support and encouragement, particularly during the final months of writing this thesis.

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I acknowledge financial support from the SERC and the Scottish Crops Research Institute.

DECLARATION

This thesis has been composed by myself and has not been used in any previous application for a degree. The results were obtained by myself, except where the contribution of others has been acknowledged. All sources of information have been specifically acknowledged by means of references.



Susan Wharam.

ABBREVIATIONS.

A	absorbance
A	adenine
AMPS	ammonium persulphate
Ap	ampicillin
ATP	adenosine triphosphate
bp	base pairs
Bla	β -Lactamase
BSA	bovine serum albumin
C	cytosine
cAMP	cyclic adenosine monophosphate
Cel	cellulase
CIP	calf intestinal alkaline phosphatase
Cm	chloramphenicol
cm	centimetre(s)
conc	concentrated
cpm	counts per min
C-terminal	carboxy-terminal
Da	Daltons
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddATP	2',3'-dideoxyadenosine 5'-triphosphate
ddCTP	2',3'-dideoxycytidine 5'-triphosphate
ddGTP	2',3'-dideoxyguanosine 5'-triphosphate
ddTTP	2',3'-dideoxythymidine 5'-triphosphate
DGA	digalacturonic acid
dGTP	2'-deoxyguanosine 5'-triphosphate

dITP	2'-deoxyinosine 5'-triphosphate
DM	MM supplemented with 0.5% (w/v) DGA
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	2'-deoxynucleotide 5'-triphosphates
DT	doubling time
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
<i>Eca</i>	<i>Erwinia carotovora</i> subsp. <i>atroseptica</i>
<i>Ecc</i>	<i>Erwinia carotovora</i> subsp. <i>carotovora</i>
<i>Ech</i>	<i>Erwinia chrysanthemi</i>
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	diaminoethanetetra-acetic acid
EMS	ethylmethylsulphonate
EtBr	ethidium bromide
EtOH	ethanol
FA	fusaric acid
g	gramme(s)
ΔG	change in free energy
G	guanine
G6-PDH	glucose 6-Phosphate dehydrogenase
gpi	growth phase independent
hr	hours
HR	hypersensitive response
IEF	isoelectric focusing
IPTG	isopropyl β -D-thiogalactopyranoside
Kb	kilo base pair(s)

Kcal	kilo calorie(s)
KDa	kilo Dalton(s)
K _M	Michaelis-Menten constant
Kn	kanamycin
l	litre(s)
LacZ	β-galactosidase
LB	Luria-Bertani medium
LPS	lipopolysaccharide
M	molar
mA	milliampere(s)
mg	milligramme(s)
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
MM	minimal medium
MMS	minimal sucrose medium
mRNA	messenger RNA
MW	molecular weight
NAD ⁺	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NADP ⁺	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NaOAc	sodium acetate
NB	nutrient broth
NBA	nutrient broth agar
nm	nanometre(s)
N-terminal	amino-terminal
OD	optical density

ONPG	O-nitro-phenol- β -D-galactose pyranoside
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
Peh	polygalacturonase
Pel	pectate lyase
Pem	pectin methylesterase
pfu	plaque forming unit
PGA	polygalacturonate
6-PGDH	6-phosphogluconate dehydrogenase
PhoA	alkaline phosphatase
PM	peI minimal medium
Pnl	pectin lyase
Prt	protease
Pul	pullulanase
R	resistant
RBS	ribosome binding site
REP	repetitive extragenic palindromic (sequence)
Rif	rifampicin
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
S	sensitive
SDS	sodium dodecyl sulphate
sec	second(s)
Sm	streptomycin
S/N	supernatant
sp	species

Sp	spectinomycin
SSC	standard sodium citrate
subsp	subspecies
T	thymine
TBE	Tris-borate-EDTA buffer
Tc	tetracycline
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
Tn	transposon
Tris	2-amino-2(hydroxymethyl)-1,3-propane diol
U	unit(s)
U	uracil
μ g	microgramme(s)
μ l	microlitre(s)
v	volt(s)
v/v	volume/ volume
w/v	weight/ volume
X-Gal	5-bromo-4-chloro-3-indolyl β -D-galacto-pyranoside
X-P	5-bromo-4-chloro-3-indolyl phosphate
YE	yeast extract
YT	yeast/tryptone medium

AMINO ACID ABBREVIATIONS

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

RESTRICTION ENDONUCLEASE SITE ABBREVIATIONS

B	<i>Bam</i> HI
Bg	<i>Bgl</i> II
C	<i>Cl</i> aI
E	<i>Eco</i> RI
H	<i>Hind</i> III
P	<i>Pst</i> I
S	<i>Sal</i> I
Sm	<i>Sma</i> I
Sp	<i>Sph</i> I
R	<i>Eco</i> RV
X	<i>Xma</i> I

CHAPTER ONE.

GENERAL INTRODUCTION.

PREFACE.

The genus *Erwinia*, belonging to the family Enterobacteriaceae, includes a large number of species, including ones which are pathogenic to humans and animals, phytopathogens and saprophytes (Starr and Chatterjee 1972).

The erwinias are Gram negative, non spore-forming short rods. They are facultative anaerobes and are motile by means of flagella. There have been many problems associated with the taxonomy of the *Erwinia* genus (Starr and Chatterjee 1972), which has been divided into various sub-groups. The 'soft-rot' group includes the species: *Erwinia carotovora*, *Erwinia chrysanthemi*, *Erwinia cyripedii* and *Erwinia rhapontici*. Since the latter two species have not been extensively studied, the soft-rot group is commonly considered to comprise *Erwinia chrysanthemi* (Ech) and the three subspecies of *Erwinia carotovora*: *carotovora* (Ecc), *atroseptica* (Eca) and *betavasculorum* (Ecb) (Lelliot and Dicky 1984).

The soft-rot erwinias decay living plant tissue (affecting both growing plants and harvested crops), by secreting a range of extracellular enzymes, which macerate host tissue. The economic cost of soft-rot (estimated at over \$100 million, World wide in 1980) shows the importance of understanding the disease. Since the secretion of the macerating enzymes has been shown to be essential for pathogenicity (Andro *et al* 1984), the study of this process is crucial for the understanding (and possible eventual prevention) of soft-rot.

The study of secretion by *Erwinia* spp. is important in its own right. The existence of a secretory pathway, not present in *E. coli*, means that the erwinias could be important as model systems for the study of bacterial secretion. In addition, the ability of *Erwinia* spp. to secrete various proteins could be exploited for use in biotechnology. The potential importance of erwinias as industrial organisms has been reviewed recently (Robert-Baudouy 1991), and will not be discussed here.

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In order to put current work in context, it is necessary to review some of the previous *Erwinia* research, concentrating on the major areas of pathogenicity and secretion.

1.1. PATHOGENICITY OF THE SOFT-ROT ERWINIAS.

1.1.1 INTRODUCTION.

Of the many bacteria which produce macerating enzymes, relatively few cause the decay of living plant tissue. These include species of *Erwinia*, *Bacillus*, *Pseudomonas*, *Clostridium* and *Flavobacterium* (see Perombelon and Kelman 1980). The interactions between hosts and soft-rot pathogens are not specific, meaning that a single species infects a wide range of crops, and a single crop may be infected by several species. However, the most important of the soft-rot pathogens are the erwinias.

The soft-rot *Erwinia* subspecies vary in their biochemical reactions, and this forms the basis of the taxonomic grouping (Perombelon and Kelman 1980). Serological studies have shown that *Ecc*, *Eca* and *Ech* are all related, but that the similarity is greatest between *Ecc* and *Eca* (Starr and Chatterjee 1972, Perombelon and Kelman 1980).

The soft-rot erwinias differ in their growth requirements, particularly in the optimum temperatures for growth (<25°C for *Eca*, <36°C for *Ecc* and <39°C for *Ech*) and so have different host ranges. *Eca* mainly affects potatoes (grown in cool climates), while *Ech* infects a range of tropical and sub-tropical crops (including maize and pineapple), in addition to greenhouse crops, such as saintpaulia and carnation, from temperate regions. *Ecc* has the widest host range of the soft-rot erwinias, infecting both tropical and temperate crops including potato, celery, cucumber and turnip. The following discussion concentrates mainly on the infection of potato plants and tubers.

1.1.2 SOFT-ROT SYMPTOMS.

Erwinia spp. can cause soft-rot (characterized by maceration and rotting of parenchyma tissue), of both field plants and stored crops. The main pathogen in the field is *Eca* which causes blackleg (Perombelon and Hyman 1989). Blackleg is associated with decay of the parent seed tuber. The pathogen enters the vascular system of the developing shoot, preventing emergence, or giving rise to a stem rot, causing leaves to turn yellow and wilt.

Soft-rot of harvested crops can be a serious problem, due to long storage times between harvesting and processing. The pathogen usually enters a potato tuber through a lenticel, but can also gain entry via the stolen end or wounded tissue. Once started, rotting rapidly spreads throughout the tuber.

1.1.3 ENVIRONMENTAL FACTORS AFFECTING PATHOGENESIS.

Soft-rot *Erwinia* spp. can remain latent on host crops, until conditions favour bacterial growth, when latency is broken (Hayward 1974).

In stored tubers, rotting is initiated under anaerobic conditions when the tuber surface is covered with water, and when the temperature is suitable for bacterial growth (see Perombelon and Kelman 1980).

Blackleg infections in field crops are most commonly associated with a decaying mother tuber, but are also dependent on the temperature and moisture of the soil: occurring most frequently in waterlogged soil.

1.1.4 SOURCES AND DISPERSAL OF INOCULA.

There is discrepancy over the length of time *Erwinia* spp. can survive in the soil, although this is known to be affected by soil temperature, water content and nutrient availability (Perombelon and Kelman 1980, Perombelon and Hyman 1989). Some bacteria may overwinter in the soil, but more probably survive in contaminated plant residues, left after harvesting. Since soft-rot bacteria can survive in lenticels for six to

seven months, vegetatively reproduced crops may become heavily infected, with pathogens spreading from seed, to progeny tubers.

Erwinia spp. spread throughout crops in various ways. Some *erwinias* (such as *Eca*) are motile, but they may also be dispersed by contaminated farm machinery, insect vectors or nematodes, or by windblown rain (following the formation of contaminated aerosols, by rain impacting on infected tissue). However, the most important means of dispersal is the soil water (Perombelon and Kelman 1980).

1.1.5 PATHOGENICITY DETERMINANTS.

The soft-rot *erwinias* are characterized by the production of a large number of macerating enzymes (and isozymes) including pectinases, cellulases and proteases (see 1.2). There are complex mechanisms for the regulation of extracellular enzyme synthesis, with pectic enzymes being induced by plant cell wall breakdown products. This induction, believed to be crucial for pathogenicity, is discussed in more detail later, together with other factors affecting the production of extracellular enzymes (see 1.3).

Mutants of *Erwinia* spp. have been isolated which synthesize, but do not secrete macerating enzymes (Andro *et al* 1984). These (Out⁻) mutants are non-pathogenic, showing that the secretory apparatus is itself a pathogenicity determinant.

Motility (Mot⁻) mutants of *Eca*, generated by transposon mutagenesis, have reduced virulence. Although the Mot⁻ strains are not fully avirulent, soft-rot symptoms do not spread, suggesting that motility is required for progression of the disease (Pirhonen *et al* 1991).

The bacterial cell surface is thought to be involved in pathogenicity. A major component of the outer membrane is lipopolysaccharide (LPS), which consists of a high molecular weight heterologous chain (o-antigen), a core oligosaccharide and lipid A. Mutants of *Ech* have been isolated, which secrete normal amounts of extracellular enzymes, but have altered LPS. Pathogenicity tests have shown that while mutants lacking only the o-antigen are wild-type for virulence, those lacking the o-antigen and

part of the core oligosaccharide (deep rough mutants) are avirulent (Kotoujansky 1987). The core oligosaccharide is therefore required for pathogenesis, possibly through an involvement in bacterial attachment to plant cells.

There is evidence to suggest that iron-uptake is a virulence factor. Micro-organisms respond to iron-limitation by producing high affinity iron-chelating agents (siderophores) and components for the specific uptake of ferric complexes. Various *Ech* mutants, deficient for iron-uptake but wild-type for secretion, were found to be unable to cause a systemic infection of *saintpaulia*, although some caused local maceration (Enard *et al* 1988). The mutants were thought to be affected in the siderophore biosynthetic pathway, the production of specific envelope receptor proteins of the iron transport system, or possibly in the internalization or dissociation of ferric complexes. Although mutants were unable to induce systemic symptoms, they were able to macerate isolated tubers and leaves. This led to the suggestion that plants may react to *Ech* infection by making some sites iron-deficient, so preventing the spread of disease (Enard *et al* 1988).

1.1.6 HOST DEFENCES.

Host responses to bacterial infection have been reviewed previously (Collmer and Keen 1986, Lyon 1989) and will be discussed only briefly here.

The susceptibility of host tissues to maceration varies, corresponding to vulnerability to degradation to pectic enzymes. This may depend on the ability to produce proteinacious pectic enzyme inhibitors, which have been isolated from the cell walls of various plants (see Collmer and Keen 1986).

There is evidence that potato tubers possess an oxygen-dependent mechanism for resistance to the pectic enzymes of *Erwinia* spp. (Maher and Kelman 1983). This might account for the greater susceptibility to soft-rot, of tubers stored in anaerobic conditions: under a film of water (1.1.3). The resistance mechanism is not understood, but the enzymes are not irreversibly inactivated, since active pectic enzymes have been

isolated from resistant tissue. Calcium ions may be involved, as an increased calcium content is correlated with decreased susceptibility of a tuber to maceration.

Host defences are believed to be elicited by molecules (thought to be oligogalacturonates) released from plant cell walls by the action of pectic enzymes. The elicitors may cause plant tissue to synthesize phytoalexins (Collmer and Keen 1986), although the true nature of the host response remains to be understood. If oligogalacturonates are host defence elicitors, it is hard to explain the success of the soft-rot erwinias, since these are sensitive to phytoalexins (Lyon 1989). *E. coli* elicits a plant defence response when carrying a cloned *Ecc* pectate lyase (*pel*) gene (Yang *et al* 1992), as do crude preparations of *Ecc Pel* (Davis and Ausubel 1989). This suggests that pectic enzymes have a dual function: macerating plant tissue and eliciting host defence responses.

Many bacterial pathogens, including species of *Pseudomonas* and *Xanthomonas* exhibit tightly regulated host specificity. Genetic analysis of plant-pathogen interactions has revealed race-specific disease resistance, specified by single bacterial avirulence (*avr*) genes which correspond to single resistance genes in the host (reviewed: Keen 1990). There is no evidence of such a 'gene-for-gene' relationship between soft-rot erwinias and their hosts, suggesting that the erwinias are primitive pathogens. *Erwinia* spp. therefore have an advantage over other bacterial pathogens, as they are not restricted by severe host recognition barriers. The soft-rot erwinias may be classified as opportunistic pathogens, because entry to the host is passive and cells remain latent until environmental factors render the host susceptible to attack (Perombelon 1982).

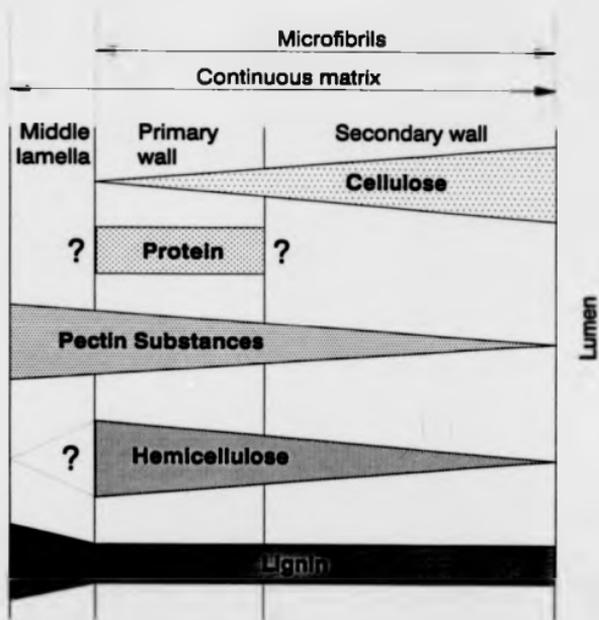
1.2 EXTRACELLULAR ENZYMES OF *ERWINIA* SPP.

1.2.1 THE SITE OF ENZYME ACTION.

The extracellular enzymes of *Erwinia* spp. attack plant cell walls: composed of a middle lamella, a primary cell wall and a secondary cell wall. Figure 1.2.1a shows a schematic representation of a plant cell wall.

Figure 1.2.1a

The Plant Cell Wall



From Bateman and Basham (1976)

The middle lamella contains pectic polymers (D-galacturonans): composed of long chains of α -1,4-linked galacturonan, which may be cross linked via side chains. The galacturonan carboxyl group is methylated in pectin and unmethylated in pectate (polygalacturonic acid, or PGA). The primary wall consists of cellulose, hemicellulose and pectic polymers. The secondary wall varies in different plants, but tends to contain proportionately more cellulose, and less hemicellulose and pectin than the primary wall (Bateman and Basham 1976).

From the above description of plant cell wall structure, it is clear that enzymes able to degrade pectin and cellulose would disrupt cell walls and hence break down plant tissue.

Soft-rot erwinias secrete pectinases, cellulases and proteases (Kotoujansky 1987), many of which exist in multiple forms. Although a complex cocktail of enzymes combines to attack host tissue, the most important enzymes in pathogenicity are the pectinases.

1.2.2 PECTINASES.

Pectinases are vital for pathogenicity: degrading components of the primary cell wall and middle lamella, causing cells to separate (Bateman and Basham 1976). There are several different types of pectinase: pectate lyase, pectin lyase, pectin methylesterase and polygalacturonase, each of which is described separately below. Figure 1.2.2a summarizes the functions of the pectinases, showing how they break down pectin, to yield a variety of products.

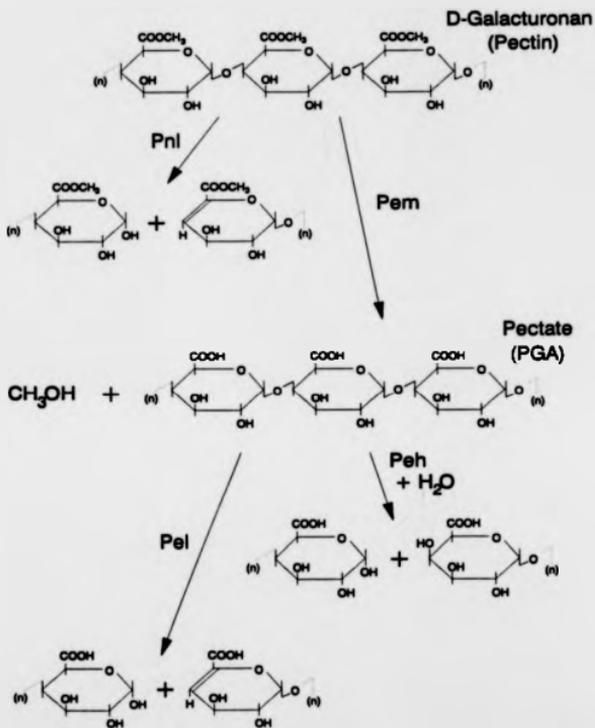
1.2.2.1 PECTATE LYASE (PEL).

Pel is produced by species of *Bacillus*, *Clostridium*, *Aeromonas*, *Pseudomonas* and *Xanthomonas*, in addition to *Erwinia*.

Pel is the most important enzyme for pathogenicity, being produced by all the soft-rot erwinias, with the exception of *Erwinia rhapontici* (Ellard *et al* pers. comm.). *Erwinia* spp. produce Pel in multiple isozymic forms: the numbers of which are dependent on both species and strain.

Figure 1.2.2a

The Breakdown of Pectin



Redrawn from Hinton (1988)

Pels, which cleave PGA α -1,4-glycosidic bonds by β -elimination, are of two main types. The more common and active form is endo-Pel (EC 4.2.2.2), which cleaves random bonds to yield oligomers. The breakdown of pectate in plant cell walls is thought to cause cell lysis under conditions of osmotic stress. The exo-form of Pel (EC 4.2.2.9) specifically attacks PGA chain termini, causing less extensive degradation (Collmer and Keen 1986).

Ech synthesizes five major isozymes of Pel (Pels A-E): differentiated by IEF, in addition to several minor isozymes. PelA is acidic (pI 4-5), Pels B and C are neutral (pI 7.0-8.5) and Pels D and E are alkaline (pI 9-10) (Bertheau *et al* 1984, Collmer *et al* 1985, Kotoujansky *et al* 1985, Ried and Collmer 1985, 1986). The five major *Ech pel* genes are arranged in two clusters: one containing *pelB* and *pelC*; the other containing *pelA*, *pelD* and *pelE* (Reverchon *et al* 1986, Kotoujansky 1987). Each *pel* gene is transcribed as an individual unit. Due to the gene organization and sequence homologies, it has been proposed that the genes for the two neutral, and two alkaline isozymes arose by gene duplication (Kotoujansky 1987). The minor protein bands with Pel activity identified by IEF, are probably not true Pels, but are produced from the major isozymes by proteolytic degradation.

Ecc and *Eca* synthesize fewer Pel isozymes than *Ech* and there is less variation between strains. The four *Ecc* Pels are named Pel A-D (Kotoujansky 1987, Hinton *et al* 1989). Pels A and B are neutral (pI 7-8) and are localized in the periplasm (Hinton *et al* 1989). This is interesting, since no periplasmic Pels have been identified in *Ech*. The *Ecc* Pels C and D are alkaline (pI 10-11) and extracellular. A third alkaline protein identified by IEF, which shows Pel activity, is thought to be a breakdown product of PelD. Little is known about the genetic organization of the *Ecc pel* genes, but *pelC* and *pelD* are thought to map together, while *pelA* and *pelB* are not linked to each other, or to *pelCD* (Hinton *et al*, unpublished).

Several *pel* genes have been cloned and sequenced. Alignment studies (Hinton *et al* 1989) suggest that there are three distinct families of Pel proteins. The PelBC family comprises extracellular Pels of *Ecc*, *Eca* and *Ech*. The PelADE family contains

extracellular Pels from *Ech* only. The third family includes intracellular Pels from *Ecc* and *Yersinia pseudotuberculosis* (see Hinton *et al* 1989).

Although categorized into different families, the twelve Pel sequences compared, all showed some homology (Hinton *et al* 1989). The consensus regions may be involved in secretion or catalytic activity.

There is uncertainty about whether all the Pels are required for virulence. Bacteria do not normally produce isozymes. Possible reasons for the production of Pel isozymes by *Erwinia* spp. include adaptation for different host pectates, or a strategy to saturate host defences (Kotoujansky 1987). All the *Ech* Pel isozymes have been detected in infected plant tissue. This is not the case for the *Ecc* Pels, but this might simply reflect a variation in their abundances (Kotoujansky 1987). For *Ech*, the alkaline isozymes attack PGA more randomly, and have a more destructive effect on plant tissue than the neutral isozymes (Keen *et al* 1984, Collmer *et al* 1985, Keen and Tamaki 1986). PelA seems to be extremely inefficient (Tamaki *et al* 1986), although it is essential for full pathogenicity (Boccora *et al* 1988). The importance of the various Pels in virulence seems to be affected by the host plant, and even by the part of the plant. The induction of the different Pels may vary, as do the temperatures for optimum activity: PelA being necessary for soft-rot symptoms at higher temperatures (Favey *et al* 1992). Differences have also been reported in the thermal stabilities of the Pels of *Ecc* and *Eca* (Hinton *et al* 1989). Little is known about the function of the *Ecc* periplasmic Pels. They may degrade galacturonate oligomers produced by extracellular Pels and transported into the periplasm. Alternatively, these enzymes might be secreted *in planta*, although sequence data do not support this idea (Hinton *et al* 1989).

1.2.2.2 PECTIN LYASE.

Pectin lyase (Pnl: EC 4.2.2.10), produced by most of the soft-rot erwinias, cleaves pectin, but not pectate (Tsuyumu and Chatterjee 1984). Although Pnl isolated from fungi is capable of macerating plant tissue, the enzyme from *Erwinia* spp. does not seem able to do this.

1.2.2.3 PECTIN METHYLESTERASE.

Pectin methylesterase (Pem: EC 3.1.1.11) de-esterifies pectin to yield PGA and methanol (Collmer *et al* 1982). The exact role of Pem is not known, although *Ech* Pem⁻ mutants have slightly reduced virulence on saintpaulia (Boccora and Chatain 1989). Certainly, the activity of Pem on partially methylated substrate is extremely low, compared to that of Pem.

1.2.2.4 POLYGALACTURONASE.

Polygalacturonase (Peh) hydrolyses internal α -1,4-glycosidic bonds in pectic polymers to generate galacturonic acid oligomers. In contrast to Pem, the enzyme is specific for pectate, showing no activity on partially esterified pectin (Nasuno and Starr 1966), and is not dependent on calcium ions, functioning in the presence of EDTA. Peh exists in two forms: the endo-form (EC 3.2.1.15) which cleaves randomly within substrate chains, and the exo-form (EC 3.2.1.82) which cleaves penultimate bonds of PGA chains to produce digalacturonic acid (DGA).

Endo-Peh has not been found in *Ech*, but an *Ech* gene for exo-Peh has been cloned and sequenced (He and Collmer 1990). Exo-Peh is thought to be involved in the induction of Pel synthesis (Collmer *et al* 1982).

Unlike *Ech*, *Ecc* and *Eca* produce and secrete endo-Peh, and genes for this have been cloned and sequenced (Lei *et al* 1985, Plastow *et al* 1986, Willis *et al* 1987, Hinton *et al* 1990, Saarilahti *et al* 1990a). The importance of endo-Peh in virulence is not known.

It was thought that *Ecc* has only one *peh* gene (Plastow *et al* 1986, Hinton *et al* 1990), but this study (7.3, 7.4) has suggested that this is not the case, and that *Ecc* SCRI193 produces exo-Peh.

1.2.3 CELLULASES.

Cellulase (Cel) breaks down cellulose polymers (β ,1-4 linked glucose). There are two types: exo- and endo-gluconases, the latter enzyme (EC 3.2.1.4) being produced by *Ech* and *Ecc*. *Ech* synthesizes two immunologically distinct endo-gluconases (CelZ

and CelY), with CelZ accounting for 80% of the total activity (Kotoujansky 1987). The minor enzyme, CelY, is thought to be periplasmic. Two Cels have been identified from different strains of *Ecc*. These enzymes: CelS (Saarilahti *et al* 1990b) and CelV (Cooper and Salmond, unpublished), are not related to each other, although CelV is homologous to the *Ech* CelZ, and a homologue is also thought to exist in *Eca* (Ellard and Salmond, unpublished). The *Ech cel* genes are thought to be constitutively expressed and subject to catabolite repression (Boyer *et al* 1984, Chambost 1986).

Endo-gluconase degrades cellulose derivatives such as carboxymethyl cellulose (CMC), to yield cellobiose. *Ech* is unable to attack crystalline cellulose, probably because it lacks exo-gluconase activity (Chambost *et al* 1897), and the importance of Cel in pathogenicity is not known.

1.2.4 PROTEASES.

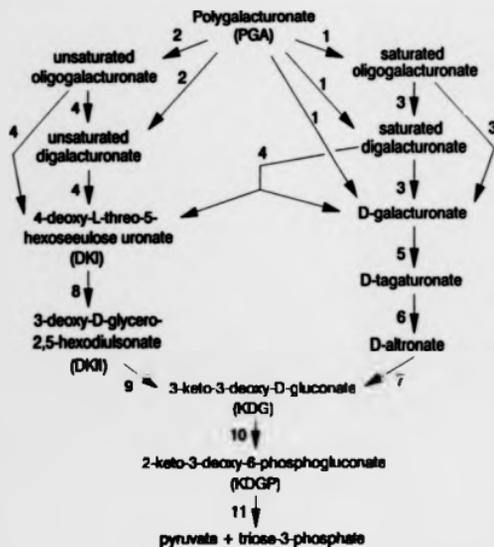
All the soft-rot erwinias produce multiple forms of extracellular protease (Prt). These may attack plant cell wall glycoproteins, or proteins involved in host defence. Although the *prt* genes of several *Erwinia* spp. have been cloned, there has been little interest in the biochemistry, or role in pathogenicity of the enzymes. Instead, most of the work has centred around the Prt secretion mechanism, which differs from that of Pel and Cel.

1.3 PECTOLYTIC ENZYME REGULATION.

1.3.1 THE IMPORTANCE OF REGULATION.

Comparisons between pathogenic and non-pathogenic pectolytic bacteria have suggested that the crucial factor, determining the ability to cause disease, is the rate of pectolytic enzyme synthesis and secretion (Zucker and Hankin 1970, Zucker *et al* 1972). A knowledge of the regulation of pectolytic enzymes in *Erwinia* spp. is therefore vital for the understanding of pathogenicity.

Figure 1.3.1a Major Pathways of PGA Catabolism



Enzymes

- 1: polygalacturonase (Pah)
- 2: pectate lyase (Pel)
- 3: oligogalacturonate hydrolase
- 4: oligogalacturonate lyase (Ogl)
- 5: uronate isomerase
- 6: D-altronate oxidoreductase
- 7: D-altronate hydrolase
- 8: 4-deoxy-L-threo-5-hexoseulose uronate dehydrogenase
- 9: 3-deoxy-D-glycero-2,5-hexodulsonate dehydrogenase
- 10: 2-keto-3-deoxygluconate kinase
- 11: 2-keto-3-deoxy-6-phosphogluconate aldolase

Adapted from Chatterjee *et al* (1985)

Regulation is complex and occurs at many levels. Some of the mechanisms described below are involved in the co-ordinated regulation of different enzymes of the pectolytic catabolite pathway. So that these may be more easily understood, the major bacterial pathways of PGA catabolism are summarized in figure 1.3.1a.

1.3.2 INDUCTION OF PEL SYNTHESIS.

The rate of Pel synthesis by *Erwinia* spp. in culture increases in response to isolated cell walls, PGA or oligogalacturonates (Zucker and Hankin 1970, Tsuyumu 1977, Chatterjee *et al* 1979, 1981, Collmer and Bateman 1981, 1982). The true inducing molecules are the breakdown products of PGA: KDG, DKII and DKI (see figure 1.3.1a), (Hugouvieux-Cotte-Pattat and Robert-Baudouy 1985, 1987, Condemine *et al* 1986, Condemine and Robert-Baudouy 1987). It is thought that a basal level of extracellular pectic enzymes breaks down PGA to yield oligogalacturonates (in particular dimers), which are transported into the cell and broken down further, to yield the inducing molecules (Tsuyumu 1977, Collmer and Bateman 1981, 1982).

Most of the research into Pel induction has been carried out in *Ech*, using gene fusions to study the expression of individual *pel* genes (Diolez and Coleno 1985, Hugouvieux-Cotte-Pattat and Robert-Baudouy 1985, Hugouvieux-Cotte-Pattat *et al* 1986, Reverchon and Robert-Baudouy 1987).

In *Ech*, induction by PGA varies for the different Pel isozymes (and possibly for different strains), with reports of a 37-fold increase in *pelC* expression in *Ech* 3937 (Diolez and Coleno 1985) and a 3-6 fold increase for *pelADE* in *Ech* B374 (Reverchon and Robert-Baudouy 1987). Inducers of *pel* genes also induce the other genes of the pectolytic pathway (see figure 1.3.1a). Greater, and more rapid induction occurs with DGA rather than PGA (Collmer and Bateman 1982). The key factors for induction in *Ech* are thought to be the relatively high basal levels of extracellular pectic enzymes, and the great sensitivity of the system, which means that $< 1 \mu\text{M}$ DGA can be effective (Collmer *et al* 1982).

The synthesis of Pel by *Ecc* is also induced by breakdown products of PGA (Tsuyumu 1977). There is great variation between strains, with the increase in *pel* expression ranging from 3.6-fold for *Ecc* 153 (Chatterjee *et al* 1979), to 32-fold for *Ecc* ATCC8061 (Zucker *et al* 1972). As with *Ech*, DGA is a better inducer than PGA (Tsuyumu 1977, Chatterjee *et al* 1979) and the true inducing molecules are thought to be DGA catabolites.

The production of Pel by different strains of *Erwinia carotovora* and non-pathogenic *Pseudomonas spp.* has been investigated. It was found that *Erwinia spp.*, unlike *Pseudomonas spp.*, produced some Pel constitutively, which would degrade PGA to cause induction. Although Pel synthesis was inducible in *Pseudomonas spp.*, a lag of several generation times occurred before maximal induction was achieved. No such lag was observed with *Erwinia spp.* Since wounded plant tissue is normally rapidly protected by the formation of an impermeable, protective layer, Pel induction in *Pseudomonas spp.* would occur too late. In addition to this, the increase in Pel synthesis upon induction was far lower in the non-pathogenic species (Zucker and Hankin 1970, Zucker *et al* 1972). The ability to induce Pel synthesis is therefore vital for pathogenicity, and accounts for why only some pectolytic bacteria cause soft-rot.

1.3.3 CATABOLITE REPRESSION OF PEL.

The synthesis of Pel by *Erwinia spp.* is subject to catabolite repression (Moran and Starr 1969), being reduced in the presence of a carbon source such as glucose, which is more easily utilized than PGA. In addition, 'self-catabolite' repression occurs, in the presence of high concentrations of inducing molecules (Tsuyumu 1979, Collmer and Bateman 1981).

Both types of catabolite repression are overcome by the addition of cAMP. Sequences homologous to the catabolite-activator-protein binding sequence of *E. coli* have been identified in the promoter regions of *pelB* and *peLE* (Keen and Tamaki 1986).

A class of *Ech* mutants (*crf*) have been isolated, which are resistant to catabolite repression (Hugouvieux-Cotte-Pattat *et al* 1986, Reverchon and Robert-Baudouy 1987).

The *crl* gene is not specific for the regulation of Pel, since it also affects the expression of Cel and Prt (Hugouvieux-Cotte-Pattat *et al* 1986).

1.3.4 NEGATIVE CONTROL OF PEL BY KDGR.

Kdgr is a repressor protein which exerts its effect by binding to a consensus DNA target sequence (Kdgr box). Kdgr boxes have been identified in *Ech* (Reverchon *et al* 1989, Condemine and Robert-Baudouy 1991), located between promoter regions and start codons of certain genes, suggesting that Kdgr binding: shown to occur *in vivo* (Reverchon *et al* 1991) may prevent transcription through steric hindrance.

The *Ech kdgR* gene has been cloned and sequenced (Reverchon *et al* 1991) and the protein has been overexpressed and purified (Nasser *et al* 1992). Gel retardation studies have shown that *Ech* and *E. coli* Kdgr proteins are not interchangeable. Kdgr is released from its binding site by KDG, but not by galacturonate (Nasser *et al* 1992), suggesting that KDG, the true inducing molecule, exerts its effect via Kdgr. Kdgr is thought to be involved in the co-ordinate regulation of all the *Ech* genes involved in pectin degradation (Hugouvieux-Cotte-Pattat *et al* 1986, Reverchon *et al* 1989). Variations in the affinity of Kdgr for different operators may account for the differential regulation of pectolysis genes (Nasser *et al* 1992).

Kdgr-mediated repression may play a similar role in *Ecc*, since Kdgr boxes have been found upstream of *Ecc pel* genes (Hinton *et al* 1989).

Kdgr⁻ mutants of *Ech* show high constitutive activities of Pel and the other enzymes of the pectolytic pathway (Hugouvieux-Cotte-Pattat *et al* 1986, Condemine and Robert-Baudouy 1987), although Cel is not affected. The level of constitutive Pel activity in the Kdgr⁻ strain varies for the different Pel isozymes. The findings suggest that *pelB* and *pelC* are weakly regulated by Kdgr and remain inducible in the absence of the repressor, while *pelADE* expression is almost optimal in the Kdgr⁻ strain without induction (Condemine and Robert-Baudouy 1987).

1.3.5 GROWTH PHASE REGULATION IN *ECH*.

In *Ech*, the synthesis of extracellular enzymes is dependent on growth phase. Enzyme production in liquid culture is very low until the end of exponential growth, when synthesis increases sharply (Collmer *et al* 1982, Hugouvieux-Cotte-Pattat *et al* 1986). In contrast, levels of Pel in *Ecc* increase in line with cell density (Moran and Starr 1969, Zucker and Hankin 1970).

Growth phase independent (*gpi*) mutants of *Ech* have been isolated, in which Pel is constitutively expressed at a high level, and production follows the growth curve (Hugouvieux-Cotte-Pattat *et al* 1986). Only the PelA,D,E isozymes were found to be constitutively expressed in *gpi* mutants, indicating that not all Pel isozymes are equally affected by growth phase in wild-type cells (Hugouvieux-Cotte-Pattat *et al* 1986). No enzymes other than Pel were affected in *gpi* mutants.

Gpi mutations are not linked to *pel* genes. The *gpiR* gene product may control a mechanism which causes cells to synthesize Pel when conditions do not favour growth. This mechanism is separate from catabolite repression.

The inactivation of GpiR meant that cells were no longer growth phase dependent, and produced Pel constitutively, suggesting that the regulator has a dual function (Hugouvieux-Cotte-Pattat *et al* 1986). The *gpiR* product may be a negative regulator with two effectors: one in PGA catabolism, the other present in growth-limiting conditions.

1.3.6 OTHER PEL REGULATORS.

Work on *Ech* has identified several regulatory genes, the precise roles of which are not yet known. These are discussed briefly below.

1.3.6.1 *PECA*.

Mutation of *pecaA* caused a decrease in the expression of all *Ech pel* genes, which were no longer inducible (Kotoujansky 1897). The *pecaA* product may be a positive regulator.

1.3.6.2 *PECL*.

The levels of Pel (all isozymes) and Pem were increased under inducing conditions when *pecl* was mutated (Hugouvieux-Cotte-Pattat and Robert-Baudouy 1989), suggesting that the gene encodes a negative regulator. The mutations had no effect on the synthesis of Pel or Pem under non-inducing conditions. The *pecl* gene is closely linked to the *pelD* and *pem* genes, and is itself induced by PGA and DGA.

1.3.6.3 *PECL*.

The *pecl* gene product is thought to be an activator of Pel, Cel and Pem, since mutating the gene results in very low levels of synthesis of these enzymes, with all the Pel isozymes being equally affected (Hugouvieux-Cotte-Pattat and Robert-Baudouy 1989). Mutations in *pecl* (which maps close to *pelBC*), are thought to suppress induction.

1.3.6.4 *PECN*.

The *pecN* gene product may be an activator, which is specific for Pel (Kotoujansky 1987).

1.3.6.5 *PECR*.

Mutation of *pecR* led to high levels of PelA synthesis in the absence of inducer (Reverchon and Robert-Baudouy 1987). *PecR* may therefore be a repressor which is specific for *pelA*. The regulation of *pelA* expression is not understood, although KdgR and GpiR are involved. Induction of PelA may only occur *in planta*.

1.3.6.6 *PECS*.

The *pecS* gene product specifically represses the *pel* genes (Reverchon *et al* 1990).

1.3.6.7 *PECY*.

The *pecY* gene (mapping between *pelD* and *pem*), encodes a protein involved in the regulation of Pem synthesis. Mutation of *pecY* causes an increased expression of *pem* (Boccarda and Chatain 1989). The negative regulator is only thought to function in the absence of inducer.

1.3.7 REGULATION OF PNL.

The regulation of Pnl in both *Ech* and *Ecc* is very different from that of Pel, with no induction occurring in the presence of pectic substances (Tsuyumu and Chatterjee 1984). Pnl is thought to be co-ordinately regulated with SOS functions: being induced by nalidixic acid, bleomycin, mitomycin C and UV irradiation. These factors also cause cell lysis, which is thought to be due to the induction of the bacteriocin carotovoricin, or of temperate phages. The link between Pnl induction and the SOS response is supported by the observation that induction does not occur in RecA⁻ strains (Zink *et al* 1985, McEvoy *et al* 1990).

Pnl, which is induced *in planta* (Tsuyumu *et al* 1985), may be involved in overcoming host plant defences. DNA damaging agents have been detected in plant tissue, and the concentration of these increases greatly upon infection (Tsuyumu *et al* 1985). Pnl may help pathogens to disperse from areas containing high concentrations of DNA-damaging agents. However, the enzyme is not inducible in several soft-rot *Erwinia* spp.

Clearly, the regulation of pectolytic enzymes is extremely complex, probably involving a cascade of events.

1.3.8 GLOBAL REGULATION.

In addition to the regulatory mechanisms described above, the soft-rot erwinias possess a system which controls the synthesis (and to a lesser extent secretion), of all the extracellular enzymes. Mutants which are deficient for this global regulatory system have several names: *sex* mutants (synthesis of exozymes) (Stephens *et al* unpublished), *Exp⁻* (Pirhonen *et al* 1991) and *Aep⁻* (Murata and Chatterjee 1990). Sequence analysis of the genes involved in global regulation has not so far revealed significant homology to any other proteins (Stephens *et al* unpublished). Temperature is known to affect the synthesis of all the extracellular enzymes (Hinton and Salmond 1987, Lanham *et al* 1991), although it is not yet known whether this effect is mediated by the *sex* genes.

1.4 SECRETION BY GRAM NEGATIVE BACTERIA: AN INTRODUCTION.

Both Gram positive and Gram negative bacteria secrete proteins such as enzymes and toxins into their environment. The Gram negative cell envelope constitutes a considerable barrier for the passage of proteins, which must cross the periplasm and outer membrane, in addition to the inner membrane, meaning that proteins exported from the cytoplasm are rarely released into the medium. Figure 1.4a shows a schematic representation of the Gram negative cell envelope. The outer membrane, which provides protection against potentially harmful factors, is a structurally unique, asymmetric bilayer. The inner layer of this is composed of phospholipid molecules, while the outer layer is predominantly LPS. The periplasm contains proteins, peptidoglycan and lipids (Hancock 1991). The constraints imposed by such a barrier mean that there are relatively few examples of secretion by Gram negative, as opposed to Gram positive bacteria.

There is some confusion with the terminology used to describe the transport of proteins across the cell envelope. For the purpose of this discussion, movement from the cytoplasm to the periplasm is defined as 'export'. The term 'secretion' refers only to release into the medium, by translocation across the outer membrane.

The two main types of secretion by Gram negative bacteria: the one-step and two-step pathways, are described below.

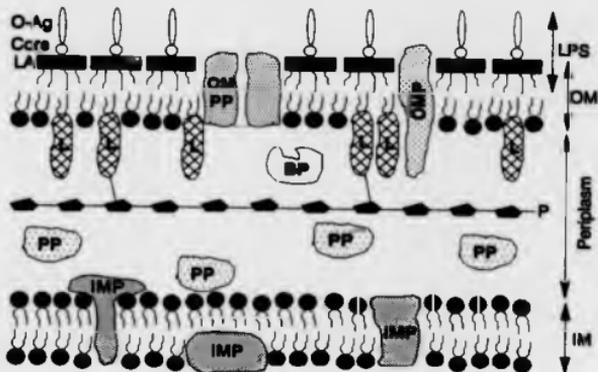
1.5 TWO-STEP SECRETION.

1.5.1 INTRODUCTION.

Examples of Gram negative two-step secretion include aerolysin, protease and amylase by *Aeromonas hydrophila* (Jiang and Howard 1991), pullulanase by *Klebsiella* spp. (Pugsley *et al* 1990a), various enzymes by *Xanthomonas campestris* (Dow *et al*

Figure 1.4a

The Gram Negative Cell Envelope

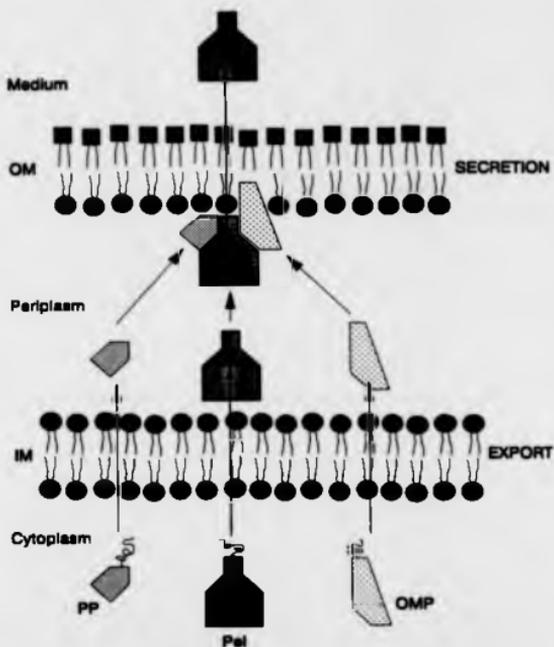


Key

- | | |
|----------------------------|-------------------------------|
| LPS: Lipopolysaccharide | OM: Outer membrane |
| O-Ag: O-Antigen | OMP: OM protein |
| Core: Core oligosaccharide | OMPP: OM Pore-forming protein |
| LA: Lipid A | IM: Inner membrane |
| L: Lipoprotein | IMP: IM protein |
| BP: Binding protein | PP: Periplasmic protein |
| P: Peptidoglycan | |

Redrawn from Reeves (1991)

Figure 1.5.1a The Two-Step Secretion Pathway



IM: Inner membrane OMP: OM protein Pel: eg of a protein secreted
 OM: Outer membrane PP: Pariplasmic protein by the two-step pathway

The diagram is highly simplified: it is now known that inner membrane proteins are also involved in the secretion step.

1987, Dums *et al* 1991) and *Pseudomonas aeruginosa* (Bally *et al* 1989, 1991), and pectinase and cellulase by *Erwinia* spp. (Andro *et al* 1984).

The two-step model for secretion proposes that the protein is first exported across the inner membrane to the periplasm: a step which usually involves the cleavage of an N-terminal signal sequence. The second step: secretion across the outer membrane, need not involve proteolytic cleavage, and is thought to require an interaction between domains of the exoprotein and proteins of the cell envelope.

The two-step secretion pathway is represented in figure 1.5.1a. The processes of export and secretion, together with evidence to support the model, are discussed below.

1.5.2 EXPORT.

The export of a polypeptide across the inner membrane, involving interaction with various Sec proteins and the cleavage of an N-terminal signal peptide, has been best characterized in *E. coli*. The major components of the Sec-dependent export apparatus are shown in figure 1.5.2a.

An exported protein is synthesized as a precursor, which has an N-terminal leader (signal) sequence. A typical signal sequence is 16-24 amino acid residues long, and consists of a basic N-terminal region and a non-helical C-terminal region. Conserved features at the C-terminal end of the leader sequence are not essential for translocation, but are required for processing by leader peptidase (1.5.2.5).

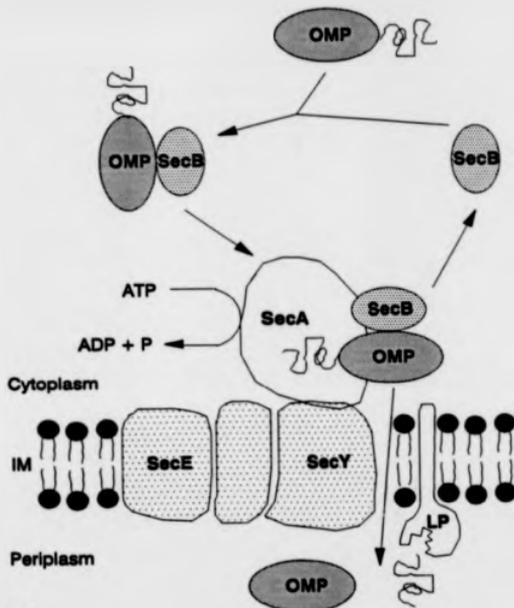
The Sec proteins, essential for export, are discussed below.

1.5.2.1 SECB.

SecB is a cytosolic 'chaperon' protein which binds to the mature domain of a protein precursor: stabilizing the protein and preventing non-productive folding, aggregation, or interaction with other proteins (Wickner *et al* 1991). Although GroEL is more abundant, SecB is the major chaperon for preprotein export as it has a second role: targeting precursors to the receptor protein SecA (Hartl *et al* 1990). However, SecB is not usually essential for Sec-dependent protein export (Bankaitis *et al* 1986), only being necessary for the export of certain proteins, such as LamB. The effects of

Figure 1.5.2a

The Sec-Dependent Export Pathway



Key

IM: Inner membrane

OMP: Outer membrane protein

LP: Leader peptidase

Adapted from Hartl *et al.* (1990)

secB mutations may be alleviated by induction of the heat shock response (Altman *et al* 1991), although SecB itself is not a heat shock protein. The precise nature of the protein able to substitute for the SecB function remains to be discovered.

1.5.2.2 SECA.

SecA acts as a receptor for the SecB/preprotein complex, having a high affinity for SecB (Hartl *et al* 1990), mature domains of precursor proteins (Lill *et al* 1990), and leader peptides. SecA, which has at least three ATP binding sites, hydrolyses ATP to provide energy for translocation (Lill *et al* 1989, Swidersky *et al* 1990), although a membrane electrochemical potential is also required. The export of a preprotein across the membrane requires many cycles of SecA binding, ATP hydrolysis, limited translocation and rebinding (Schiebel *et al* 1991).

SecA is thought to form the peripheral domain of the membrane 'translocase' complex, the integral part of which consists of the SecY/E protein. However, SecA may also exist in the cytoplasm, or associated with ribosomes (Liebke 1987, Cabelli *et al* 1991). It is possible that SecA interacts with the SecB/preprotein complex while in solution. The association of SecA with the membrane, requires SecY/E and anionic phospholipids (Hendrick and Wickner 1991).

1.5.2.3 SECY/E.

SecY/E is an integral membrane protein consisting of three subunits: SecY (Ito *et al* 1983, 1984), SecE (Riggs *et al* 1988, Schatz *et al* 1989) and a third, uncharacterized polypeptide (Brundage *et al* 1990), although this is not thought to be essential for translocation (Akimaru *et al* 1991).

SecE and SecY are both integral transmembrane proteins (Akiyama and Ito 1987, Schatz *et al* 1989). They are exceptionally hydrophobic and may exist as oligomers. The C-terminal region of SecE is required for the interaction of the two proteins (Nishiyama *et al* 1992). SecY/E is thought to bind SecA (Hartl *et al* 1990) and interact directly with the leader peptide during translocation. The preprotein may be conducted through the centre, or along the surface of SecY/E, perhaps using energy, transferred

from SecA (Wickner *et al* 1991). Alternatively, the energy from ATP may be used by SecA to actively unfold preproteins (Wickner *et al* 1991).

1.5.2.4 SECD/SECF.

SecD and SecE are also integral proteins of the cytoplasmic membrane, involved in protein translocation. The *secD* and *secE* genes form an operon and are co-transcribed (Gardel *et al* 1990). Sequence analysis has shown that the two proteins are homologous, and it has been suggested that they form a complex, possibly with another protein (Gardel *et al* 1990).

SecD and SecE are associated with the periplasmic face of the inner membrane (Gardel *et al* 1990). They may catalyse protein folding, or clear the periplasmic surface of the SecY/E protein, allowing further rounds of translocation (Wickner *et al* 1991).

1.5.2.5 CLEAVAGE OF LEADER SEQUENCES.

Leader peptides are thought to allow preproteins to interact with SecB by retarding folding. The signal sequence is then recognized by SecA and SecY, and so directs translocation. Once the polypeptide has reached the periplasm, the signal sequence is no longer required, and is usually removed by a peptidase. Peptidases: leader peptidase I and leader peptidase II (lipoprotein peptidase) recognize specific amino acid residues (discussed in more detail later: 6.4.3, 7.4.1). Leader peptidase is an integral membrane protein, with its catalytic site in the periplasmic domain. Since it is anchored to the membrane, the enzyme is able to cleave preprotein target sequences as they emerge through the membrane (Dalbey 1991).

The function of cleavage is not known. Processing is not normally required to allow the exported protein to adopt its active conformation, and it is not thought to make translocation irreversible. Leader sequences may retard folding, making proteins susceptible to proteolytic attack. Processing might then reduce the time taken for a protein to achieve a stable conformation. The primary function of cleavage is thought to be the release of polypeptides from the inner membrane, so that they may be properly localized. The exceptions are proteins which remain attached to the membrane: anchored by uncleaved signal sequences.

1.5.2.6 SORTING OF EXPORTED PROTEINS.

In *E. coli*, exported proteins either remain in the periplasm, or go to the outer membrane. The information required for this sorting is carried in the sequence of the mature protein (Benson *et al* 1984). In Gram negative bacteria, such as *Erwinia* spp., which are capable of secreting certain proteins from the periplasm to the culture medium, the situation is more complicated. The second step of secretion, which involves several accessory proteins, is discussed in later sections (1.7, 1.8).

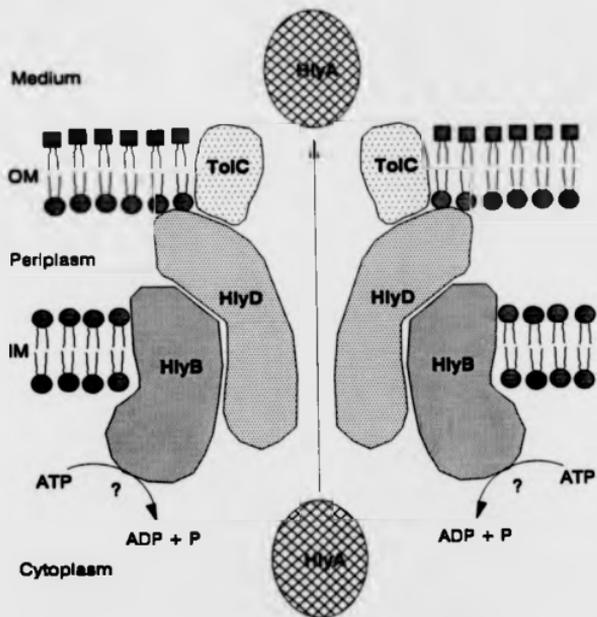
1.6 ONE-STEP SECRETION.

The one-step pathway allows a protein to be moved from the bacterial cytoplasm to the culture medium in a single step, through 'pores': fusion zones between the inner and outer membranes (Lory *et al* 1983). Most of the proteins secreted by this pathway are highly homologous. Examples include *Pasteurella haemolytica* leukotoxin (Strathdee and Lo 1989), *Bordetella pertusis* adenylyl cyclase-haemolysin (Glaser *et al* 1988), colicin V: secreted by *E. coli* (Gilson *et al* 1990), *Actinobacillus pleuropneumoniae* (Gygi *et al* 1990), *Morganella morganii* (Koronakis *et al* 1987) and *Proteus vulgaris* (Koronakis *et al* 1988). The best characterized example is the secretion of α -haemolysin by *E. coli* (discussed below). Other proteins, secreted by the same pathway, but not homologous to α -haemolysin are *Serratia marcescens* metalloprotease (Nakahama *et al* 1986), *Pseudomonas aeruginosa* alkaline protease (Guzzo *et al* 1991) and *Erwinia* spp. proteases (Letoffe *et al* 1990, 1991). The rest of this section will concentrate on the secretion of α -haemolysin by *E. coli*.

α -haemolysin does not cross the membrane via the general export pathway. The protein lacks an N-terminal signal sequence and is not subject to proteolytic cleavage (Hartlein *et al* 1983). Secretion is independent of the Sec apparatus (Holland *et al* 1990, Gentschev *et al* 1990), and does not proceed via a periplasmic intermediate (Gray *et al* 1986, Koronakis *et al* 1989). Figure 1.6a shows the components of the one-step secretory apparatus.

Figure 1.6a

Apparatus for the One-Step Secretion of Haemolysin



The structural gene for α -haemolysin (*hlyA*), and the genes encoding the secretory proteins (*hlyB, C, D*) map to a contiguous 7.5 Kb fragment of DNA and are co-expressed (Noegel *et al* 1979, Hartlein *et al* 1983, Welch *et al* 1983, Mackman *et al* 1985).

The HlyA protein is post-translationally modified by the cytoplasmic protein HlyC: a step which is required for α -haemolysin toxicity, but not for secretion (Nicaud *et al* 1985). Secretion is then directed by a 'signal sequence' at the C-terminus of HlyA (Nicaud *et al* 1986), which interacts with the HlyB and HlyD proteins. The C-terminal 'signal sequence' is 113 amino acid residues long, and may be analogous to the presequences required for import into mitochondria (Koronakis *et al* 1989). Secretion does not require the entire signal sequence. It has been proposed that the final 27 residues are essential (Koronakis *et al* 1989). However, various point mutations in this region had little effect on secretion and it was found that just 4 residues, dispersed throughout the last 46, are vital for optimal secretion (Kenny *et al* 1992). These 'critical contact' residues are believed to be required for the interaction between HlyA and the membrane translocator. Fusion proteins have been generated, in which the HlyA C-terminal 'signal sequence' directs the secretion of other proteins such as LacZ (Kenny *et al* 1991). LacZ cannot normally be secreted, as it folds into a secretion-incompetent conformation in the cytoplasm. The ability of the HlyA 'signal sequence' to secrete LacZ therefore suggests that HlyA may be folded prior to secretion.

Secretion is thought to involve direct interaction between the HlyA 'signal sequence', HlyB and possibly HlyD. This may require 'spacers' in HlyA to maintain a protein conformation in which the signal is optimally exposed for interaction with HlyB (Kenny *et al* 1991).

Gene fusion and cellular fractionation studies have shown HlyB and HlyD to be associated with the inner membrane (Mackman *et al* 1985, Wang *et al* 1991), although unlike classical inner membrane proteins, they also fractionate to the outer membrane in small amounts (Wang *et al* 1991). Topology studies using TnlacZ and TnphaA have suggested that HlyB has eight membrane-spanning domains (Wang *et al* 1991,

Gentschev and Goebel 1992), while HlyD has only one (Wang *et al* 1991). The two proteins are thought to interact, to form a transmembrane channel.

HlyB, having ATP-binding domains (Higgins *et al* 1986, Gerlach *et al* 1986), is thought to provide energy for translocation, although an electrochemical gradient across the membrane is also required during the passage of the C-terminal 'signal sequence' (Koronakis *et al* 1991). HlyB belongs to a group of bacterial transport proteins (all of which bind ATP), which transport a diverse range of molecules including polypeptides and polysaccharides (Blight and Holland 1990). HlyD homologues have so far only been identified in systems which transport large polypeptides, suggesting that this protein might determine specificity (Blight and Holland 1990).

In addition to the Hly proteins, TolC, a minor outer membrane protein is specifically required for α -haemolysin secretion (Wandersman and Deleplaire 1990). TolC may allow the HlyB/HlyD complex to interact with the outer membrane (Wandersman and Deleplaire 1990).

It is proposed that the proteins described above, interact to create a 'revolving door', in which conformational changes in the translocator result in the movement of HlyA across the membrane (Blight and Holland 1990).

There are some differences between the machinery for HlyA secretion and other related systems. The secretion of metalloproteases (PrtB and PrtC) by *Ech* requires three proteins: PrtD, PrtE and PrtF, homologous to HlyB, HlyD and TolC respectively (Letoffe *et al* 1990). These are encoded by genes which map upstream of the structural *prt* genes, and have their own promoters, meaning that regulation must differ from that of the *E. coli hly* genes (Letoffe *et al* 1990).

Only limited cross complementation occurs between the different one-step secretory apparatus. The *Ech* Prt export machinery is capable of secreting metalloprotease from *Serratia* sp., unlike the *E. coli* α -haemolysin machinery (Letoffe *et al* 1990). The specificity of the systems is thought to act at the level of recognition of the C-terminal 'signal sequence' by the translocator (Wang *et al* 1991).

1.7 EXTRACELLULAR ENZYME SECRETION BY *ERWINIA* SPP.

1.7.1 ONE-STEP OR TWO-STEP SECRETION?

The first evidence of a specific secretory system in *Erwinia* spp. was obtained by fractionating *Ech* cultures and assaying for enzyme activity (Andro *et al* 1984). Pel and Cel were found to be extracellular, while the marker enzymes Bla and LacZ were localized in the periplasm and cytoplasm respectively, showing that lysis had not occurred. Similar results were later obtained to show that Peh and Prt are also specifically secreted (Wandersman *et al* 1986).

The study of secretion by *Erwinia* spp. is based on the analysis of various mutants. Mutagenesis (chemical and insertional) has produced a range of mutants, defective for extracellular enzymes (Andro *et al* 1984, Thurn and Chatterjee 1985, Salmond *et al* 1986, Hinton and Salmond 1987). An interesting class of mutants (termed Out⁻) synthesize, but do not secrete Pel and Cel (Andro *et al* 1984). The Pel⁻Cel⁻ phenotype of Out⁻ mutants shows that both enzymes are secreted by the same pathway. Since the secretion of Prt is not affected in Out⁻ mutants (Andro *et al* 1984), this must occur via a different pathway.

Prt secretion is independent of the general export (Sec-dependent) pathway. The *Erwinia* spp. Prt enzymes are synthesized without N-terminal signal sequences, and are thought, like α -haemolysin, to have C-terminal sequences required for secretion (Delepelaire and Wandersman 1990). Sequence analysis revealed homologies between proteins involved in the secretion of *Ech* Prt, and of *E. coli* HlyA (Letoffe *et al* 1990). All the evidence suggests that *Erwinia* spp. secrete Prt by the one-step mechanism.

The activity of Pel, Cel and Peh accumulates in the periplasm of Out⁻ mutants (Andro *et al* 1984, Thurn and Chatterjee 1985). This suggests that secretion normally occurs via the periplasm, although it must be considered that mutations might cause a re-routing to the periplasm. However, there is other evidence for a secretory route via the periplasm. A transient build-up of Pel and Cel has been detected in the periplasm of wild-type *Ech* cells early in the growth curve: before secretion occurs (Andro *et al*

1984, Ji *et al* 1987). The existence of periplasmic intermediates of Pel, Cel and Peh suggests that secretion is via the two-step pathway.

Pulse-chase labelling showed that in *Ech*, Pel is processed and localized in the periplasm, prior to being translocated across the outer membrane. Almost all of the Pel synthesized, was secreted into the supernatant within 2 min (He *et al* 1991b). Overexpression of Pel led to an accumulation of the enzyme in the periplasm (He *et al* 1991b). This not only supported the two-step hypothesis, but also showed that the apparatus for secretion across the outer membrane of *Ech* is saturable.

Genes for the production and secretion (*owt*) of Pel from *Ech* (see 1.7.2) were introduced into a strain of *E. coli* which was a temperature sensitive (Ts) Sec mutant (He *et al* 1991b). At the non-permissive (but not the permissive) temperature, pre-Pel accumulated inside the cell, independent of the presence of *owt* genes. This proved that Pel is exported across the inner membrane via the Sec-dependent pathway, and that Pel-processing is dependent on Sec, rather than Out proteins. Entry to the Out pathway is therefore thought to occur only after Sec-dependent export.

The structural genes for Pel from *Ech* and *Ecc* have been cloned into *E. coli*. The enzymes were synthesized, exported and processed as in *Erwinia* spp., but then remained in the periplasm (Keen *et al* 1984, Collmer *et al* 1985, Thurn and Chatterjee 1985, Zink and Chatterjee 1985, Ji *et al* 1987). These results support the hypothesis that the first step in Pel secretion uses the general export pathway present in *E. coli*, while showing that *E. coli* is not capable of directing Pel to the supernatant. The existence of a secretory mechanism in *Erwinia* spp. which is not present in *E. coli* makes these bacteria important for the study of Gram negative secretion.

1.7.2 THE *OUT* GENES OF *ERWINIA* SPP.

Ecc owt genes were identified by complementing *Out*⁻ mutants to the *Out*⁺ phenotype, using a wild-type *Ecc* library. The complementing DNA fragments, shown to overlap by restriction analysis, defined the *owt* gene region. *Out*⁻ mutants fell into several distinct complementation groups, suggesting that the region comprises a cluster

of *out* genes (Gibson *et al* 1988, Murata *et al* 1990). Similar work identified the *Ech out* gene cluster, which, as in *Ecc*, is unlinked to the extracellular enzyme structural genes (He *et al* 1991a).

The *out* cluster of *Ecc* 71 has been used to complement *Out*⁻ mutants of other strains of *Ecc* and of *Ech* and *Eca* (Murata *et al* 1990), although it could not confer the *Out*⁺ phenotype on *E. coli*. An *E. coli* strain carrying *out*⁺ and *pel* constructs retained *Pel* activity in the periplasm (Murata *et al* 1990). This might have been because the *out*⁺ construct did not encode all the necessary *Out* proteins. Alternatively, the *out* genes might not have been correctly expressed in *E. coli* (Murata *et al* 1990). The ability of a cluster of *out* genes from *Ech* to allow the secretion of *Pel* from *E. coli* (He *et al* 1991a) might be because in this case the entire *out* cluster was present, or because the *Ecc* and *Ech* clusters are regulated differently.

Heterologous systems were used to test the abilities of the *Ech out* cluster to secrete *Ecc Pel*, and the *Ecc out* cluster to secrete *Ech Pel*, from *E. coli* (He *et al* 1991a). The heterologous proteins were not secreted, suggesting that a component of the *Out* apparatus is species specific. Further support for this theory comes from observations that the *Ech* cellulase EGZ cannot be secreted by *Ecc*, and the *Ecc* cellulase *CelV* cannot be secreted by *Ech* (Py *et al* 1991b). This species specificity may have arisen by the co-evolution of the secretory apparatus and extracellular enzymes in the different bacterial species.

While the study described here was in progress, the DNA sequence of the *Ecc out* cluster was being analysed (Reeves *et al* in press). The cluster was found to consist of several genes, transcribed in the same direction, and probably organized into an operon. Data bank searches revealed a high degree of homology (in terms of general gene organization and sequence identity) with a cluster involved in the secretion of pullulanase (*Pul*) by *Klebsiella oxytoca* (Pugsley *et al* 1990a). Homologies were also found with various other genes and gene clusters from a range of Gram negative bacteria.

All the homologues identified are involved in the translocation of macromolecules across cell membranes. This family of proteins, which will be referred to collectively as 'traffic wardens', probably define the major apparatus for the translocation of macromolecules across the outer membrane of Gram negative bacteria (Salmond and Reeves 1992).

Most of the rest of this introduction will deal with examples of two-step secretion by Gram negative bacteria, and the traffic warden proteins involved.

1.8 THE SECRETION OF PULLULANASE BY *KLEBSIELLA* SP.

The best characterized example of a Gram negative, two-step secretion system is that for the secretion of pullulanase (Pul) by *Klebsiella oxytoca* (formerly *K. pneumoniae*). The system has been reviewed elsewhere (Pugsley *et al* 1990a). In the following sections, the knowledge of the secretion pathway will be summarized and particular attention will be paid to the most recent research, concentrating on the traffic warden proteins and their possible functions.

1.8.1 THE LIPOPROTEIN: PULLULANASE.

Klebsiella spp. utilize pullulan: an $\alpha(1-6)$ linked maltotriose polymer, as a carbon source. Since the polymer is too large to diffuse through the membrane, it is broken down outside the cell by Pul: a starch debranching enzyme. Pul cleaves $\alpha(1-6)$ glucosidic linkages in polymers such as pullulan, producing linear dextrans made up of $\alpha(1-4)$ linked glucose residues and maltotriose, which can be imported into the cell.

Pullulanase (EC 3.2.1.41) is a 117 KDa (approx.) protein (encoded by the gene *puIA*), and is synthesized as a precursor with a signal sequence (Pugsley *et al* 1986). The processing site is characteristic of a lipoprotein (Chapon and Raibaud 1985, Murooka and Ikeda 1989), in which the N-terminal Cys residue (position +1 of the mature protein) is modified to glycercylcys, then fatty acylated (Pugsley *et al* 1986). The modification and processing of lipoproteins is discussed in more detail later (7.4,

7.7). Evidence that Pul is a lipoprotein comes from studies involving labelling fatty acid groups with ^3H glycerol and ^3H palmitate. In addition, treatment with globomycin (a specific inhibitor of lipoprotein peptidase), causes the accumulation of prepullulanase (Pugsley *et al* 1986, Murooka and Ikeda 1989).

After secretion, Pul is initially anchored to the outer membrane by the fatty acid groups. The enzyme is then released into the extracellular medium (Pugsley *et al* 1990a), and large protein micelles are formed, held together by interactions between fatty acyl groups (Pugsley *et al* 1990b). Other bacterial extracellular lipoproteins which exist as free and cell bound forms include an endogluconase produced by *Fibrobacter succinogenes* (McGavin *et al* 1989) and some of the penicillinases of Gram positive bacteria (see Pugsley *et al* 1986).

1.8.2 THE PULLULANASE SECRETION PATHWAY.

Pul is secreted via the two-step pathway (1.5). It remains bound to the outer membrane, before being gradually released. The three stages leading to the release of Pul into the extracellular medium, outlined below, are illustrated in figure 1.8.2a.

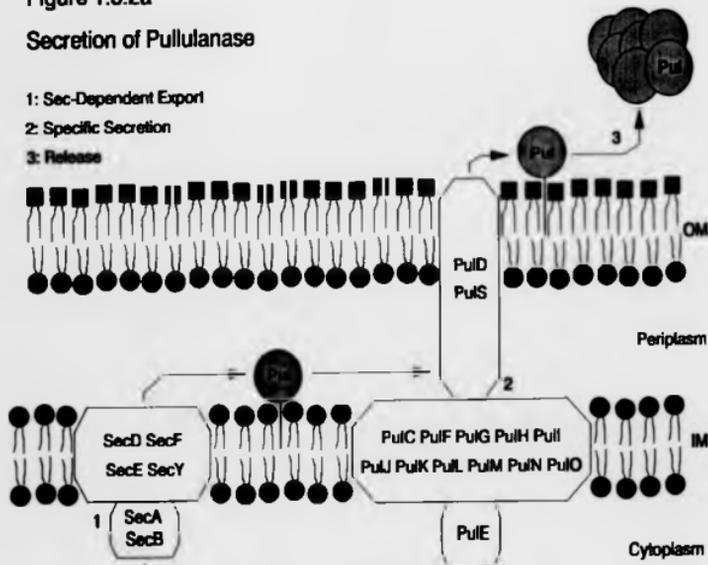
Pul, synthesized as a precursor with an N-terminal signal sequence (Chapon and Raibaud 1985, Pugsley *et al* 1986, Kornacker and Pugsley 1989) which is required for secretion (Pugsley *et al* 1990b), is exported from the cytoplasm via the Sec-dependent (1.5), general export pathway (Pugsley *et al* 1986, 1990b, d'Enfert *et al* 1987b). Much of the work investigating Pul export has used systems reconstituted in *E. coli*. It was shown that a block in the *E. coli* general export pathway prevented the processing and export of Pul, regardless of whether genes of the *pul* cluster (1.8.3) were present (Pugsley *et al* 1990b). This was not a secondary effect due to the inhibition of the secretion of *pul* gene products, as these are stable, and would have survived from the time before export was blocked (Pugsley *et al* 1991b).

After export, prepullulanase is processed by lipoprotein signal peptidase. The N-terminal amino acid sequence of a mature lipoprotein determines where the protein is targeted. The presence of Asp at position +2 of Pul would suggest that this protein is

Figure 1.8.2a

Secretion of Pullulanase

- 1: Sec-Dependent Export
- 2: Specific Secretion
- 3: Release



Adapted from Pugsley *et al* (1991b)

sorted to the inner membrane (Yamaguchi *et al* 1988). It is thought that Pul is transiently anchored to the inner membrane before the second step of secretion occurs (Pugsley *et al* 1990a). Evidence for this inner membrane intermediate comes from protease accessibility, fractionation and immunochemistry studies (Pugsley *et al* 1990b). The intermediate has a different conformation from the mature, extracellular protein, but is at least partially folded (Pugsley *et al* 1990b). Removal of the Asp residue (+2) by site directed mutagenesis, caused the lipoprotein sorting apparatus to transfer Pul to the inner face of the outer membrane, in the absence of any other Pul secretion factors (Pugsley and Kornacker 1991). In this case, the protein did not reach the cell surface, even if other secretion factors were expressed later. This suggested that Pul must enter the specific secretion pathway before it associates with the outer membrane (Pugsley and Kornacker 1991), and hence, that some vital secretion factor(s) may be associated with the inner membrane.

In *E. coli* carrying the cloned structural gene *pulA*, Pul is synthesized, processed and fatty acylated, and exported to the periplasm (Michaelis *et al* 1985, Pugsley *et al* 1990a), suggesting that Pul is not targeted to the outer membrane like typical outer membrane proteins. The secretion of Pul across the outer membrane requires the expression of genes mapping either side of *pulA* on the *Klebsiella* sp. chromosome (d'Enfert *et al* 1987b, 1989, d'Enfert and Pugsley 1989, Pugsley and Reys 1990). The products of some of these genes may interact with Pul during export, although their absence does not prevent translocation across the inner membrane (Pugsley *et al* 1990b).

The region of DNA required for Pul secretion, contains the structural gene *pulA* and 15 other genes: *pulB*, *pulS* and *pulC-O*. Most work has concentrated on the *pulC-O* operon. At least 12 of the *pulC-O* genes encode proteins essential for Pul secretion. With the exception of the cytoplasmic protein(s): PulE (and PulB), all the *pul* gene products are located in the cell envelope: mainly associated with the inner membrane, although PulD is an outer membrane protein (Pugsley *et al* 1990a). Topology studies have shown that most of the membrane-associated Pul secretion proteins have a type II

configuration (with the C-terminal bulk exposed to the periplasm), although PulL spans the membrane only once, and PulF and PulO span it many times (d'Enfert *et al* 1989, Pugsley and Reysa 1990, Reysa and Pugsley 1990). Although Pul uses the general export pathway, PulE is cytoplasmic, and PulL, F and O all have large cytoplasmic domains. It is possible that one (or more) of these proteins provides energy for the secretion step (Pugsley *et al* 1990a). The localization of so many of the *pul* gene products in the inner membrane, is in agreement with the finding that Pul must enter the specific secretion pathway before it associates with the outer membrane (see above).

The export and secretion of Pul are distinct and independent events. These may be experimentally uncoupled, by expressing *pulC-O* only after Pul synthesis has been stopped, without preventing the correct targeting of Pul to the cell surface (Pugsley *et al* 1991b). The Pul inner membrane intermediate is at least partially folded (Pugsley *et al* 1991b), suggesting that if the Pul secretory apparatus recognizes a specific signal, this might be conformational, and not a simple primary amino acid sequence. The Pul secretory apparatus is discussed in more detail later (1.8.3).

Localization studies (using tests for proteinase K susceptibility, cell fractionations and immunostaining) have shown that Pul is firmly anchored to the outer membrane of exponential phase cells (d'Enfert *et al* 1987a), due to its fatty acyl groups (Murooka and Ikeda 1989).

The free, extracellular form of Pul is lipid-modified (as determined by ^3H palmitate labelling studies) and has the same MW as the cell-associated form, suggesting that release is not achieved by proteolysis, or the removal of the fatty acyl groups (Pugsley *et al* 1986). The release of Pul from the outer membrane seems to occur spontaneously, and is not affected by mutations in the *pul* gene cluster (Pugsley *et al* 1990a). Release of Pul is specific, and is not associated with an increased level of other outer membrane proteins or lipids, as would be expected if vesicles were released from the cell membrane (d'Enfert *et al* 1987a, 1987b).

Extracellular Pul exists free, or as aggregates: micelles formed by interaction between fatty acyl groups (Pugsley *et al* 1990b). Extracellular Pul cofractionates with

the metalloporin LamB, and the two proteins are thought to be physically associated (d'Enfert *et al* 1987a). This is interesting, in view of the fact that the breakdown products of pullulan are imported into the cell via LamB (Wandersman *et al* 1979).

Having described the general features of the Pul secretion pathway, the gene cluster encoding the secretory apparatus will be discussed in more detail.

1.8.3 THE *PUL* GENE CLUSTER OF *KLEBSIELLA OXYTOCA*.

The organization of genes involved in the secretion of Pul by *Klebsiella oxytoca* is shown in figure 1.8.3a. The genes *pulC-O* are transcribed as a single unit, with *pulO* being the end of the operon (Pugsley and Reys 1990).

The *pul* region contains four palindromic sequences: predicted to form stem loop structures in the mRNA transcripts. The palindrome lying downstream of *pulO* is followed by three Ts, and is therefore likely to be a rho-independent transcription terminator. Downstream of this sequence there are three and a half almost perfect direct repeats, the function of which is not known. The functions of the other predicted stem loop forming sequences (not believed to be involved in termination) are also unknown.

The products of the various *pul* genes have been visualized, and their cellular localizations determined. The current knowledge of the Pul secretory apparatus proteins is summarized in table 1.8.3a.

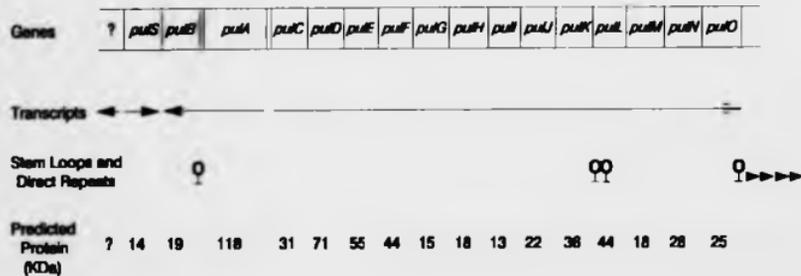
One of the most important discoveries is the peptidase activity of PulO. This is specific for proteins with the prepilin peptidase (MePhe) consensus cleavage site: thought to include PulG,H,I and J. Processing by PulO is extremely rapid and may be co-translational (Dupuy *et al* 1992, Pugsley and Dupuy 1992).

PulO removes only the first few amino acid residues from its substrate polypeptides, leaving most of the N-terminal signal sequence intact (Dupuy *et al* 1992). This processing is thought to allow proteins to be transported to different locations within the cell.

The assembly of pili in *Pseudomonas aeruginosa* involves homologues of PulO,G,H,I and J (see 1.9.3). Comparison of the two systems suggests that, when

Figure 1.8.3a

The *pul* Cluster of *Klebsiella oxytoca*



Adapted from Pugsley *et al* (1990a)

TABLE 1.8.3a: PROTEINS ENCODED BY THE *PUL* GENE CLUSTER OF *KLEBSIELLA OXYTOCA*.

PROTEIN	ESSENTIAL FOR SECRETION?	LOCATION	POSSIBLE ROLE/ OTHER INFORMATION	REFERENCES
?	No	ND		d'Enfert & Pugsley (1989)
S	Yes	OM	Anchored lipoprotein. Gene not part of maltose regulon: still expressed when <i>pulA</i> is not.	d'Enfert & Pugsley (1989)
B	No	C	Unknown function. Gene co-transcribed with <i>pulA</i> .	d'Enfert & Pugsley (1989)
A		OM/EC	Pullulanase, lipoprotein	see 1.8.1
C	Yes	IM	Old name: MalX	d'Enfert <i>et al</i> (1989)

TABLE 1.8.3a continued

PROTEIN	ESSENTIAL FOR SECRETION?	LOCATION	POSSIBLE ROLE/ OTHER INFORMATION	REFERENCES
D	Yes	OM/P	Synthesized with signal sequence. May interact with PulS.	d'Enfert <i>et al</i> (1989)
E	Yes	C	Binds ATP: possibly a kinase. May act as a chaperone. Toxic at high levels.	Possot <i>et al</i> (1992)
F	Yes	IM	Major domain in cytoplasm. May interact with PulE. Poorly expressed.	Possot <i>et al</i> (1992)

TABLE 1.8.3a continued

PROTEIN	ESSENTIAL FOR SECRETION?	LOCATION	POSSIBLE ROLE/ OTHER INFORMATION	REFERENCES
G	Yes	IM	May be processed by PulO.	Reyss & Pugsley (1990) Pugsley & Dupuy (1992)
H	Yes	IM	May be processed by PulO. Poorly expressed.	Reyss & Pugsley (1990) Pugsley & Dupuy (1992)
I	Yes	IM	May be processed by PulO.	Reyss & Pugsley (1990) Pugsley & Dupuy (1992)
J	Yes	IM	May be processed by PulO.	Reyss & Pugsley (1990) Pugsley & Dupuy (1992)

TABLE 1.8.3a continued

PROTEIN	ESSENTIAL FOR SECRETION?	LOCATION	POSSIBLE ROLE/ OTHER INFORMATION	REFERENCES
K	Yes	IM		Pugsley <i>et al</i> (1990a)
L	Yes	IM		Pugsley & Reys (1990)
M	Yes	IM		Pugsley & Reys (1990)
N	Yes	IM		Pugsley & Reys (1990)
O	Yes	IM	Transmembrane peptidase: processes PulG-J. Poorly expressed.	Pugsley & Dupuy (1992)

KEY: OM: Outer membrane, IM: Inner membrane, C: Cytoplasm, P: periplasm, EC: Extracellular, .ND: Not determined

processed by PulO, the PulG-J proteins interact (via their hydrophobic signal sequences) to form a pilus-like structure. This might aid secretion by bringing the membranes together, or it might form a platform for the assembly of other proteins involved in secretion. The other systems homologous to that for Pul secretion are discussed in more detail later (1.9), together with the possible implications of the homologies.

1.8.4 REGULATION OF THE *PUL* CLUSTER.

When *pulA* was cloned into *E. coli*, it was shown to be maltose-inducible (Michaelis *et al* 1985). Studies of *E. coli* maltose regulons have shown the positive effect of maltose to be mediated by a DNA-binding protein: MalT (Cole and Raibaud 1986). The inducibility of *pulA* in *E. coli* (with constitutive *pulA* expression being detected in hosts constitutive for the expression of maltose regulons), suggests that the *E. coli* and *Klebsiella oxytoca* MalT proteins are functionally interchangeable (Michaelis *et al* 1985).

Promoters controlled by MalT have a consensus sequence: thought to form part of a MalT binding site. Sequence analysis identified two such promoter regions in the *pul* cluster. These are in opposite orientations between *pulA* and *pulC*, and are named *pulA_p* and *malX_p* (Chapon and Raibaud 1985). The region between *pulA* and *pulC* shows a high degree of symmetry and may constitute a common control element for the two operons (Chapon and Raibaud 1985).

It can therefore be concluded that the genes required for the secretion of Pul are co-expressed with *pulA*, being positively regulated by maltose (d'Enfert *et al* 1987b). The exception to this is *pulS*, which is not part of the maltose regulon, being independently expressed from its own promoter (d'Enfert and Pugsley 1989).

An additional positive regulator: a complex of the *crp* product and cAMP (Chapon 1982), is also involved in *pul* regulation, meaning that catabolite repression occurs in the presence of glucose (Michaelis *et al* 1985).

1.9 'TRAFFIC WARDENS'.

1.9.1 MEMBERS OF THE TRAFFIC WARDEN FAMILY.

Sequence analysis and data bank searches have identified a number of proteins which are homologous to those encoded by the *pul* genes of *Klebsiella oxytoca*, as shown in figure 1.9.1a. It should be noted that the proteins are aligned according to homology, and that this does not necessarily represent gene organization, which is discussed in the text. Much of the information shown in figure 1.9.1a has not yet been published, and was obtained while this study was in progress. Some of the data presented later in this report (Ecc OutC: 6.2) is also included, for the sake of completeness. Further information on the Pul homologues is given in table 1.9.1a.

In the following sections, each of the systems involving Pul homologues will be considered separately. This is necessary, in order to understand the diversity of members of the traffic warden family, and at the same time, to identify any conserved functions between the proteins.

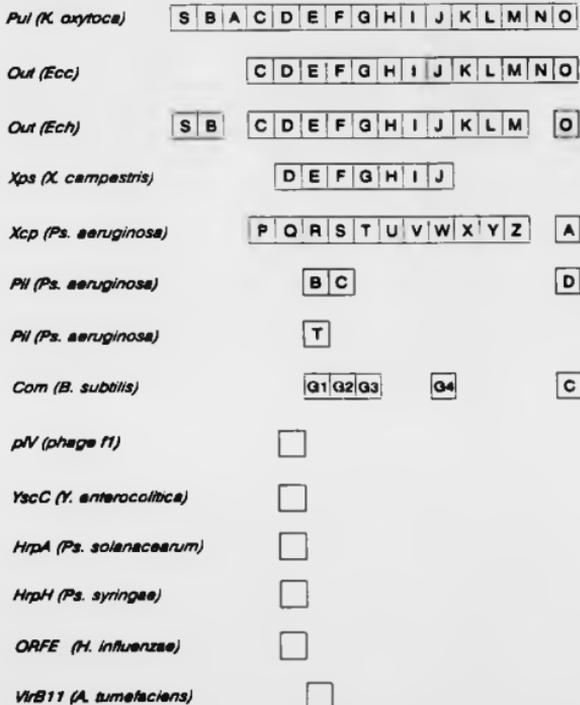
1.9.2 THE SECRETION OF ENZYMES AND TOXINS

BY *PS. AERUGINOSA*.

Pseudomonas aeruginosa, an opportunistic pathogen of humans (causing death in patients suffering from diseases such as cystic fibrosis), secretes several enzymes and toxins, some of which are important in pathogenicity. Most strains secrete at least two proteases (elastase and alkaline protease), a lipase, a phospholipase, alkaline phosphatase and exotoxin A. All these are secreted by the two-step mechanism (1.5), except alkaline protease, which uses the one-step pathway (1.6).

Ps. aeruginosa xcp (extracellular protein deficient) secretion mutants accumulate mature, processed exoproteins in the periplasm (Wretling and Pavlovskis 1984, Filloux *et al* 1987). The organization of the *xcp* genes differs from that of the *pul* cluster. The *xcp* genes map to three loci which are dispersed on the chromosome (Wretling and Pavlovskis 1984). No homologues of *pulN* or *pulO* have been found downstream of

Figure 1.9.1a Traffic Warden Protein Homologies



Proteins are aligned according to homology, rather than gene organization.

TABLE 1.9.1a: THE FAMILY OF TRAFFIC WARDENS.

SPECIES	GENE/ CLUSTER	FUNCTION	REFERENCES
<i>K. oxytoca</i>	<i>pul</i>	Secretion of Pul	Reviewed: Pugaley <i>et al</i> (1990a)
<i>Ecc</i>	<i>ou1</i>	Secretion of Pel, Cel and Peh	Reeves <i>et al</i> in press
<i>Ech</i>	<i>ou1</i>	Secretion of Pel, Cel and Peh	He <i>et al</i> (1991) Condemine <i>et al</i> (1992)
<i>Ps. aeruginosa</i>	<i>xcp</i>	Secretion of enzymes	Bally <i>et al</i> (1989, 1992) Filloux <i>et al</i> (1990)
<i>Ps. aeruginosa</i>	<i>pil</i>	Pilus assembly	Nunn <i>et al</i> (1990) Whitchurch <i>et al</i> (1990)
<i>X. campestris</i>	<i>xpu</i>	Secretion of enzymes	Dow <i>et al</i> (1987) Dums <i>et al</i> (1991) Hu <i>et al</i> (1991)
<i>B. subtilis</i>	<i>com</i>	Competence in DNA uptake	Albano <i>et al</i> (1989) Mohan <i>et al</i> (1989)
<i>H. influenzae</i>	ORFE	Competence in DNA uptake	Tomb <i>et al</i> (1991)
<i>A. tumefaciens</i>	<i>virB11</i>	Transfer of bacterial DNA to host tissue	Ward <i>et al</i> (1988) Christie <i>et al</i> (1989)
<i>Yersinia</i> sp.	<i>ycsG</i>	Secretion of Yops	Michiels <i>et al</i> (1991)
<i>Ps. syringae</i>	<i>hrp</i>	Hypersensitive response	Collmer pers. comm.
<i>Ps. solanacearum</i>	Hrp	Hypersensitive response	Boucher pers. comm.
Phage f1	<i>gpIV</i>	Filamentous phage assembly	Russell (1991)

xcpZ, which is followed by a palindromic sequence (likely to form a stem loop structure in the transcript), and direct repeat sequences (Bally *et al* 1991), reminiscent of those found downstream of *pulO* (1.8.3). The *xcpA* gene, which maps to a different chromosomal locus, is homologous to *pulO*. This gene was found to be identical to *pilD* (sequenced previously: Nunn *et al* 1990), which is essential for pilus assembly (see 1.9.3).

The predicted products of *xcpT*, *U*, *V* and *W* are highly homologous (in their N-terminal regions) to prepilins (see 1.9.3), and are thought to be synthesized as precursors, which are processed by the XcpA/PilD (PulO homologue) peptidase. Certainly, XcpU is cleaved by XcpA (Bally *et al* 1992). It is proposed that XcpT, *U*, *V* and *W* (like pilin sub-units) form a helical structure: held together by their hydrophobic N-termini.

PulO can complement mutations in *xcpA*, although PulN and PulM are not functionally interchangeable with XcpY and XcpZ (Bally *et al* 1992). XcpR has an ATP-binding motif, in agreement with findings for PulE, and other homologues of this (see below).

Although *xcp* genes of *Ps. aeruginosa* are associated with pathogenicity, they have also been identified in non-pathogenic strains of *Pseudomonas*, and may be involved in protein secretion by certain strains of the bacteria which promote plant growth (de Groot *et al* 1991).

1.9.3 PILUS ASSEMBLY IN *PS. AERUGINOSA*.

The pili of Gram negative bacteria are hollow tubes, composed of a helical array of pilin sub-units. *Ps. aeruginosa* pili are important for colonization: mediating cell attachment.

Ps. aeruginosa pili are similar to those found in *Neisseria gonorrhoeae* (Seifert *et al* 1990, Dupuy *et al* 1991), *Moraxella bovis* (Marrs *et al* 1985) and *Bacteroides nodosus* (McKern *et al* 1983), all of which have a polar location and give rise to a twitching motility, involved in pathogenicity. The amino acid sequence of the pilin sub-units is

similar, with a high degree of identity at the N-terminus of the molecule (Dalrymple and Mattick 1987). A short (6-7 amino acids) N-terminal signal sequence is followed by a highly conserved stretch of about 30 hydrophobic residues (Strom and Lory 1987). The group are collectively referred to as type IV methylphenylalanine (MePhe) piliins, as processing involves the removal of the short leader, and attachment of a methyl group to the new N-terminal (Phe) residue (Marrs *et al* 1985).

When the *Ps. aeruginosa* structural pilin gene (*pilA*) is expressed in *E. coli*, the product is exported but does not reach the cell surface, and pili are not assembled (Strom and Lory 1986). The genes *pilB*, *C* and *D* are required for pilus assembly, but not for pilin synthesis (Nunn *et al* 1990).

PilD is a peptidase which cleaves prepilin, to generate mature pilin (Nunn and Lory 1991, Strom and Lory 1991). Purified PilD from *Ps. aeruginosa* is capable of processing prepilin from *Bacteroides nodusus* and *Neisseria gonorrhoeae*, and total membrane preparations from these species cleave *Ps. aeruginosa* prepilin (Mattick *et al* 1987, Nunn and Lory 1991), showing that PilD is not species-specific. The role of PilD is not restricted to pilus assembly, but extends to the export of various proteins (see 1.9.2).

The gene *pilT*, mapping to a different chromosomal locus from *pilA-D*, is involved in twitching (Whitchurch *et al* 1991). PilT, thought to be a cytoplasmic nucleotide binding protein, is homologous to PilB. The relationship between these two proteins is not known. They may act antagonistically, with PilB causing polymerization of pilin sub-units (via their hydrophobic sequences) and PilT causing disassembly, and hence retraction. Alternatively, the proteins could act as positive and negative regulators (Whitchurch *et al* 1991).

The organization of the *pil* genes, which differs considerably from that of the *pil* genes, is shown in figure 1.9.3a.

Figure 1.9.3a

**Organization of the *pil* genes of
*Pseudomonas aeruginosa***



Taken from Whitchurch *et al.* (1991)

1.9.4 EXTRACELLULAR ENZYME SECRETION BY *XANTHOMONAS* SP.

Xanthomonas campestris pv. *campestris*, which causes black rot of crucifers, secretes various enzymes including endogluconases, pectinases and proteases (Dow *et al* 1987). The *xps* gene cluster, required for pathogenicity, is involved in secretion, with *xps* (*Xanthomonas* protein secretion) mutants accumulating enzymes in the periplasm (Dow *et al* 1987).

XpsE is predicted to be a soluble, nucleotide binding protein. In addition to the homologies with traffic warden proteins (see figure 1.9.1a), homology was also reported with PrtD (*Ech*) and HlyB (*E. coli*). XpsF is also homologous to proteins involved in one-step secretion: PrtE (*Ech*) and HlyD (*E. coli*), in addition to those involved in two-step secretion (Dums *et al* 1991). Although the significance of these findings is not known, it is interesting to note that unlike the *Erwinia* spp. *out* clusters, the *xps* cluster directs the secretion of proteases, even though these are not thought to have N-terminal signal sequences. The *xcp* genes are more closely related to the corresponding *pul* genes than to those encoding one-step secretion proteins (Dums *et al* 1991).

Although the *xps* genes probably form an operon, the organization differs from that of the *pul* cluster. Upstream of *xpsE* there is a putative promoter, preceded by a possible transcription terminator, suggesting that *xpsE* is the first gene of the *Xanthomonas* sp. secretion operon (Dums *et al* 1991).

XpsD (required for secretion) is homologous to PulD, but appears to be a lipoprotein (Hu *et al* 1992). The organization of the *xps* cluster in the region of *xpsD* is very different from that of the *pul* cluster. *XpsD* is the third gene of an operon. The predicted products of the two genes upstream of *xpsD* were not found to be homologous to any other proteins, and it is not yet known whether they are required for secretion.

1.9.5 COMPETENCE IN *BACILLUS SUBTILIS*.

The state of competence, which enables *B. subtilis* to bind, take up and integrate exogenous DNA, develops in response to nutritional and growth stage-specific signals, which are poorly understood. Seven loci (*comA-G*) are involved in competence: the 'early' *com* genes (*A* and *B*) being required for the expression of the 'late' (*C-G*) genes, early in the stationary phase of growth (Dubnau 1991).

The *comG* locus includes seven ORFs, transcribed from a single promoter (Albano and Dubnau 1989). Putative transcription terminators upstream of *comG1* and downstream of *comG7* indicate that these delimit the operon (Albano *et al* 1989).

ComG mutants are unable to bind DNA. ComG1 is a soluble nucleotide binding protein. It is directly involved in the transfer and processing of DNA, but also has a regulatory role: being required for the full expression of *comE*. The role of ATP hydrolysis is not certain, since DNA uptake is not thought to be driven by ATP (Dubnau 1991). ComG1 may act as an energy coupler or chaperon for the transport of components of the competence apparatus (Dubnau 1991).

The ComG3-7 proteins are all small and fairly similar to each other. ComG3-5 are McPhe proteins homologous to pilins, and may interact with each other to form a pilus-like structure (Albano *et al* 1989, Breiting and Dubnau 1990).

ComC is a highly hydrophobic membrane protein (Mohan *et al* 1989), likely to be involved in assembling the competence apparatus.

The regulation of competence is highly complex and has been reviewed previously (Dubnau 1991). An interesting observation (in view of the results with the *Ecc ou* cluster: see 4.4), is that DNA from the promoter regions of *comG* and *comC* represses the expression of late *com* genes, when present in multicopy (Albano *et al* 1989, Mohan and Dubnau 1990).

1.9.6 COMPETENCE IN *HAEMOPHILUS INFLUENZAE*.

H. influenzae becomes competent for DNA uptake in the late log phase of growth, or in response to nutritional signals. Competence is characterized by the formation of specialized surface vesicles (transformasomes), which are involved in DNA binding and uptake. In addition, recombination capacity is increased and single stranded gaps appear in the chromosome (Tomb *et al* 1989).

A locus involved in competence contains ten putative ORFs, several of which might constitute an operon (Tomb *et al* 1991). One of these: ORFE, is predicted to encode a protein which is homologous to PulD and pIV. The function of this protein is not yet known. The predicted products of the other ORFs of the competence locus are not homologous to Pul proteins.

1.9.7 VIRULENCE OF *AGROBACTERIUM TUMEFACIENS*.

A. tumefaciens causes crown gall: a neoplastic disease of dicotyledons, by genetically transforming host cells. A specific segment of DNA (T-DNA) is transferred from the Ti plasmid to the plant nucleus, by functions encoded by the *vir* region of the Ti plasmid. There are six *vir* loci. Some of these are constitutive, while others are induced by phenolic compounds present in plant extracts.

VirB is the largest of the *vir* operons, containing eleven genes (Ward *et al* 1988), many of which are thought to encode membrane proteins. *VirB* is required for virulence, is plant inducible, and is transcribed as a single unit (Stachel and Nester 1986). The *virB* gene products are thought to form a pilus-like structure (Engstrom *et al* 1987). This might deliver T-DNA, or interact with proteins of exposed plant cell membranes: stabilizing bacterial/ cell interactions, or forming membrane fusion points (Ward *et al* 1988).

VirB11 is associated with the inner face of the cytoplasmic membrane (Christie *et al* 1989) and is thought to be involved in the transfer, rather than processing of T-DNA. The purified protein binds ATP, has ATPase activity, and is autophosphorylated (Christie *et al* 1989). Phosphorylation may change the conformation of *VirB11*,

allowing it to bind to other proteins. Alternatively, VirB11 might be a kinase which activates other proteins. Conformational changes in a membrane apparatus could create a protein channel and so allow the passage of DNA through the membrane.

1.9.8 SECRETION OF YOP PROTEINS BY *YERSINIA ENTEROCOLITICA*.

Yersinia spp. are human pathogens. Under certain conditions they secrete eleven proteins (Yops), which are essential for pathogenicity. Yops are secreted in such large amounts that they form visible aggregates in culture.

Yops are not thought to be secreted by either the Hly-like one-step, or the two-step pathway. The proteins do not have a classical N-terminal sequence, but secretion does involve the N-terminal region of the proteins, rather than the C-termini, which would be required for the one-step mechanism.

The loci: *virA*, *B*, *C* and *F* are involved in pathogenicity. Of these, *virA* and *virC* are involved in Yop secretion, with mutants accumulating Yops intracellularly (Michiels *et al* 1991). The *virC* locus comprises thirteen genes (*ysc*: *Yersinia* secretion), arranged in a single operon. One of these genes: *yscC* is predicted to encode a PulD homologue (Michiels *et al* 1990). Although *yscJ* encodes a lipoprotein, no other Pul homologues have been identified. This, together with the lack of Yop N-terminal signal sequences, suggests that the Yop secretion mechanism is different from those described previously. If YscC is involved in a unique export mechanism, and yet is homologous to PulD and pIV, this would raise interesting questions concerning the evolution of diverse transport mechanisms.

1.9.9 THE HYPERSENSITIVE RESPONSE.

The hypersensitive response (HR), which occurs when a pathogenic bacterium invades a non-host plant, is characterized by the release of a range of toxins, enzymes and antimicrobial phytoalexins, from the plant cells. The responding plant cells die within 8-24 hr, and the multiplication and spread of the pathogen is arrested (Klement 1982). The genes (designated *hrp*) required for the initiation of the HR response in

non-host plants, by various phytopathogenic bacteria, have been reviewed recently (Willis *et al* 1991). It is thought that in addition to their involvement in HR initiation, *hrp* genes are required for pathogenicity in host plants, although other genes are also necessary for this (Huang *et al* 1988).

Pseudomonas syringae pv. *syringae*, which causes brown spot disease of beans, has a large cluster of *hrp* genes which have been cloned and characterized (Huang *et al* 1988, Mukhopadhyay *et al* 1988, Huang *et al* 1991, Xiao *et al* 1992). There are thought to be thirteen *hrp* genes: probably organized into eight transcriptional units (Xiao *et al* 1992), and the majority of these genes are thought to be induced in the presence of plant extracts. Sequence analysis has suggested that some genes of the *hrp* locus encode membrane-associated proteins (Huang *et al* 1991), and one Hrp protein is homologous to PulD (Collmer pers. comm.).

Recent studies have revealed similarities between the *hrp* cluster of *Ps. syringae* pv. *syringae* and those of other species, including *Ps. solanacearum* (Boucher pers. comm.), *Ps. syringae* pv. *phaseolicola* (Rahme *et al* 1991, Panopoulos pers. comm.), *Xanthomonas campestris* pv. *vesicatoria* (Bonas pers. comm.) and *Yersinia* spp. In each case, genes have been identified which are thought to encode membrane-associated or exported proteins, and these are believed to function in the secretion of molecules involved in the interaction with the plant.

1.9.10 FILAMENTOUS PHAGE ASSEMBLY.

A general review of filamentous phage assembly has been published recently (Russel 1991). The following discussion is limited to those areas which are relevant to the study of traffic wardens.

Unlike most bacterial viruses which are assembled in the cell cytoplasm and released by lysis, filamentous phages are continuously assembled and extruded from the cell.

Phage f1, which infects *E. coli* via F pili, is composed of five phage- encoded proteins. The DNA is enclosed in a hollow tube: formed from approximately 2700

monomers of major coat protein (pVIII) in a helical array. The minor coat proteins, arranged at the ends of these tubes, are pIII and pVI (involved in adsorption and penetration respectively), and pVII and pIX which have a role in phage assembly (Russel 1991).

Three other proteins are essential for encapsidation and extrusion of phage. These are pI and pIV (phage encoded) and the *E. coli* protein, thioredoxin. Mutations in the genes for pI and pIV do not affect the synthesis of phage DNA or proteins, but prevent the production of progeny, suggesting that pI and pIV have a role in morphogenesis.

The pIV protein is synthesized with an N-terminal signal sequence. After processing, the mature protein is transiently soluble in the periplasm before integrating into the membrane (Brissette and Russel 1990), although it does not appear to have an anchor sequence. Although fractionation experiments suggest that pIV is localized in both the inner and outer membranes, the protein is thought to be predominantly in the outer membrane (Brissette and Russel 1990). There are some similarities between pIV, which is thought to affect the cytoplasmic membrane, and outer membrane porins (Russel 1991). Little is known about the function of pIV, although it causes *E. coli* host cells to synthesize Psp (phage shock protein), and is lethal when overexpressed (Brissette *et al* 1990). It is possible that the N-terminal domain of the transient periplasmic form of pIV disrupts the cytoplasmic membrane: allowing access for a new phage particle, although this would not account for the estimate that fifty pIV molecules are required for every phage particle assembled (Brissette and Russel 1990).

1.9.11 THE TRAFFIC WARDENS: COMMON THEMES.

Having considered each member of the traffic warden family separately, it is clear that there are some common features. These are important in understanding the evolutionary development and functioning of the different systems, and are summarized below

The genes are probably normally organized as polycistronic operons.

Many of the encoded proteins are predicted to be membrane associated.

Many of the loci encode a cytoplasmic, ATP-binding protein.

Several genes have been identified which encode small, pilin-like proteins with MePhe cleavage sites.

Genes encoding MePhe peptidases have also been identified.

The systems described in the preceding sections are responsible for the transfer of a wide range of macromolecules across biological membranes, and yet have many features in common. It is clear that a specialized, yet widespread bacterial transport system has been identified. This raises an interesting question as to the evolution of the systems. One possibility is that a mechanism for the transfer of a phage was captured by host cells, then evolved, acquiring other functions (Dubnau 1991).

1.9.12 TRAFFIC WARDEN FUNCTION IN HETEROLOGOUS SYSTEMS.

As stated previously (1.7.2), the Out systems of *Erwinia* spp. are species-specific for the enzymes they secrete (He *et al* 1991a, Py *et al* 1991b). The abilities of traffic wardens to function in other heterologous systems are outlined below.

Pseudomonas aeruginosa is unable to secrete plasmid-encoded *Klebsiella oxytoca* pullulanase (de Groot *et al* 1991).

PuL and PuM are unable to complement mutations in *xcpY* and *xcpZ* (de Groot *et al* 1991). This might be because the proteins are required to form a complex structure, but cannot interact with proteins from a different species.

When the phage $\phi 1$ *pIV* gene is expressed in *E. coli*, it induces the synthesis of phage shock protein (Brissette *et al* 1990). The same response was observed with cloned *puLD*, suggesting that *pIV* and *PuLD* have some function in common (Russel 1991).

PuO has a broad specificity. It complements mutations in *xcpA* (Bally *et al* 1992) and processes gonococcal type IV pilin (Dupuy *et al* 1992). *XcpA* also processes a

range of proteins, provided they have the consensus MePhe cleavage site (Strom and Lory 1991).

In summary, although homologous, the different members of the traffic warden family show specificity for the molecules they transport, even though some transport a range of apparently unrelated proteins. No specific recognition signals have yet been identified. However, since there is evidence to suggest that proteins may be folded before being secreted across the outer membrane (Pugsley *et al* 1990b), recognition could involve a particular 3-dimensional structure. This would obviously be more difficult to identify than a simple signal sequence.

Another possibility which must be considered is that specificity is due to differential regulation of the gene clusters encoding the various systems.

1.10 WHY RESEARCH INTO *ECC* SCRI193 IS IMPORTANT.

As discussed previously (1.1), *Ecc* is a plant pathogen of great economic significance. This means that there are obvious incentives for gaining a better understanding of the organism.

A vital factor in pathogenicity is the secretion of extracellular enzymes by *Ecc*. The enzymes Pel, Cel and Prt can easily be assayed, either spectrophotometrically, or by the use of assay plates. Secretion can be studied by using cell fractionations, to determine the localization of enzyme activity.

Prior to this study, *Ecc* mutants defective in the secretion of Pel and Cel had been generated and cosmid complementation had identified the chromosomal locus involved in secretion. A wide range of techniques are available for the characterization of this (*out*) locus, since SCRI193 is extremely genetically amenable, as outlined below.

Because *Erwinia* spp. are taxonomically close to *E. coli*, many of the techniques developed for *E. coli* can be used. Although certain techniques are strain dependent (Ellard *et al* 1989), *Ecc* SCRI193 has been found to be particularly amenable. SCRI193 is suitable for chromosome mobilization (Chatterjee *et al* 1985, Forbes and Perombelon

1985), plasmid transformation (Hinton *et al* 1985) and electroporation. The introduction of *lamB* into SCRI193 (generating HC131) allows phage λ to be used as a delivery vehicle for transposons and cosmids, enabling transposon mutagenesis (Salmond *et al* 1986) and cosmid complementation (Murata *et al* 1990, Reeves 1991) to be performed. Generalized transduction is possible using ϕ KP (Toth 1992).

Homologies between the secretory apparatus proteins of *Ecc* and other Gram negative bacteria were discovered while this study was in progress. The homologies make *Ecc* an important model for the study of a mechanism for the transfer of diverse macromolecules by eubacteria.

1.11 PROJECT AIMS.

The aim of the project was to study the secretion of extracellular enzymes by *Ecc* SCRI193, paying particular attention to the *out* gene cluster and its regulation.

The first part of the work involved the characterization of wild-type secretion, to form a basis for the study of *Out*⁻ mutants.

The *out* gene cluster was to be characterized, and its regulation investigated, using transposon mutagenesis and gene fusions. This was to be followed by the molecular characterization of the putative regulatory region.

CHAPTER TWO.

MATERIALS AND METHODS.

The strains and plasmids used are listed in tables 2a and 2b respectively.

TABLE 2a: STRAINS.

STRAIN	CHARACTERISTICS	PLASMID PHENOTYPE	SOURCE	REFERENCE
<i>E. coli</i>				
CC118	<i>araD139::(ara-lex)7697</i> <i>lacX74, phoAV20, galE</i> <i>galK, thi, rpsL, rpsB</i> <i>argE(Am), recA1</i>		D. Gill	Mason & Beckwith (1985)
DH1	F', <i>recA1, endA1,</i> <i>th-1, hsdR17, gyp196,</i> <i>(r_g⁻mg⁻), supE44, rtdA1</i>		D. Gill	Misra (1982)
Q342	R64, <i>dnd11</i> pLVC9	Tc ^R Ca ^R	D. Gill	van Haese <i>et al</i> (1983)
RM101	<i>(lac-pro), supE,</i> <i>thi, F' proD16, proAB,</i> <i>lac^f, ZdeltschM15</i>	F'	J. Hinton	Yanisch-Perron <i>et al</i> (1985)
K12	wild-type		D. Gill	Beckmann (1983)
K38	K12, H5C		F. Hayes	Tabor & Richardson (1985)

TABLE 2a continued

STRAIN	CHARACTERISTICS	PLASMID PHENOTYPE	SOURCE	REFERENCE
LE392	F ⁻ , <i>AndRS14</i> , (<i>rg⁺mlc⁺</i>), <i>supE44</i> , <i>supF38</i> , <i>lacY1</i> , <i>galK2</i> , <i>galT22</i> , <i>metB1</i> , <i>trpR35</i>		J. Hinton	Manassis (1982)
TQ1	λ M101 <i>ecoK</i> derivative	F ⁻	J. Hinton	Carter <i>et al</i> (1985)
<i>E. coli</i>				
SCR1193	wild-type		J. Hinton	Forbes & Perombelon (1985)
HC131	SCR1193(pHCF2)	Ap ^R , LamB	J. Hinton	Salmond <i>et al</i> (1986)
GB2001	HC131 <i>pho::TnJ0</i>	Ap ^R , Tc ^R LamB	J. Hinton	Hinton & Salmond (1987)
GB4000	HC131 <i>lac::TnJ0</i>	Ap ^R , Tc ^R LamB	J. Hinton	Hinton & Salmond (1987)
AC4000	HC131 <i>ami::TnJ0</i>	Ap ^R , Tc ^R LamB	Lab stock	Unpublished
HJN1000	HC131 <i>ami⁺</i> (EM8)	Ap ^R , LamB	N. Housby	Unpublished

TABLE 2a continued

STRAIN	CHARACTERISTICS	PLASMID PHENOTYPE	SOURCE	REFERENCE
PR33	GB2001 <i>out</i> ::Taphed	Ap ^R , Km ^R LamB	P. Reeves	Unpublished
PR54	GB2001 <i>out</i> ::Taphed	Ap ^R , Km ^R LamB	P. Reeves	Hinton & Salmon (1987)
RJP122	HC131 <i>out</i> ' (EM8)	Ap ^R , LamB	P. Reeves	Reeves (1991)
RJP159	HC131 <i>out</i> ' (EM8)	Ap ^R , LamB	P. Reeves	Reeves (1991)
RJP190	HC131 <i>out</i> ' (EM8)	Ap ^R , LamB	P. Reeves	Reeves (1991)
RJP208	HC131 <i>out</i> ' (EM8)	Ap ^R , LamB	P. Reeves	Reeves (1991)
RJP220	HC131 <i>out</i> ' (EM8)	Ap ^R , LamB	P. Reeves	Reeves (1991)
RJP221	HC131 <i>out</i> ' (EM8)	Ap ^R , LamB	P. Reeves	Reeves (1991)
RJP240	HC131 <i>out</i> ' (EM8)	Ap ^R , LamB	P. Reeves	Reeves (1991)
RJP251	HC131 <i>out</i> ' (EM8)	Ap ^R , LamB	P. Reeves	Reeves (1991)
RJP253	HC131 <i>out</i> ' (EM8)	Ap ^R , LamB	P. Reeves	Reeves (1991)

TABLE 2b: PLASMIDS AND PHAGE

PLASMID/ COSMID	CHARACTERISTICS	PHENOTYPE	SOURCE	REFERENCE
pBR322	multi-copy cloning vector	Ap ^R , Tc ^R	Amerham Int.	Boivar <i>et al</i> (177)
pHC79	pBR322::cos	Ap ^R , Tc ^R	J. Hinton	Hahn & Collins (1980)
pHCP2	pBR322:: <i>kan^R</i>	Ap ^R , Tc ^R	J. Hinton	Clement <i>et al</i> (1982)
pSP6	multi-copy cloning vector <i>cat</i> , <i>mob</i>	Sp ^R , Sm ^R	P. Raevus	Salvarij <i>et al</i> (1984)
pUC8	multi-copy cloning vector <i>lacZ</i>	Ap ^R	Lab stock	Vinje & Messing (1982)
pUC18	multi-copy cloning vector <i>lacZ</i>	Ap ^R	Lab stock	Yanish-Perron <i>et al</i> (1985)
pUC19	multi-copy cloning vector <i>lacZ</i>	Ap ^R	Lab stock	Yanish-Perron <i>et al</i> (1985)

TABLE 2b continued

PLASMID/ COSMID	CHARACTERISTICS	PHENOTYPE	SOURCE	REFERENCE
pIC-19R	multi-copy cloning vector <i>lacZ</i>	Ap ^R	Lab stock	Yanisch-Perron <i>et al</i> (1985)
cHIL122	pHC79:: <i>ona</i> ⁺	Ap ^R , Tc ^R	P. Reeves	Reeves (1991)
cHIL208	pHC79:: <i>ona</i> ⁺	Ap ^R , Tc ^R	P. Reeves	Reeves (1991)
cHIL208/4	pSF6:: <i>ona</i> ⁺	Sp ^R , Sm ^R	P. Reeves	Unpublished
cHIL208/5	pSF6:: <i>ona</i> ⁺	Sp ^R , Sm ^R	P. Reeves	Unpublished
cHIL251/1	pSF6:: <i>ona</i> ⁺	Sp ^R , Sm ^R	P. Reeves	Unpublished
cHIL253	pHC79:: <i>ona</i> ⁺	Ap ^R , Tc ^R	P. Reeves	Reeves (1991)
cHIL208/L (1-8)	cHIL208:: <i>TyphoA</i>	Ap ^R , Tc ^R , Km ^R		This study
cHIL208/P (1-14)	cHIL208:: <i>TyphoA</i>	Ap ^R , Tc ^R , Km ^R		This study
pDBK519	multi-copy cloning vector	Km ^R	A. Chatterjee	Koen <i>et al</i> (1988)
pAKC501	pDBK519, <i>ona</i> ⁺	Km ^R	A. Chatterjee	Murata <i>et al</i> (1991)

TABLE 2b continued

PLASMID/ COSMID	CHARACTERISTICS	PHENOTYPE	SOURCE	REFERENCE
pMG54	pBR322, <i>cat::Taphan</i>	Ap ^R , Tc ^R K ^R	M. Gibson	Unpublished
cMG54	pMC79:: <i>cat</i> ⁺	Ap ^R , Tc ^R	M. Gibson	Unpublished
pE4	pUC8, <i>puh</i>	Ap ^R	Lab stock	Plasnow <i>et al</i> (1986)
J36161	pUC19, <i>pefD</i>	Ap ^R	Lab stock	Hinton <i>et al</i> (1988)
J36197	pUC19, <i>pefC</i>	Ap ^R	Lab stock	Hinton <i>et al</i> (1989)
pT7-5	T7 ϕ 10 promoter	Ap ^R	F. Reeves	Tabor & Richardson (1985)
pT7-6	T7 ϕ 10 promoter	Ap ^R	F. Reeves	Tabor & Richardson (1985)
pOP1-2	T7 polymerase	K ^R	F. Reeves	Tabor & Richardson (1985)
pT7-5EW100	pT7-5, <i>catC</i>	Ap ^R		This study
pT7-5W200	pT7-5, <i>catX</i>	Ap ^R		This study
pT7-5W0	pT7-5W200 <i>4catX</i>	Ap ^R		This study

TABLE 2b continued

PLASMID/ COSMID	CHARACTERISTICS	PHENOTYPE	SOURCE	REFERENCE
pT7-55W201	pT7-55W200 Δ <i>HindIII</i>	Ap ^R		This study
pT7-55W202	pT7-55W200 Δ <i>SalI</i>	Ap ^R		This study
pT7-55W203	pT7-55W200 Δ <i>EcoRI</i>	Ap ^R		This study
p8W1	pHC-19R, <i>omcC</i>	Ap ^R		This study
p8W2	pBR322, <i>omcX</i>	Ap ^R , Tc ^R		This study
p8W2B	pBR322, <i>omcX</i>	Ap ^R , Tc ^R		This study
PHAGE				
M13 mp18	sequencing vector		Amersham Int	Manning & Vieira (1982)
M13 mp19	sequencing vector		Amersham Int	Manning & Vieira (1982)
λ_{cI} p157	thermoinducible		J. Hinton	Vollenweider <i>et al</i> (1980)
λ_{cI} T4lacZ			F. Keeves	Simon <i>et al</i> (1989)

TABLE 2b continued

PLASMID/ COSMID	CHARACTERISTICS	PHENOTYPE	SOURCE	REFERENCE
$\Delta::\text{TaphoA}$			F. Reeves	Mancil & Beckwith (1985)
ΦKP	SCR1193 bacteriophage		I. Toth	Toth (1992)

Media and solutions are listed in table 2c

TABLE 2c: MEDIA AND SOLUTIONS

Constituents are shown per litre unless stated otherwise. Items shown in brackets were added after autoclaving (121°C, 20 min), from sterile stocks.

Nutrient Broth (NB)	13 g Oxoid nutrient broth
Luria Broth (LB)	10 g Bacto tryptone 5 g Bacto yeast extract (YE) 5 g NaCl (pH 7.2)
SOB	20 g Bacto tryptone 5 g Bacto YE (10 ml 1 M NaCl) (2.5 ml 1 M KCl) (10 ml 1 M MgSO ₄) (10 ml 1 M MgCl ₂) (pH 6.8)
SOC	A _s SOC (+ 20 ml 1 M glucose)
Double Difco (DD) Broth	20 g Bacto tryptone 8 g NaCl

DD: For λ work	As DD (+ 10 ml 20% (w/v) maltose) (+ 10 ml 1 M $MgSO_4$)
2YT	16 g Bacto tryptone 10 g Bacto YE 5 g NaCl
Minimal Medium (MM)	(20 ml 50x phosphate buffer) (10 ml 10% (w/v) $(NH_4)_2SO_4$) (10 ml 1% (w/v) $MgSO_4 \cdot 7H_2O$) (10 ml 50% (w/v) glycerol) (An alternative energy source could be used e. g. 10 ml 20% (w/v) sucrose)

If necessary the above media were solidified with Bacto agar
(added at 16 g per litre)

M9 Salts (10x)	60 g Na_2HPO_4 (anhydrous) 30 g KH_2PO_4 (anhydrous) 10 g NH_4Cl 5 g NaCl (pH 7.4)
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M9 MM	(100 ml 10x M9 salts) (890 ml H ₂ O) (0.1 ml 1 M CaCl ₂) (2 ml ml 0.1 M MgSO ₄) (10 ml 20% glucose) (pH 7.4)
Fusaric Acid (FA) Medium	16 g Bacto agar 10 g Bacto tryptone 5 g Bacto YE 10 g NaCl 2 g glucose 0.05 g chlortetracycline hydrochloride 10 g NaH ₂ PO ₄ ·H ₂ O (6 ml 2 mg/ml fusaric acid) (5 ml 20 mM ZnCl ₂)
50x Phosphate Buffer	= 500 ml 2.7 M K ₂ HPO ₄ = 500 ml 2.7 M KH ₂ PO ₄ Solutions titrated → (pH 6.9-7.1)
Pel Phosphate Buffer	15 g Na ₂ HPO ₄ (anhydrous) 0.7 g NaH ₂ PO ₄ ·H ₂ O (pH 8.0)

Pel MM	(5 ml 20% (w/v) Bacto YE) (10 ml 10% (w/v) $(\text{NH}_4)_2\text{SO}_4$) (1 ml 1 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) (10 ml 50% (v/v) glycerol) (250 ml 2% (w/v) PGA (pH 5.5)) (20 ml 50x phosphate buffer)
Pel Assay Medium	16 g Bacto agar (5 ml 20% (w/v) Bacto YE) (10 ml 10% (w/v) $(\text{NH}_4)_2\text{SO}_4$) (1 ml 1 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) (10 ml 50% (w/v) glycerol) (250 ml 2% (w/v) PGA (pH 5.5)) (200 ml Pel phosphate buffer)
Peh Assay Medium	As Pel assay medium (+ 8 ml 0.25 M EDTA)
Cel Assay Medium	10 g carboxymethyl cellulose (CMC) dissolved by autoclaving (30 mins) 16 g Bacto agar (25 ml 20% (w/v) Bacto YE) (4 ml 50% (v/v) glycerol) (20 ml 50x phosphate buffer) (10 ml 10% (w/v) $(\text{NH}_4)_2\text{SO}_4$) (10 ml 1% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)

Prt Assay Medium	13 g Oxoid nutrient broth 30 g Oxoid gelatin 16 g Bacto agar
Stab Medium	As NB + 7 g Bacto agar
Freezing Medium (2x)	126 g K_2HPO_4 (anhydrous) 0.9 g Sodium citrate 0.18 g $MgSO_4 \cdot 7H_2O$ 1.8 g $(NH_4)_2SO_4$ 3.6 g KH_2PO_4 (anhydrous) 88 g glycerol
Phage Buffer	10 mM Tris base 10 mM $MgSO_4$ 0.01% (w/v) gelatin (pH 7.4)
TE Buffer	10 mM Tris base 1 mM EDTA Adjusted to required pH with HCl
STET Buffer	8% (w/v) sucrose 5% (w/v) Triton-X100 50 mM EDTA 50 mM Tris-HCl (pH 8.0) Not autoclaved

TBE Buffer (10x)	10.8% (w/v) Tris base 5.5% (w/v) Boric Acid 0.93% (w/v) EDTA
Ligation Buffer (10x)	4 mM ATP 66 mM MgCl ₂ 0.1 M DTT 0.66 M Tris-HCl (pH 7.6)
Calf Intestinal Alkaline Phosphatase (CIP) Buffer (10x)	500 mM Tris-HCl (pH 9.0) 10 mM MgCl ₂ 1 mM ZnCl ₂ 10 mM spermidine
SSC (20x)	3 M NaCl 0.3 M Sodium citrate
Blotto	0.5% (w/v) dried milk (Cadbury's Marvel) 1% (w/v) SDS 6% (w/v) PEG 8000 0.27 M NaCl 1 mM EDTA 1.5 mM Sodium phosphate (pH 7.7)
DNA Loading Buffer	0.25% bromophenol blue 40% (w/v) sucrose Stored at 4°C

Gel Mixes: SDS-PAGE.

Acrylamide Stock	30% (w/v) acrylamide 0.8% (w/v) bisacrylamide Filtered (0.45 μ m filter) Stored at 4°C
Lower Buffer (4x)	1.5 M Tris-HCl (pH 8.8) 0.4% (w/v) SDS 0.1% (v/v) TEMED (pH 9.0)
Upper Buffer (4x)	0.5 M Tris-HCl (pH 6.8) 0.4% (w/v) SDS 0.2% (v/v) TEMED (pH 6.8)
Lower Gel (12%)	10 ml 4x lower buffer 16 ml acrylamide stock 13.8 ml H ₂ O 0.2 ml 10% (w/v) ammonium persulphate (AMPS) (freshly prepared)
Stacking Gel (3%)	2.5 ml 4x upper buffer 1.0 ml acrylamide stock 6.4 ml H ₂ O 0.2 ml 10% (w/v) AMPS

Cracking Buffer (2x)	60 mM Tris-HCl (pH 6.8) 1% (v/v) β -mercaptoethanol 10% (v/v) glycerol 0.01% (w/v) bromophenol blue
Running Buffer Stock (RBS) (4x)	1.2% (w/v) Tris base 5.76% (w/v) glycine
Running Buffer (RB) (1x)	1 l 4x RBS 3 l H ₂ O 40 ml 10% (w/v) SDS

Gel mixes: Sequencing Gels

Acrylamide Stock	38% (w/v) acrylamide 2% (w/v) NN-methylenebisacrylamide 2% (w/v) Amberlite mixed bed resin Filtered. Stored at 4°C.
0.5x TBE 6% Gel Mix (500 ml)	75 ml 40% acrylamide stock 25 ml 10x TBE 230 g urea (BRL: ultrapure) Filtered. Stored at 4°C: 3-4 weeks.
5.0x TBE 6% Gel Mix (200 ml)	30 ml 40% acrylamide stock 100 ml 10x TBE 92 g urea (BRL: ultrapure) 10 mg Bromophenol blue

Chemicals were obtained from Sigma, BDH or Fisons, unless stated otherwise.

Antibiotics used, their abbreviations and the concentrations used are listed in table 2d.

TABLE 2d: ANTIBIOTICS

ANTIBIOTIC	ABBREV.	FINAL CONC. ($\mu\text{g/ml}$)
Sodium ampicillin	(Ap)	50
Chloramphenicol	(Cm)	50
Kanamycin sulphate	(Kn)	50
Rifampicin	(Rif)	50
Spectinomycin	(Spc)	50
Streptomycin sulphate	(Str)	100
Tetracycline	(Tc)	10

All antibiotics were prepared as 100x final concentration stocks. Rif, Tc and Cm were dissolved in 50% (v/v) ethanol (EtOH) and stored, in the dark at -20°C . The other antibiotics were dissolved in sterile ELGA water and stored at 4°C .

The size markers used in the electrophoresis of DNA and proteins are listed in table 2e.

TABLE 2e: SIZE MARKERS

SIZE MARKERS FOR AGAROSE GEL ELECTROPHORESIS OF DNA (KDa)

λ *Hind*III/ *Eco*RI

21	1.65
5	1.38
4.6	0.95
4.2	0.8
3.5	0.565
2	0.125

1 Kb Ladder (BRL)

12.216	6.108	1.018	0.220
11.198	5.090	0.517	0.201
10.180	4.072	0.506	0.154
9.162	3.054	0.396	0.134
8.144	2.036	0.344	0.075
7.126	1.1636	0.298	

SIZE MARKERS FOR SDS-PAGE OF PROTEINS

Rainbow Markers (Amersham: Mid Range)

PROTEIN	COLOUR	KDa
Myosin	Blue	200
Phosphorlase b	Brown	97.4
Bovine serum albumin	Red	69
Ovalbumin	Yellow	46
Carbonic anhydrase	Orange	30
Trypsin inhibitor	Green	21
Lysozyme	Magenta	14.3

2.1 PLATE ASSAYS FOR ENZYME ACTIVITY.

2.1.1 INOCULATION AND INCUBATION.

Single, fresh colonies were picked on to assay media with sterile tooth picks. Alternatively, as discussed later (7.3.2), wells (5 mm diameter, made in the plates using a Pasteur pipette), were filled with 28 μ l samples of culture supernatant or sonicate. The plates were incubated at 30°C overnight and developed as described below.

2.1.2 DEVELOPING ASSAY PLATES.

2.1.2.1 PROTEASE (PRT) ASSAYS.

The method, derived from that of Thum and Chatterjee (1985), involved flooding the plates with 4M $(\text{NH}_4)_2\text{SO}_4$. Pr^+ colonies produced clear zones on an opaque white background.

2.1.2.2 CELLULASE (CEL) ASSAYS.

The method was modified from that of Gilkes *et al* (1984). Plates were flooded with 0.2% (w/v) congo red (Sigma) solution for 20 min, bleached with 1M NaCl for 15 min, then stained with 1M HCl for 5 min. Cel⁺ colonies were identified by translucent red halos on a dark blue background.

2.1.2.3 PECTATE LYASE (PEL) ASSAYS.

The method, derived from that of Andro *et al* (1984), involved flooding the plates with 7.5% (w/v) copper acetate solution for 1-2 hr. Pel⁺ colonies produced double, cream halos on a translucent blue background.

2.1.2.4 POLYGALACTURONASE (PEH) ASSAYS.

This assay is discussed in more detail later (7.3.2). The plates were developed as for the Pel assay. The addition of EDTA to the media allowed a distinction to be made between Peh and Pel activity. Peh⁺ colonies gave single, cream halos on the blue background.

2.2 DETECTION OF GENE FUSIONS USING INDICATOR PLATES.

2.2.1 ALKALINE PHOSPHATASE (PHOA).

Colonies were streaked on to NBA (Bacto agar) plates containing the appropriate antibiotic(s) and 40 µl/ml XP: 5-bromo-4-chloro-3-indolyl phosphate (Nova Biochem., freshly prepared as a 20 mg/ml stock in DMF). After incubation overnight (30°C for *Ecc*, 37°C for *E.coli*), PhoA⁺ colonies were blue.

2.2.2 β-GALACTOSIDASE (LACZ).

Colonies were streaked on to NBA (Bacto agar) plates containing the appropriate antibiotic(s) and 40 µl/ml X-Gal: 5-bromo-4-chloro-3-indolyl β-D-galacto-pyranoside

(Nova Biochem., freshly prepared as a 20 mg/ml stock in DMF. After incubation overnight, LacZ⁺ colonies were blue.

2.3 SAMPLING THROUGHOUT THE GROWTH CURVE OF *ECC*.

2.3.1 FOLLOWING THE GROWTH CURVE.

An overnight culture of HC131 was grown up in 5 ml PM with Ap selection (New Brunswick Rollordrum, 30°C, 275rpm) and 1 ml of this was used to inoculate 50 ml PM in a 250 ml flask. The culture was incubated at 30°C, 275 rpm (New Brunswick Aquatherm), removing 1 ml samples at hourly intervals. Optical density (600 nm) was measured (Corning Spectrophotometer 258) and a graph was plotted, showing absorbance (600 nm) against time (hr) after inoculation. Samples (5 ml) were removed for fractionation at early, mid and late log and stationary phase (as determined from the graph).

2.3.2 CELL FRACTIONATION BY COLD OSMOTIC SHOCK.

This method was derived from that of Neu and Heppel (1965). A 5 ml sample (an overnight culture, or a sample removed from a larger culture as described above) was combined with 0.5 ml 0.5 M Tris-HCl (pH 7.8) and incubated at room temperature (RT) for 10 min, before being centrifuged (MSE Chilspin, 5,000 rpm, 5 min, 4°C). The resulting supernatant (S/N) fraction was stored on ice, while the pellet was washed in PM, then resuspended in 800 µl sucrose solution (30 mM Tris-HCl pH 7.8, 40% sucrose, 2 mM EDTA). The sample was transferred to an Eppendorf tube, incubated at 30°C for 10 min, then centrifuged (MSE Micro Centaur, high speed, 1 min). The S/N was carefully removed and discarded. The pellet was immediately resuspended in 500 µl ice-cold water, incubated on ice for 10 min, then centrifuged (MSE Micro Centaur, high speed, 1 min). The S/N, i.e. the periplasmic fraction, was removed and stored on ice.

The pellet was resuspended in 5 ml Tris-HCl (50 mM pH 7.8) and transferred to a 25 ml glass beaker on ice. The sample was sonicated (MSE sonicator, 3/4 inch probe, amplitude: 6 microns) for 3x 30 seconds (sec) with a cooling interval of 30 sec between bursts. Cell debris was removed by centrifugation (MSE Chilspin, 5,000 rpm, 10 min, 4°C) and the resulting sonicate (cytoplasmic fraction) was stored on ice.

Samples were either used fresh or frozen at -20°C, in several aliquots to avoid re-freezing.

For some experiments, only S/N and sonicate samples were required. After the S/N had been removed, the cell pellet was washed, then resuspended in 5 ml Tris-HCl and sonicated as above.

2.3.3 CELL FRACTIONATION BY THE PRODUCTION OF SHOCKATES.

A 5 ml culture was mixed with 0.5 ml 0.5 M Tris-HCl (pH 7.8), incubated at room temperature for 10 minutes, centrifuged (MSE Chilspin, 5,000 rpm, 5 min, 4°C), and the S/N was decanted and stored on ice. The cell pellet was washed with 5 ml salt wash buffer (10 mM Tris-HCl pH 7.5, 30 mM NaCl), resuspended in 6 ml buffered sucrose (30 mM Tris-HCl pH 7.3, 20% sucrose) and incubated in a shaking waterbath (25°C, 275 rpm, 10 min). After centrifugation (MSE Chilspin, 5,000 rpm, 5 min, 4°C), the pellet was resuspended in 2 ml cold 0.5 mM MgCl₂ and incubated in a shaking ice/water bath (275 rpm, 10 min). Centrifugation (MSE Chilspin, 5,000 rpm, 5 min, 4°C) produced the 'shockate' or periplasmic fraction (stored on ice) and a cell pellet, which was resuspended in 5 ml Tris-HCl (50 mM, pH 7.8) then sonicated. Sonication, and the subsequent removal of cell debris, were carried out as described in the cold shock protocol. Samples were used fresh, or frozen in aliquots at -20°C.

2.3.4 CONCENTRATION OF SUBCELLULAR FRACTIONS.

A 10 ml culture was centrifuged (MSE Chilspin, 5,000 rpm, 10 min, 4°C) and the S/N was decanted into a centriprep tube (Amicon MW 30,000 cut off), used according to the manufacturer's instructions. After three successive centrifugations (MSE Hi-Spin

21, 3,000 rpm, 10 min, 4°C) the 10 ml sample was concentrated down to 0.5 ml (approx.). Centrifugation speeds higher than those recommended by the manufacturer were required, because of the viscosity of PM caused by the PGA.

The cell pellet, (stored on ice) was resuspended in 0.5 ml Tris-HCl (50 mM pH7.8) and transferred to a bijou bottle. After sonication (MSE sonicator, 1/8 inch probe, amplitude: 12 microns) for 6x 30 sec with a 30 sec cooling interval between bursts, the sample was transferred to an Eppendorf tube and centrifuged (MSE Micro Centaur, high speed, 2 min) to remove cell debris. Samples were frozen, in aliquots at -20°C.

Had it been necessary, the periplasmic fraction could have been isolated separately as described previously (2.3.2, 2.3.3) and concentrated using centriprep tubes.

2.4 DETERMINATION OF PROTEIN CONCENTRATION.

The protein content of a sample was calculated by a method based on that of Bradford (1976). Bio-Rad protein assay reagents were used, following the manufacturer's instructions for the microassay: designed for protein samples of 1-20 μg ($< 25 \mu\text{g/ml}$).

Standards were prepared by making a series of bovine serum albumin (BSA) solutions in the range 0-20 $\mu\text{g/ml}$. To 0.8 ml standard solution or sample in an Eppendorf tube, 0.2 ml dye reagent (a mixture of a dye, phosphoric acid and methanol) was added. The tubes were mixed by inversion and left at RT for 5-60 min before the optical densities (OD), (595 nm) were read (Philips PU 8720 UV/vis scanning spectrophotometer). The BSA readings were used to construct a standard curve, from which the sample protein concentrations were calculated. The results were expressed as mg protein /ml.

2.5 SPECTROPHOTOMETRIC ENZYME ASSAYS.

Enzyme assays were carried out on samples of S/N, periplasm and cytoplasm (obtained as described previously: 2.3.2 and 2.3.3).

2.5.1 β -LACTAMASE (Bla).

The method was based on that of O'Callaghan *et al* (1972). Sample volume depended on the cell fraction being assayed: 100 μ l of S/N or cytoplasm, 2 μ l of periplasm, or 20 μ l if the entire cell sample (including the supernatant) had been sonicated together. The volume was made up to 800 μ l with 0.1 M phosphate buffer (pH 7.0). 20 μ l of the chromogenic substrate nitrocephin (Becton Dickinson Microbiology Systems (Glaxo), freshly prepared at 4 mg/ml in DMSO) was added and the OD (500 nm) was followed at 30°C on a Philips PU 8720 spectrophotometer (integration time: 30 sec). The control sample consisted of 800 μ l phosphate buffer and 20 μ l nitrocephin. After correcting for this 'rate', activity was expressed as $\Delta A_{(500)}/\text{min}/\text{ml}$.

2.5.2 β -GALACTOSIDASE (LacZ).

The method was based on that of Miller (1972). The basic assay procedure is set out below; various modifications to this are discussed later (3.3.3)

Samples (250 μ l S/N, 5 μ l periplasm, or 50 μ l sonicate) were added to Z-buffer, to give a final volume of 500 μ l. The blank consisted of 500 μ l Z-buffer only.

Aliquots (100 μ l) of ONPG (orthonitrophenyl- β -D-galactopyranoside, Sigma: 4 mg/ml, freshly prepared in Z-buffer) were multidispensed into the samples, noting the time. Samples were incubated (37°C) until they became a faint yellow colour (typically between 140 min for supernatants and 40 min for sonicates). Reactions were stopped by the addition of 250 μ l 1M Na_2CO_3 , and the time was again noted.

The ODs of the samples were read at 420 nm (RT, Philips PU 8720 spectrophotometer) and activities were expressed as $\Delta A_{(420)}/\text{min}/\text{ml}$, after correcting for the blank.

Z-Buffer (constituents per litre).

Na ₂ HPO ₄ (anhydrous)	8.52 g
NaH ₂ PO ₄ ·2H ₂ O	6.24 g
KCl	0.75 g
MgSO ₄ ·7H ₂ O	0.25 g
β-mercaptoethanol	2.7 ml
(pH 7.0)	
Not autoclaved.	

2.5.3 GLUCOSE 6-PHOSPHATE DEHYDROGENASE (G6-PDH).

The assay, based on that of Langdon (1966) measured NADP⁺ reduction via the increase in absorbance at 340 nm.

The sample (60 μl) was added to 120 μl reaction mix and 720 μl sterile ELGA water in a quartz cuvette. The OD was followed at 340 nm (UV, room temperature, Philips PU 8720 spectrophotometer, integration time: 30 sec). Activity was expressed as Δ A₍₃₄₀₎/min/ml, after correction for the blank (containing growth medium instead of the sample).

G6-PDH Reaction Mix (constituents per 4ml)

1 M Tris-HCl (pH 7.5)	1 ml
25 mM G6-P (fresh)	1 ml
0.2M MgCl ₂	1 ml
2 mM NADP ⁺ (pH 7.5, fresh)	1 ml

Freshly prepared.

2.5.4 6-PHOSPHOGLUCONATE DEHYDROGENASE (6-PGDH).

The assay, based on that of Ng and Dawes (1973) measured NADP⁺ reduction via the increase in absorbance at 340 nm.

The sample (60 μ l) was added to 495 μ l reaction mix and 345 μ l sterile ELGA water in a quartz cuvette. The OD was followed at 340 nm (UV, RT, Philips PU 8720 spectrophotometer, integration time: 2 min). Activity was expressed as $\Delta A_{(340)}/\text{min/ml}$, correcting for the blank as above.

6-PGDH Reaction Mix (constituents per 6.85 ml)

120 mM Tris-HCl (pH 7.6)	5.25 ml
10 mM 6-phosphogluconate (fresh)	0.1 ml
10 mM NADP ⁺	0.75 ml
259 mM MgCl ₂	0.75 ml

Freshly prepared.

2.5.5 PECTATE LYASE (Pel).

The assay, based on that of Starr *et al* (1977) measured the production of 4,5-unsaturated breakdown products of polygalacturonic acid (PGA), via an increase in absorbance at 235 nm.

The sample (21.5 μ l) was added to 876 μ l reaction mix (preheated to 37°C) in a quartz cuvette and the OD was followed at 235 nm (UV, 37°C, Philips PU 8720 spectrophotometer, integration time: 1 min). Activity was expressed as $\Delta A_{(235)}/\text{min/ml}$, after correcting for the blank (containing growth medium instead of the sample).

Pel Reaction Mix (constituents per 7.78 ml)

0.575% PGA (pH 5.5)	3.20 ml
H ₂ O (ELGA)	1.13 ml
Pel reaction buffer	3.45 ml

PeI Reaction Buffer (constituents per 100 ml)

1 M Tris-HCl (pH 8.5)	23 ml
1 M CaCl ₂	78 μ l
H ₂ O (ELGA)	77 ml

2.5.6 POLYGALACTURONASE (Peh).

The increase in reducing groups, occurring when oligo-galacturonates were released from PGA, was detected using the copper arsenomolybdate reagent (Nelson 1944, Somogyi 1952). The optimization of this procedure is discussed later (7.3.1). The standard method, as described previously (Collmer *et al* 1988) is given here.

The sample (50 μ l) was combined with 450 μ l Peh assay buffer and 500 μ l 1% PGA (pH 5.5), and mixed by vortexing. A 200 μ l aliquot was removed (zero time sample) and added to 200 μ l copper reagent (in a 2 ml Eppendorf tube). The remaining reaction mixture was incubated (30°C, 30 min), before a further sample was removed and mixed with copper reagent.

Once combined with copper reagent, samples were heated in a boiling water bath (15 min) then allowed to cool. Colour reagent (200 μ l) was added and the samples were shaken carefully until effervescence had stopped. ELGA water (1.2 ml) was added and the samples were centrifuged (MSE Micro Centaur, high speed, 2 min). The S/Ns were removed and their ODs were measured at 500 nm (Philips PU 8720 spectrophotometer). Activity was expressed as $\Delta A(500)/\text{min.}/\text{ml}$, after correcting for the blank (containing growth medium instead of the sample).

Peh Assay Buffer (constituents per 180 ml)

4 M NaCl	20 ml
1 M Na Acetate (pH5.0)	20 ml
0.5 M EDTA	1.6 ml
H ₂ O	138.4 ml

Peh Copper Reagent (constituents per litre)

Na_2CO_3	24 g
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Potassium sodium

tartrate	12 g
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Dissolved in 250 ml H_2O

10% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	40 ml
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NaHCO_3	16 g
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Added to above solution

Na_2SO_4	18 g
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Boiled in 500 ml H_2O (30 min) then added to above solution

Made up to 1 litre. Not autoclaved.

Peh Colour Reagent (constituents per 500 ml)

Ammonium molybdate	25 g
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Dissolved in 450 ml H_2O (30 min)

Conc. H_2SO_4	21 ml
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Added to above solution.

Sodium arsenate	3 g
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Dissolved in 25 ml H_2O , then added to above solution.

Incubated (37°C, 48 hr) to allow yellow/green colour to develop.

2.5.7 ALKALINE PHOSPHATASE (PhoA).

The method was based on that of Brickman and Beckwith (1975). The sample (100 μl) was mixed with 900 μl 1 M Tris-HCl (pH 8.0) and 100 μl Sigma 104[®] (4 mg/ml,

freshly prepared in 1 M Tris-HCl pH 8.0) was added, noting the time. The mixture was incubated (37°C) until a faint yellow colour appeared (typically 30-140 min), when the reaction was stopped by the addition of 100 μ l 1 M K_2HPO_4 (filter sterile), noting the time. The OD was measured at 420 nm (Philips PU 8720 spectrophotometer) and activity was expressed as $\Delta A_{(420)}/\text{min}/\text{ml}$, after correcting for the blank (containing growth medium instead of the sample).

* Sigma 104: p-nitrophenyl phosphate, disodium salt

2.6 PREPARATION OF λ LYSATES.

2.6.1 HIGH TITRE LYSATES.

A phage stock was prepared by resuspending two fresh plaques in 1 ml phage buffer. Chloroform (50 μ l) was added and the sample was vortexed (Scientific Industries Inc. Vortex Mixer VM20, 1 min) and centrifuged (MSE Micro Centaur, high speed, 2 min). The S/N was recovered and stored at 4°C.

Lysates were prepared on the suppressing host LE392. This was grown in 10 ml LB (10 mM $MgSO_4$) until late log phase, when 200 μ l samples were removed and infected with 10-100 μ l phage stock (prepared as described above). After incubation (RT, 10 min), 3 ml molten 0.25% DDA agar (45°C) was added and the samples were overlaid on to thick, wet, fresh DDA plates. Lysis was observed after incubation at 37°C overnight.

The top agar was washed off the plate with 2 ml phage buffer, combined with 0.3 ml chloroform, then vortexed for 30 min and centrifuged (MSE Chilspin, 5,000 rpm, 10 min, 4°C). The supernatant was decanted and stored over chloroform at 4°C. The lysate titre was determined by infecting 200 μ l of a late log phase culture of LE392, with 100 μ l of serially diluted lysate samples and making 0.75% DDA overlays. The lysate titre was calculated after incubation at 37°C overnight. The method yielded $1-5 \times 10^{10}$ plaque forming units (pfu) per ml.

2.6.2 THE GENERATION OF LYSATES BY SUPERINFECTION.

A 25 ml culture of *E. coli* CC118 (carrying the cosmid to be packaged) was grown to mid log phase in LB with antibiotic selection in a 200 ml flask (New Brunswick Aquatherm waterbath, 37°C, 300 rpm). As fast growth was essential, good aeration was required and antibiotics which retard the growth of resistant strains e.g. tetracycline (Tc) were avoided where possible.

Magnesium was added to 10 mM, along with 250 μ l λ cl⁸⁵⁷ lysate stock and the culture was incubated at 30°C, 275 rpm for 20-30 min. The cells were heat shocked (stationary waterbath, 42°C, 20 min), then incubated (38°C, 275 rpm) for about 30 min after the first signs of lysis were detected (3-4 hr in total). The sample was transferred to a universal tube with 1 ml chloroform and vortexed (Scientific Industries Inc. Vortex Mixer VM20) for 1 hr. After centrifugation (MSE Chilspin, 5,000 rpm, 10 min, 4°C), the S/N was decanted into a fresh tube and magnesium was added to 10 mM. The lysate was stored over 1 ml chloroform, at 4°C.

The titre of lysates prepared by this method tended to be slightly lower and more variable than for lysates prepared by the plate method. The main advantage of superinfection was that large volumes of the lysate could easily be obtained. If necessary, the titre could be raised by PEG precipitation.

2.6.3 PEG PRECIPITATION OF PHAGE.

The lysate was mixed with 25% PEG, 3 M NaOH solution (1 ml/4 ml lysate) in an Oakridge tube and incubated on ice (1 hr). Phage were pelleted by centrifugation (MSE Hi-Spin 21, 15,000 rpm, 10 min, 4°C), resuspended in a suitable volume of phage buffer and stored over chloroform at 4°C.

2.7 TRANSDUCTION.

The method was essentially the same for the transduction of *E. coli* and *Erwinia*, the only difference being the media used: LBM (LB containing 10 mM Mg²⁺ and 0.2% maltose for *E. coli*; LB with added Mg²⁺ only for *Ecc*).

The recipient strain was grown to mid log phase in 10 ml supplemented LB with antibiotic selection. The cells were pelleted (Labor-50M, 4,500 rpm), resuspended in 1 ml LBM and infected with 100 μ l λ lysate (1x 10¹⁰ pfu/ml approx.). After incubation (30°C, stationary, 1 hr), 10 ml LBM was added and incubation was continued (30°C, 275rpm, 1 hr). The cells were then pelleted, resuspended in 450 μ l LB, divided into three samples and spread on to LB plates with antibiotic selection. Plates were incubated at 30°C (*Erwinia*) or 37°C (*E. coli*) overnight.

2.8 PREPARATION OF PLASMID DNA

2.8.1 RAPID PLASMID ISOLATION FROM *E. COLI* BY BOILING.

The method was based on that of Holmes and Quigley (1981). A 5 ml overnight (grown in NB broth with antibiotic selection, at 37°C, 275 rpm), was centrifuged, (Labor-50M, 4,500 rpm, 10 min) and the cell pellet was resuspended in 300 μ l STET buffer in an Eppendorf tube. Alternatively, several inoculating loopfuls of cells were scraped off a NBA antibiotic plate and resuspended in 200 μ l STET buffer.

Lysozyme was added (10 μ l, 10 mg/ml), the sample was incubated on ice for 20-30 min, then heated in a 95°C waterbath for 3 min. The tube was immediately centrifuged (MSE Micro Centaur, high speed, 10 min) and the gelatinous pellet was carefully removed with a sterile toothpick and discarded.

The DNA was precipitated with 0.8 volume of cold isopropanol (an isopropanol: DNA solution ratio of less than 1:1 was used to avoid the precipitation of RNA and protein in addition to DNA). The dried DNA pellet was resuspended in 100 μ l TE buffer (pH 8.0), extracted with 0.5 volume of phenol/chloroform, then reprecipitated by the addition of 8 μ l 3 M NaOAc (pH 5.5) and 200 μ l cold ethanol, followed by

incubation at -20°C for 30 min and centrifugation (MSE Micro Centaur, high speed, 5 min). The resulting DNA pellet was dried (55°C , 10 min) and resuspended in TE buffer (pH 8.0), containing DNase-free pancreatic RNase (Sigma, $20\ \mu\text{g}/\text{ml}$). The volume chosen for resuspension depended on the copy number of the plasmid; for example $100\ \mu\text{l}$ was used for pUC-based plasmids, while $80\ \mu\text{l}$ was used for plasmids based on pBR322.

DNA prepared by this method was suitable for restriction analysis, using $4\ \mu\text{l}$ of the DNA solution per digest.

2.8.2 RAPID PLASMID ISOLATION FROM *ERWINIA* SPP.

$2.5\ \text{ml}$ of an overnight culture (grown in NB broth with antibiotic selection, at 30°C , 275 rpm) was centrifuged (Labor 50-M, 4,500 rpm, 5 min). The cell pellet was washed in $2.5\ \text{ml}$ phage buffer, resuspended in $500\ \mu\text{l}$ lysozyme mix (0.3 M sucrose, 25 mM Tris (pH 8.0), 25 mM EDTA + lysozyme: 2 mg/ml, added immediately before use) and transferred to an Eppendorf tube. The sample was incubated on ice for 20 min, then at 37°C for 10 minutes. After gentle mixing, $250\ \mu\text{l}$ freshly prepared alkaline SDS solution (0.3 M NaOH, 2% SDS) was added. The tube was vortexed (VM20) until cell lysis had occurred, then incubated, with the lid off, at 65°C for 10 min, vortexing briefly after 5 min. The sample was cooled on ice, then extracted with $80\ \mu\text{l}$ acid phenol/chloroform (i.e. phenol/chloroform not equilibrated with buffer).

At this stage, the DNA could be used for restriction analysis, although the following purification steps were normally carried out. The DNA was precipitated by the addition of $70\ \mu\text{l}$ unbuffered NaOAc and $700\ \mu\text{l}$ isopropanol, followed by incubation at RT for 5 min and centrifugation (MSE Micro Centaur, high speed, 2 min). The DNA pellet was dried (55°C , 10 min.) and dissolved in $50\ \mu\text{l}$ TE buffer (pH 8.0). The sample was extracted with $25\ \mu\text{l}$ neutral phenol/chloroform, then precipitated with $5\ \mu\text{l}$ 3 M unbuffered NaOAc and $50\ \mu\text{l}$ isopropanol. The DNA pellet was redissolved in TE buffer and the phenol extraction and precipitation steps were

repeated. The resulting DNA pellet was dried (55°C, 10 min.), resuspended in 50 μ l TE buffer (pH 8.0), containing DNase-free RNase (20 μ g/ml) and stored at 4°C. For restriction digests, 5 μ l samples of the DNA solution were used.

2.8.3 LARGE SCALE PLASMID ISOLATION FROM *E. COLI* USING CAESIUM CHLORIDE GRADIENT PURIFICATION.

A 250 ml culture was grown in NB with antibiotic selection (New Brunswick incubator shaker, 37°C, 275 rpm). Plasmids based on pBR322 were amplified by the addition of 75 mg spectinomycin when the culture O.D. (600 nm, Corning spectrophotometer 258) had reached 0.4 (approx.), followed by incubation for a further 4-5 hours.

The culture was centrifuged (MSE Hi-Spin 21, 8,000 rpm, 20 min, 4°C). The cell pellet was resuspended in 9 ml Tris-sucrose solution (0.05 M Tris, 25% sucrose, pH 8.0) and transferred to an Oakridge tube (Nalgene) on ice. Lysozyme was added (2.5 ml at 5 mg/ml: freshly prepared in 0.25 M Tris-HCl pH 8.0) and the tube was left on ice for 5 min. EDTA (2.25 ml, 0.25 M, pH 8.0) was added and the sample returned to ice for 5 min. 9 ml lysis mix (0.05 M Tris, 0.063 M EDTA, 2% Brij 58, 0.4% Na Deoxy, pH 8.0) was added and the tube was incubated at 42°C, with occasional inversion, until cell lysis had occurred.

The sample was centrifuged (MSE Hi-Spin 21, 18,000 rpm, 30 min, 4°C) and 15 ml S/N was pipetted off, taking care to avoid the fragile pellet. CsCl was added (1 g/ml DNA solution), mixed, and EtBr was added (0.8 ml of 10 mg/ml solution per 10 ml CsCl/DNA solution). The sample was loaded into 5 ml Quick Seal tubes (Beckman), which were sealed (Beckman tube sealer) and centrifuged (Beckman L8-70M Ultracentrifuge, Vti65 rotor, 60,000 rpm, overnight i.e. 16 hr approx., 22°C).

The band of plasmid DNA, easily visible under long wave UV light (with a greater density than that of the chromosomal DNA), was removed with a syringe and 0.8 mm hypodermic needle, using a 0.6 mm needle in the top of the tube to prevent the formation of a vacuum. EtBr was removed from the DNA by treatment with water-

saturated 1-butanol: added in equal volume, mixed and removed, repeatedly until the DNA solution was no longer pink.

CsCl was normally removed by precipitation: mixing with 2 volumes sterile ELGA water and 6 volumes ethanol, incubating at -20°C for 10 min, then centrifuging (MSE Hi-Spin 21, 10,000 rpm, 10 min, 4°C). The DNA pellet was washed with 70% ethanol, dried (55°C , 10 mins.) and resuspended in 1 ml TE buffer (pH 8.0). For restriction digests, 2 μl samples were used.

For experiments requiring particularly pure DNA, CsCl was removed by dialysis. Dialysis tubing, cut into strips of the required length, was twice boiled for 10 min in 1 mM EDTA, rinsing in ELGA water in between. Once cool, the tubing was stored in 1 mM EDTA at 4°C , and rinsed thoroughly in ELGA water before use. DNA was dialysed in pre-prepared tubing in 2 l ELGA water, stirring gently at 4°C . The water was changed every hr for 3-4 hr, then replaced by TE buffer (pH 8.0) and dialysis was continued overnight.

2.9 SPECTROPHOTOMETRIC QUANTITATION OF DNA.

The method, that of Maniatis *et al* 1982, required that the DNA sample was reasonably pure (i.e. without significant amounts of protein, phenol or agarose).

The absorbance (of a 10^{-2} dilution) was read at 260 nm and at 280 nm (Philips PU 8720 spectrophotometer). The nucleic acid concentration was calculated from the value obtained at 260 nm. An OD of 1.0 corresponds to 50 $\mu\text{g}/\text{ml}$ (approx.) for double stranded DNA, 40 $\mu\text{g}/\text{ml}$ for single stranded DNA or RNA and 20 $\mu\text{g}/\text{ml}$ for oligonucleotides. An estimate of nucleic acid purity was obtained from the ratio of $\text{OD}_{260}/\text{OD}_{280}$: pure samples of DNA and RNA giving values of 1.8 and 2.0 respectively. Low values, due to contamination with protein or phenol indicated that the quantitation of nucleic acid was inaccurate.

2.10 INTRODUCTION OF FOREIGN DNA INTO CELLS.

2.10.1 TRANSFORMATION OF *E. COLI*.

The procedure was carried out on ice where possible, using pre-chilled solutions. A 20 ml culture (grown to exponential phase in NB broth, 25 ml flask, New Brunswick G24 Incubator Shaker, 37°C, 275 rpm) was chilled on ice for 20 min, before being centrifuged (MSE Chilspin, 5,000 rpm, 10 min, 4°C). The cells were washed in 0.1 M MgCl₂, resuspended in 0.1 M CaCl₂ (Sigma Grade I), chilled on ice for 20 min, pelleted as before and resuspended in 1 ml 0.1 M CaCl₂.

The competence of the cells improved if they were kept on ice for 2 hr before use, and did not decrease significantly if stored for up to 2 days at 4°C. For longer storage the cell suspension was made up to 15% glycerol and kept at -70°C. Competent cells prepared by this method were suitable for transformation with DNA isolated by the small scale, or caesium chloride gradient techniques (2.8).

The DNA (10-50 ng, typically 2 µl from a small scale prep.) was added to 100 µl competent cells and mixed briefly by vortexing (VM 20). After incubation on ice for 30 min (with occasional mixing), the cells were 'heat shocked' at 42°C for 2 min. If gene expression time was required, 1 ml NB was added and the sample incubated for 1 hr (37°C, 275 rpm) before being plated on to selective media.

2.10.2 PLASMID MOBILIZATION INTO *ERWINIA* SP.

pBR-plasmids were introduced into *Ecc* via *E. coli* GJ342, by a method based on that of van Haute *et al* (1983). GJ342 carries two helper plasmids: pLVC9 (a Cm^R version of pGJ28: used by van Haute *et al*) and R64*drd*11 (Tc^R), providing the ColE1 *mob* and *tra* functions respectively.

GJ342 was transformed (2.10.1) with the desired plasmid, plating on to NBA containing Cm, Tc and an antibiotic (usually Ap) to select for the pBR-based plasmid, and growing at 37°C overnight. The donor strain obtained was patch mated with the

Ecc recipient: mixing an inoculating loopful of each on a NBA plate, and incubating at 30°C overnight.

To select for *Erwinia* transconjugants, several inoculating loopfuls of cells were streaked on to minimal sucrose (MMS) Ap plates. After incubation at 30°C for 2-3 days, single colonies were picked and restreaked (on to MMS, Ap) to ensure that there was no *E. coli* carry-over.

2.10.3 ELECTROPORATION.

Extracellular DNA may be introduced into recipient cells by treating them with high-voltage pulses: a method which has been shown to be successful in a variety of species (Dower *et al* 1988, Luchansky *et al* 1988). For the introduction of DNA into *Erwinia*, electroporation was used in favour of both transformation and conjugation, due to the relative speed and efficiency of the procedure. The method was also used for *E. coli* when high efficiency was required.

A 10 ml culture (grown overnight in NB with antibiotic selection at 30°C (for *Erwinia*), 275 rpm) was centrifuged (Labor-50M, 4,500 rpm, 10 min). The cells were washed twice with 10 ml sterile ELGA water, then twice with 1 ml 10% glycerol. Finally the cells were resuspended in 200 µl sterile ELGA water, divided into 40 µl aliquots ready for use, and placed on ice until they were needed. This protocol could be scaled up to use a 1 l culture, resuspending the cells in 2-3 ml (total), 10% glycerol (divided into 100 µl aliquots ready for use), for storage at -70°C. These cells could be kept for up to 6 months without any significant loss of electroporation efficiency.

As DNA used for electroporation had to be free from salts, it was resuspended in sterile ELGA water rather than TE buffer as usual. The DNA (5-50 ng) was added to an aliquot of cells and incubated on ice for 1-10 min. The mixture was then transferred to a pre-chilled electroporation cuvette (0.2 cm electrode, Bio-Rad), shaken down so that it formed an even layer over the cuvette base without any bubbles, and replaced on ice.

Electroporation was carried out using a Bio-Rad Gene Pulser, according to the manufacturer's instructions (pulse controller resistance: 200 ohms, capacitance: 25 μ FD, voltage: 2.5 Kv). Ideally, time constants of 4.8 were obtained. Immediately after pulsing, 1 ml 2YT medium was added to each sample, which was incubated (1 hr at 30°C, 200 rpm) to allow recovery, before being plated on to selective media.

2.11 SELECTION FOR PLASMID LOSS IN *ERWINIA* SP.

For the removal of pHCP2 from *Ecc*, a culture was grown up (5 ml NB, without Ap, at 30°C, 275 rpm, overnight), then subcultured and incubated as before. Samples of the resulting culture were streaked on to NBA plates (without Ap), which were incubated at 30°C overnight. Single colonies were picked and screened for sensitivity to Ap (i.e. for the loss of pHCP2). This method gave a percentage curing of 20-25%.

2.12 FUSARIC ACID SELECTION FOR TRANSPOSON LOSS (for use against transposons encoding Tc resistance, to create deletions).

The method (used to remove *Tn10*), was derived from that of Bochner *et al* (1980). It relied on the fact that the Tc in the fusaric acid (FA) medium was not toxic (having been autoclaved), but still induced Tc resistance genes. Any cells producing the Tc^R proteins were killed by FA. $Tn10^-$ strains were therefore FA^R .

The strain of *Ecc* carrying a *Tn10* insertion was grown in 10 ml LB overnight (275 rpm, 30°C). The culture was serially diluted and 100 μ l aliquots of each dilution (neat, 10^{-2} , 10^{-4} , 10^{-6} , and 10^{-8}) were spread on to FA plates, which were incubated at 30°C until colonies appeared (16-48 hrs). FA^R colonies were streaked on to FA plates, incubating at 30°C as before. When single colonies were screened, 90-100% were found to be Tc^S , showing that *Tn10* had been lost. Further screening was then carried out to confirm that the loss of *Tn10* had created a deletion.

Control strains were always plated out for comparison. A $Tn10^+$ (Tc^R) strain should have been sensitive to FA, whereas a $Tn10^-$ (Tc^S) strain should have been FA^R .

2.13 RESTRICTION ENDONUCLEASE DIGESTION OF DNA.

Digests were set up according to the method of Maniatis *et al* 1982. The appropriate 'React' buffer (BRL 10x concentration) was added to the DNA solution, followed by the restriction enzyme(s). Sterile ELGA water was added to bring the enzyme concentration to 10%. Samples were normally incubated at 37°C for 1 hr, although vector DNA to be used for cloning was left for at least 3 hr. *Sma*I digests were incubated at 30°C overnight.

2.14 PHENOL EXTRACTION OF DNA.

Phenol/chloroform was prepared by dissolving 100 g phenol and 100 mg 8-hydroxyquinoline in a mixture of 100 ml chloroform and 4 ml iso-amyl alcohol. The solution was equilibrated with two changes of 0.2 volume 1 M Tris-HCl (pH 8.0), then two changes of 0.2 volume TE buffer (pH 8.0) and stored in the dark at 4°C.

A DNA sample was combined with an equal volume of phenol/chloroform in an Eppendorf tube, and mixed to form an emulsion. After incubation at RT for 10 min (with occasional mixing), the tube was centrifuged (MSE Micro Centaur, high speed, 2 min). The upper, aqueous layer was removed without disturbing the interface, and re-extracted. To maximise recovery of DNA from small sample volumes, the phenolic layer was saved after the removal of the aqueous layer, mixed with an equal volume of TE buffer and centrifuged. The DNA-containing layer was removed and combined with that obtained previously.

2.15 ETHANOL PRECIPITATION OF DNA.

A DNA solution was combined with 0.1 volume of 3 M NaOAc (pH 5.5) and 2 volumes of cold ethanol, mixed by vortexing and chilled at -20°C for 20 min. The DNA was pelleted by centrifugation (MSE Mico Centaur, high speed, 5 min.) and the S/N was discarded. The pellet was washed with 70% ethanol, dried (55°C, 10 min) and resuspended in the required volume of TE buffer (pH 8.0).

Recovery of DNA, especially from particularly pure samples, could be improved using glycogen. This was made up at 10 mg/ml, stored at 4°C and added (at 1 µl/500 µl DNA solution) just prior to incubation at -20°C.

2.16 PHOSPHATASE TREATMENT OF VECTOR DNA.

The DNA was resuspended in 8 µl TE buffer (pH 8.0), and 1 µl calf intestinal alkaline phosphatase (CIP) buffer (Boehringer Mannheim) was added, followed by CIP (Boehringer Mannheim) at 0.1 U/ 10 pmol of ends.

For DNA with 5' terminal phosphates, incubation was carried out at 37°C for 1 hr, with a second aliquot of enzyme being added after 30 min. For DNA with blunt or recessed 5' termini, samples were incubated at 37°C for 15 min then at 56°C for 15 min. These incubation steps were repeated after the addition of more phosphatase. Reactions were stopped by the addition of 1 µl 0.5 M EDTA. The phosphatase was removed by phenol extraction (repeated 2-3 times) and the DNA was obtained by ethanol precipitation.

2.17 DNA LIGATION.

The method was essentially that described by Maniatis *et al* (1982). Vector DNA was digested (2.13) with the required restriction enzyme (which was then removed by phenol extraction (2.14)), and treated with CIP (2.16). The phenol extraction procedure was repeated and the DNA was resuspended in TE buffer (pH 8.0). The DNA to be ligated (previously cut with a compatible enzyme, phenol extracted and resuspended in TE buffer at a concentration of >50 µg/ml), was mixed with the vector in a 9:1 fragment: vector ratio. Appropriate amounts of 10x ligation buffer and T4 DNA ligase (BRL) were added (typically using 1-2 U ligase/ligation) and the volume was made up with sterile ELGA water. Samples were incubated, either at 15°C overnight, or at RT for 3-4 hr.

Recircularization was promoted by reducing the DNA concentration to 10 $\mu\text{g}/\text{ml}$.

For the ligation of blunt ends, T4 DNA ligase (New England Biolabs) was used (100 U/ligation).

2.18 AGAROSE GEL ELECTROPHORESIS OF DNA.

Digested DNA, mixed with a 0.1 volume of loading buffer, was loaded (with suitable size markers) on to a horizontal slab gel (usually 0.6% agarose (Sigma) in TBE buffer, containing 1 $\mu\text{g}/\text{ml}$ EtBr: prepared as a 10 mg/ml stock, stored in the dark at RT), in a tank of TBE buffer (1 $\mu\text{g}/\text{ml}$ EtBr). The gel was electrophoresed at 80-120 volts (v) for a few hr, or at 7-25 v overnight (voltage chosen according to gel size). DNA was visualized on a short wave UV (260 nm) transilluminator and photographed using Polaroid type 665 film.

By plotting standard curves of the log of the distance travelled (mm) against size (Kb), it was possible to determine the size of each restriction fragment. The standards used were λ EcoRI/HindIII (a mixture of λ cut with EcoRI, and cut with HindIII), and a commercial 1 Kb ladder (BRL). The sizes of the standard fragments are listed in table 2.e.

Restriction mapping was performed as described by Maniatis *et al* (1982).

2.19 ISOLATION OF DNA FRAGMENTS FROM AGAROSE GELS.

DNA fragments were separated by electrophoresis and the bands were visualized on a long wave UV (330 nm) transilluminator (to minimize DNA damage). A small trough (1-2 mm wide) was made below the desired fragment and the gel was replaced in the electrophoresis tank, containing just enough buffer to maintain electrical contact, without covering the gel. The trough was filled with fresh TBE buffer and electrophoresis was continued at 90-100 v for bursts of 30-40 sec. Between each burst, the trough was drained and filled with fresh buffer. The process was continued until all of the required fragment had been collected, as determined by examination under long

wave UV light. The samples were pooled, extracted with phenol/ chloroform (2.14), precipitated with ethanol (2.15) and resuspended in TE buffer (pH 8.0).

2.20 SOUTHERN HYBRIDIZATIONS.

2.20.1 FILTER PREPARATION.

Agarose gel electrophoresis was performed as described previously (2.18), running at a low voltage to ensure clear band separation. The gel was photographed (with a ruler for scale), trimmed to size, then soaked in depurination solution (0.25 M HCl) for 7 min. After being rinsed in ELGA water, the gel was soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 min, rinsed again, then placed in neutralizing solution (0.5 M Tris-HCl pH 8.0, 1.5 M NaCl) for 30 min.

The pretreated gel was placed on 3 mm Whatman paper, with wicks dipping into a tank of 20x SSC. Some 'Hybond N' (Amersham) filter, (cut to size and prewetted with 3x SSC) was placed on the gel, (excluding air bubbles), followed by 3-4 pieces of 3 mm Whatman paper (also prewetted with 3x SSC), a stack of paper towels and a weight. The blot was normally left overnight, but DNA transfer could be achieved in 3 hr by replacing the paper towels every 10-15 min.

After transfer, the filter was washed in 3x SSC for 5 min. DNA was cross-linked to the nylon by exposure to short wave (260 nm) UV light (3 min), with a single layer of Saran wrap between the source and nylon. At this stage the filter could be stored (in Saran wrap), to be probed at a later date.

2.20.2 PROBE SYNTHESIS.

2.20.2.1 'RANDOM PRIMED' LABELLING OF OLIGONUCLEOTIDES.

The random primed DNA labelling kit (Boehringer Mannheim) was used, according to the manufacturer's instructions.

Heat-denatured DNA (25 ng in 2 μ l) was mixed with 3 μ l dNTP mix (dATP, dGTP and dTTP each at 1.7 mM in Tris-HCl buffer), 2 μ l reaction mix (hexanucleotide mix in

10x reaction buffer), 3 μ l (30 μ Ci) [α - 32 P] dCTP (aqueous solution, Amersham), 9 μ l sterile ELGA water and 1 μ l DNA Poll 'Klenow fragment' (2 U/ μ l, in glycerol). After incubation at 37°C for 30 min, the reaction was stopped by adding 2 μ l EDTA (0.2 M, pH 8.0) and heating the sample at 65°C for 10 min. The expected incorporation using this procedure was 65%.

2.20.2.2 LABELLING OF SINGLE STRANDED DNA.

Single stranded template DNA, cloned into M13 for sequencing was labelled by primer extension. DNA (1 μ g in 7 μ l) was combined with -40 primer (0.5 pmol in 1 μ l) and 2 μ l reaction buffer (200 mM Tris-HCl pH 7.5, 100 mM MgCl₂, 250 mM NaCl), both obtained from US Biochemical. The sample was heated at 65°C for 2 min, then allowed to cool slowly (over a period of about 30 min) to 30°C. Annealing was then complete. The DNA, placed on ice, had to be used within 4 hr.

The labelling reaction was set up by adding the following solutions to the 10 μ l annealed template-primer: 1 μ l 0.1 M DTT (dithiothriitol), 3 μ l dNTP mix (dATP, dGTP and dTTP: each at 1.7 mM in Tris-HCl buffer), 2 μ l (20 μ Ci) [α - 32 P] dCTP (aqueous solution, Amersham) and 2 μ l Sequenase Version 2.0 (US Biochemical), previously diluted 1:8 in ice-cold enzyme dilution buffer (10 mM Tris-HCl pH 7.5, 5 mM DTT, 0.5 mg/ml BSA, US Biochemical). After incubation at 37°C for 20 min the reaction was stopped by the addition of 1 μ l 0.5 M EDTA.

2.20.3 PROBING A FILTER.

To prevent non-specific hybridization, the filter was blocked by being incubated in 'blotto' at 65°C in a shaking waterbath (New Brunswick Aquatherm) for at least 3 hr.

The probe was denatured in a boiling waterbath (8 min) and transferred to a bag (Salton polythene roll) containing the filter in 20 ml fresh 'blotto' (preheated to 65°C). Once any air bubbles had been excluded, the bag was carefully sealed (Tavak Modex SMT200 bag sealer) and incubated at 65°C with gentle shaking (New Brunswick Aquatherm) overnight.

The filter was washed to remove unbound probe. All wash steps used solutions which had been preheated to 65°C. The filter was rinsed briefly four times with low stringency wash solution (3x SSC, 0.1% SDS), then washed twice in the same solution (65°C shaking waterbath, 10 min). The filter was then washed, usually four times (65°C shaking waterbath, 15 min) with a higher stringency wash solution (0.5x SSC, 0.1% SDS), checking for background levels of radiation using a Geiger counter. The filter was blotted dry with 3mm Whatman paper, wrapped in Saran wrap and exposed to autoradiography using Fuji medical X-ray film. The required exposure time was calculated according to an equation which is based on the fact that 12 hr is sufficient for a filter with 5000 cpm:-

$$\text{Exposure Time (hr)} = 12(5000 \div \text{cpm})$$

When necessary, exposure time was reduced by the use of calcium-tungstate-phosphor intensifier screens (Dupont-Cronex Lightening-Plus), exposing the film at -70°C. One screen increased sensitivity 4-5 fold, while two screens increased sensitivity 8-10 fold.

2.21 DNA SEQUENCING.

2.21.1 RANDOM SEQUENCING OF SINGLE STRANDED DNA.

2.21.1.1 PREPARATION OF DNA.

The required DNA was obtained by agarose gel electrophoresis (2.18) of suitable restriction digests, followed by fragment isolation from the gel (2.19). The DNA (10 µg approx.) was self-ligated to generate closed circles and/ or chains (depending on the fragment ends), any of which could be used to generate random fragments.

The ligated DNA (in 30 µl) was sheared using a cup horn sonicator (Heat Systems Ultrasonics W-380), filled with water to a depth of 3 cm (approx.), with the sample clamped 1 mm above the probe. The DNA was sonicated for two bursts (maximum

output, 80 sec). Between bursts the sample was centrifuged briefly (MSE Micro Centaur, high speed, 3 sec) and the water surrounding the probe was replaced to improve cooling. The extent of shearing was checked by electrophoresis of a 1-2 μ l sample of the DNA. If the peak size was not in the required size range (300-1000 bp), sonication was continued as necessary.

The sub-fragments' ends were repaired by treatment with polymerases. The sheared DNA (in 30 μ l) was combined with 2 μ l dNTP mix (dATP, dCTP, dGTP and dTTP, (Boehringer Mannheim): all at 0.5 mM in TE buffer), 10 U DNA Pol I 'Klenow fragment' (Boehringer Mannheim), 10 U T4 DNA Pol I (BRL) and 1 μ l 250 mM $MgCl_2$ (a cofactor of both enzymes), and incubated at RT for 40 min.

Agarose gel electrophoresis and elution into a trough were used to isolate DNA fragments in the required size range (normally 300-800 bp), which were resuspended in 50 μ l TE buffer after phenol extraction and ethanol precipitation.

2.21.1.2 GENERATION OF M13 PLAQUES.

M13 (usually mp18) DNA (50 ng, New England Biolabs) was digested with *Sma*I (at 30°C, overnight), treated with CIP (2.16) and ligated with the blunt ended, size fractionated DNA fragments (2.17), incubating at 15°C overnight.

E. coli TG1 was transformed with the ligated DNA (20 μ l ligation mix: 200 μ l competent cells), as described previously (2.10.1). After the heat shock, the cells were added to 3 ml molten agar (containing 25 μ l 2% (w/v) X-Gal in DMF and 25 μ l 2.5% (w/v) IPTG in H₂O) at 45°C, mixed and overlaid on to a 2YT plate. When the agar had set (15 min, RT), the plate was inverted and incubated at 37°C overnight.

2.21.1.3 PLAQUE HARVESTING AND TEMPLATE PREPARATION.

White plaques, harvested with sterile toothpicks, were either used immediately or stored in 200 μ l phage buffer (4°C, up to 4 months)

A fresh overnight culture of TG1 was diluted 1:100 in 2YT, and 2 ml aliquots were dispensed into sterile phage tubes. The cultures were infected with M13 by the addition

of either a single plaque, or 50 μ l of phage buffer used for plaque storage. After incubation (New Brunswick G24 incubator shaker, 300 rpm, 37°C, 5 hr), 1.5 ml of each culture was pelleted (MSE Micro Centaur, high speed, 5-10 min) and 1 ml of the S/N was transferred to a fresh Eppendorf. M13 DNA was precipitated by the addition of 250 μ l 20% PEG/NaCl, followed by incubation at RT for 20-60 min and centrifugation (MSE Micro Centaur, high speed, 10 min). The S/N was carefully removed by aspiration and the phage pellet was left to resuspend in 200 μ l TE buffer (pH 8.0) at room temperature for 15 minutes. After phenol extraction (2.14) and ethanol precipitation (2.15) the DNA was resuspended in 30 μ l TE buffer (pH 8.0) and stored at -20°C.

2.21.2 SEQUENCING REACTIONS.

Chain termination sequencing reactions were set up using the Sequenase Version 2.0 kit (US Biochemical), according to the manufacturer's instructions.

The template and primer were annealed by mixing 7 μ l (1 μ g approx.) of template DNA with 1 μ l -40 primer (0.5 pmol/ μ l) and 2 μ l reaction buffer (200 mM Tris-HCl pH 7.5, 100 mM MgCl₂, 250 mM NaCl), incubating at 65°C for 2 min, then cooling slowly (over a period of 30 min approx.) to 30°C. Annealing was then complete and the sample was stored on ice (for up to 4 hr before use).

The labelling reaction was set up on ice, by adding the following solutions to the template/primer (10 μ l): 1 μ l 0.1 M DTT, 2 μ l labelling mix (7.5 μ M dCTP, 7.5 μ M dGTP, 7.5 μ M dTTP, diluted 5 fold with sterile ELGA water just before use), 0.5 μ l [α -³⁵S] dATP (10 μ Ci/ μ l and 10 μ M) and 2 μ l Sequenase Version 2.0 (diluted 1:8 with ice-cold enzyme dilution buffer: 10 mM Tris-HCl pH 7.5, 5 mM DTT, 0.5 mg/ml BSA, immediately before use). After mixing, the sample was incubated at RT for 2-5 min.

The termination reactions were set up in a micro test assay plate (Falcon). For each template being sequenced, four wells were labelled: G, A, T, C and 2.5 μ l of the corresponding termination mix was added to each well (eg in well G, ddG mix: 80 μ M dCTP, 80 μ M dGTP, 80 μ M dTTP, 8 μ M ddGTP, 50 mM NaCl). The plate was

prepared before the labelling reactions had been started and was incubated at 37°C for at least 1 min during the labelling reaction time. The labelling mix was then aliquoted into the wells of the micro test plate: 3.5 μ l into each of the four wells prepared for that template. The plate was centrifuged (MSE Mistral 2000, 2,600 rpm, 1 min) and incubated at 37°C for 3-5 min before the addition of 4 μ l stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) to each well (spun down, MSE Mistral 2000, 2,600 rpm, 1 min). The samples could be stored at 4°C (up to 4 hr), or at -20°C (up to 1 week) before use.

2.21.3 PREPARING AND RUNNING SEQUENCING GELS.

A pair of gel plates (50 cm, Raven) was cleaned thoroughly with ethanol, then acetone and the front plate (eared) was siliconized ('Repelcote', BDH), before being cleaned with ethanol once more. The plates, separated by 0.4 mm 'Plastikard' spacers, were taped together using 'tuck tape' (New Rochelle).

Acrylamide solutions were prepared by mixing 45 ml 0.5 xTBE 6% gel mix (white) with 80 μ l each of 25% (w/v) AMPS (ammonium persulphate, Bio-Rad) and TEMED (Sigma), and 7 ml 5.0 x TBE 6% gel mix (blue), with 14 μ l AMPS and TEMED. 35 ml of the white mix was put on one side (in a 50 ml syringe), while the remaining solution was taken up in a pipette, followed by the blue mix. A single bubble was introduced (to mix the interface) and the acrylamide was run down one side of the gel plates (held at an angle of 45°). The gradient was completed by the addition of the white mix from the syringe. A sharks tooth comb (BRL) was put in place, the apparatus was clamped together with bulldog clips, and the gel was left to polymerize (2 hr (approx.) at RT).

The vertical slab gel apparatus (Raven) was assembled, filling the reservoirs with TBE buffer. Sequencing reactions were heated (80°C, 15 min) and immediately loaded on to the sequencing gel with the tracks for each template being in the order: A C G T. The gel was run (constant power: 37 watts) until the lower dye front was just off the bottom (2-2.5 hr approx.)

The gel was fixed in 10% (v/v) glacial acetic acid (15 min), transferred to 3 mm Whatman paper, covered in Saran wrap and dried under vacuum (Bio-Rad Slab Gel Drier, 80°C, 20-30 min). The dried gel was exposed to (Fuji, medical) X-ray film (RT overnight).

Gels were read with the aid of a digitizer (Science Accessories Corporation) and sequences were recorded and merged using the Microgenie computer package, (Beckman) which was also used for simple sequence analysis and homology studies. More complex studies were carried out using the Seqnet system.

2.21.4 MODIFICATIONS TO THE SEQUENCING PROTOCOL.

2.21.4.1 SEQUENCING OF DOUBLE STRANDED DNA.

The DNA fragment to be sequenced was cloned into a vector such as pUC18, pUC19 or pBR322 (high copy number, commercially available primers) and DNA was prepared using CsCl gradient purification (2.8.3).

The method was derived from that of Mierendorf and Pfeffer (1987). The DNA (3 μ g) was mixed with 3 μ l 1 M NaOH, 1 mM EDTA (freshly prepared) and sterile ELGA water, bringing the final volume to 15 μ l. After incubation at 37°C for 30 min, the NaOH was neutralized with 1.2 μ l 3 M NaOAc (pH 4.8) and the DNA was precipitated by the addition of 36 μ l ethanol, followed by incubation at -70°C for 15 min and centrifugation (MSE Micro Centaur, high speed, 10 min). The DNA pellet was washed (70% EtOH), dried (55°C, 10 min) and resuspended in 6 μ l sterile ELGA water. The annealing reaction was set up by the addition of 2 μ l sequenase reaction buffer (Boehringer Mannheim) and 2 μ l primer (at 1.5 ng/ μ l), and incubated at 37°C for 15-30 min. The sample was stored on ice (up to 4 hr) and used for sequencing reactions exactly as described previously (2.21.2).

2.21.4.2 DIRECTED SEQUENCING IN M13.

The required fragment, isolated from an agarose gel was cloned into the M13 polylinker. Plaques were generated on TG1, and the DNA was prepared and sequenced exactly as described previously. By cloning a fragment into M13 mp18 and M13 mp19 (New England Biolabs), the sequence of both strands was obtained.

2.21.4.3 SEQUENCING USING OLIGONUCLEOTIDES.

Oligonucleotide seventeeners were used: chosen to have a G/C content of as near 50% as possible.

The DNA was resuspended in sterile ELGA water to give a concentration of 1.5 ng oligonucleotide DNA/ μ l (approx.). Sequencing reactions were set up as described previously (2.21.2), using the oligonucleotides in place of the -40 primer.

2.21.4.4 EXTENSION REACTIONS.

By changing the reaction conditions, it was possible to obtain sequence in the 200-800 nucleotide range, at the expense of that nearer the primer. The labelling reaction was altered to give a 3-5 fold increase in the concentration of dNTPs present, i.e. the labelling mix was used undiluted. The amount of labelled dATP was also increased (to 1-1.5 μ l of 10 μ M, 10 μ Ci/ μ l aqueous solution per reaction) and the labelling reaction time was lengthened to 5 min. Sequencing was otherwise carried out as described previously, except that the gel was run for longer. When the upper dye front had reached the bottom of the gel, a sample of stop solution was loaded, and the gel was run until this lower dye front had reached the bottom (5-6 hr in total).

2.21.4.5 READING CLOSE TO THE PRIMER.

Manganese ions affect the activity of Sequenase Version 2.0, reducing the average length of DNA synthesized during the termination step of the sequencing reaction, so that the sequence near the primer is intensified.

Sequencing reactions were set up exactly as described previously (2.21.2), except that 1 μ l Mn buffer (United States Biochemical) was added to the labelling reaction immediately before the sequenase enzyme. The gel was run until the first blue dye had run 80% (approx.) of the way down the gel (1-2 hr).

2.21.4.6 READING THROUGH COMPRESSIONS.

Both strands of DNA were read: a practice which usually sorted out ambiguities in one strand. For some compressions however, it was necessary to destabilize the secondary structure of the DNA by substituting dITP for dGTP.

Sequencing reactions were set up as described previously, except that labelling and termination mixes containing dITP instead of dGTP were used. It was sometimes helpful to run the gel at a higher power than usual (39 watts, rather than 37), as the increased temperature reduced DNA secondary structure formation. Standard dGTP reactions were always set up with, and run alongside those with dITP. This was because dITP sequence was of a generally low standard, so comparison with normal tracks was useful.

2.21.4.7 READING THROUGH PAUSES.

The Taquenase kit (US Biochemical) was used, according to the manufacturer's instructions. The sequencing reactions were similar to those for Sequenase version 2.0 (2.21.2), except that the annealing step was carried out at a higher temperature: 70°C. The labelling reaction was set up by the addition of 2 μ l Taquenase labelling mix to the ice-cold template-primer (in 13 μ l), followed by 0.5 μ l [α -³⁵S] dATP (10 μ M, 10 μ Ci/ μ l) and 2 μ l Taq polymerase (diluted 1:8 in ice-cold Taq enzyme dilution buffer). The mixture was incubated at 45°C for 5 min. For the termination reaction, 4 μ l of the labelling reaction mix was combined with 4 μ l of each of the termination mixes, in wells of a microtest plate, which was incubated at 70°C for 5 min. After cooling, stop solution was added and the samples were used as normal.

2.22 GENE PRODUCT IDENTIFICATION.

2.22.1 THE T7 RNA POLYMERASE/PROMOTER EXPRESSION SYSTEM.

Plasmid-encoded proteins were identified by the method developed by Tabor and Richardson (1985).

The gene(s) of interest were cloned into pT7-5, such that expression was under the control of the T7 RNA polymerase promoter. The plasmid was transformed into *E. coli* K38, which was already carrying pGP1-2: encoding T7 RNA polymerase, expressed from a lambda promoter, at 42°C only, due to the inactivation of the λ cl⁸⁵⁷ repressor. Colonies were grown on LB plates, with Ap and Kn selection, at 30°C.

Single colonies of K38 (pT7-5; pGP1-2) were grown up in 10 ml LB (Ap, Kn) in a 50 ml sidearm flask (New Brunswick water Aquatherm, 30°C, 275 rpm), until the OD (600 nm, Corning Spectrophotometer 258) was 0.4 (2-2.5 hr approx.), when a 1 ml sample was removed and centrifuged (Labor-50M, 4,500 rpm, 5 min). The cells were washed with 5 ml M9 media, resuspended in 5 ml M9 media supplemented with 0.02% of 18 amino acids (minus cysteine and methionine), and returned to a 50 ml flask for further incubation (30°C, 275 rpm, 60 min.).

Gene expression was induced (42°C, 275 rpm, 20 min), then rifampicin (Rif:Sigma, prepared as a 20 mg/ml stock in methanol; stored in the dark at 4°C, up to 3 weeks) was added to a final concentration of 200 µg/ml. The culture was left at 42°C for a further 10 min before being returned to 30°C (275 rpm, 20 min).

A 0.5 ml sample of the culture was transferred to a 2 ml Eppendorf tube, 10 µCi of ³⁵S-labelled methionine was added, and the tube incubated (New Brunswick G24 incubator shaker, 30°C, 250 rpm, 5 min). The cells were pelleted (MSE Micro Centaur, high speed, 20 sec), resuspended in 150 µl cracking buffer and kept frozen at -20°C until required when aliquots (40 µl) of the samples were boiled (5 min) and analysed by SDS-PAGE (2.23).

2.22.2 PULSE-CHASE LABELLING OF PROTEINS.

The stability of proteins expressed using the T7 RNA polymerase/ promoter system was determined as set out below. The normal protocol (as described above: 2.22.1) was followed, except that four 0.5 ml samples were removed from the induced, Rif-treated culture. Each sample was labelled with ^{35}S -methionine for 1 min (rather than the usual 5 min). This pulse was followed by a chase, using non radioactive methionine (added to a final concentration of 0.5%). The samples were incubated (New Brunswick G24 incubator shaker, 30°C, 250 rpm), for 0, 5, 15 or 60 min. They were then centrifuged, and the cell pellets were resuspended in cracking buffer and heat-denatured as described above.

2.22.3 THE 'ZUBAY' EXPRESSION SYSTEM.

Bacterial cell-free coupled transcription-translation, as described by de Vries and Zubay (1967) and later modified (Zubay 1973) was performed, using the prokaryotic DNA-directed translation kit (Amersham), according to the manufacturer's instructions. The gene(s) of interest was cloned into a suitable vector: normally pBR322. Samples, prepared using CsCl gradient purification (2.8.3) had to be free from contamination with RNase, phenol, CsCl and EtBr.

A 5 μg sample of DNA (in TE buffer, pH 8.0) was made up to 12.5 μl with dilution buffer (Amersham). This was combined with 7.5 μl supplement solution (a mixture of nucleotides, tRNA, an energy-generating system and inorganic salts), 3 μl amino acids solution (an equimolar mixture, minus Met), 2 μl ^{35}S -Met (15 $\mu\text{Ci}/\mu\text{l}$) and 5 μl S-30 extract (S/N fraction from *E. coli* MRE 600, RNaseI⁻). The sample was mixed gently and incubated at 37°C. After 60 min, 5 μl Met chase solution was added and incubation was continued for a further 5 minutes at 37°C, to allow the completion of any protein chains prematurely terminated due to the limitation of ^{35}S -Met. The reaction was terminated by placing the sample on ice. The sample was mixed with an equal volume of cracking buffer, boiled (5 mins.) and analysed by SDS-PAGE (2.23): loading 20-30 μl of sample per track.

2.23 PROTEIN ELECTROPHORESIS.

The protocol was based on that of Silhavy *et al* (1984). The gel apparatus (Bio-Rad: Protean 16 cm) was set up according to the manufacturer's instructions, cleaning the gel plates thoroughly, with ethanol and acetone before use. A 12% acrylamide gel mix was prepared and poured between the gel plates, leaving 3 cm (approx.) for the stacking gel. A layer of water-saturated butanol was added with a Pasteur pipette, and the gel was left to polymerize (1-2 hr at RT, or 30-60 min: 42°C oven). Once the butanol had been poured off and the gel rinsed with water, 3% stacking gel was poured on top. The comb (Bio-Rad, pre-wetted with 10% w/v AMPS) was introduced and the gel was left to polymerize (1-2 hr at RT).

The gel was placed in a tank of 1x running buffer, standing on a magnetic stirrer. The wells were rinsed out and protein samples (40 μ l), previously boiled (5 min) in cracking buffer, were loaded into them. Samples were run into the stacking gel at 40 mA, then electrophoresis was continued at 7 mA until the blue dye front had reached the bottom of the gel (16 hr approx.).

The gel, removed from the gel plates, was fixed in a mixture of isopropanol, water and glacial acetic acid (25:65:10), shaking gently (Luckam Ltd. shaker, half speed) for 30 min. After being transferred to 3 mm Whatman paper, the gel was dried under vacuum (Bio-Rad Slab Gel Drier, 60°C, 3-4 hr) and exposed to X-ray film (Hyperfilm- β max, Amersham: used according to the manufacturer's instructions). Similar results, but slightly poorer resolution, were obtained using normal X-ray film (Fuji, medical), if the gel was treated with 'Amplify' (Amersham), between being fixed and dried.

CHAPTER THREE.

**THE LOCALIZATION OF ENZYME ACTIVITY DURING THE
GROWTH CYCLE OF HC131.**

3.1 INTRODUCTION

As discussed previously (1.1.5), the major pathogenicity determinants of *Erwinia* spp. are a range of extracellular enzymes including pectate lyase, protease, cellulase and polygalacturonase. In order for these enzymes to macerate host tissue, causing soft-rot, they must be translocated from the cytoplasm of the pathogen to the extracellular medium: a process which involves crossing both the inner and outer membranes. Mutants of *Ecc* defective in secretion, but not the production of Pel and Cel (*Out*⁻ mutants) are unable to cause soft-rot (Andro *et al* 1984), showing that the secretory apparatus itself is an important pathogenicity 'determinant' in soft-rot *Erwinia* spp. A detailed knowledge of enzyme secretion is therefore crucial for the understanding (and perhaps eventual inhibition) of soft-rot.

Apart from its critical role in pathogenicity, the secretion of extracellular enzymes is an important area of study in its own right. *Erwinia* sp, possessing a secretory apparatus which is not present in *E. coli*, is an important model for the analysis of protein translocation across the membranes of Gram-negative bacteria.

The secretion-defective (*Out*⁻) mutants are critical in the study of secretion and hence pathogenicity, but before they can be analysed in any meaningful way, the synthesis and localization of exoenzymes in the wild-type strain must be understood. It was therefore necessary to make a detailed study of secretion by HC131. Pel was chosen as the representative extracellular enzyme because, in addition to being the major enzyme involved in pathogenicity, Pel can be assayed relatively easily and reproducibly, compared to some of the other macerating enzymes.

Previous work demonstrated that SCRI193 synthesizes Pel from early on in the exponential phase. This is in contrast to the situation with *Ech*, which produces Pel only after entry to the stationary phase (Hugouvieux-Cotte-Pattat *et al* 1986). In SCRI193, maximum Pel activity is found in mid exponential phase ($A_{600}=2.0$), after which point in the growth cycle approximately 90% of the enzyme activity was located in the culture supernatant (Hinton 1986). The data indicated that Pel accumulated

intracellularly when the culture density was 0.5-2.0 OD₍₆₀₀₎ units, suggesting that initially, Pel was synthesized but not secreted. An intracellular build up of Pel activity has also been reported in *Ech*: towards the end of the exponential growth phase (Collmer and Bateman 1982).

It seemed likely that the Pel secretory apparatus was regulated; being switched on at a particular point in the growth cycle, possibly in connection with a build-up of Pel within the cell. In order to investigate this possibility, further characterization of the intracellular build-up was required.

As described previously (1.5, 1.6), two distinct models have been proposed for secretion by Gram-negative bacteria, with the two-step hypothesis being favoured for the secretion of Pel, Cel and Peh by *Erwinia* spp. (1.7). It was therefore important to determine whether Pel accumulation early in the growth cycle was cytoplasmic or periplasmic. Evidence of a periplasmic build-up would not only support the two-step hypothesis, but would also indicate that secretion was regulated at the post-export stage.

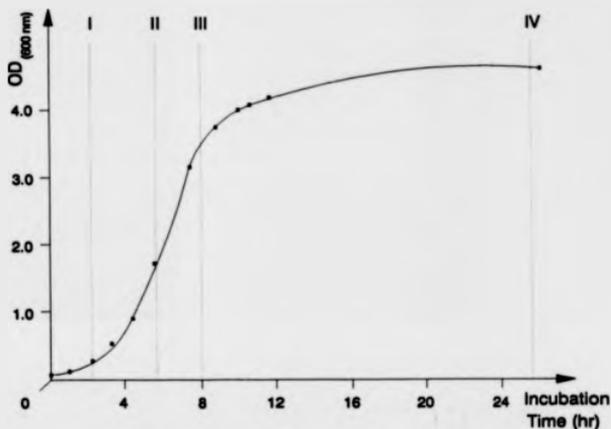
3.2 PEL LOCALIZATION DURING THE GROWTH CYCLE OF HC131.

HC131 was grown in PM, with Ap selection, in a 250 ml flask at 30°C, 275 rpm. Growth curves were obtained, taking samples for cell fractionation as described previously (2.3).

Figure 3.2.1a shows the growth curve of HC131. The Pel activities (as a % of the total) found in each fraction at the time points sampled are shown in figure 3.2.1b. The total Pel activity, plotted against culture OD_(600 nm) is shown in figure 3.2.1c. The data confirmed previous findings, showing that Pel was synthesized from early in the growth cycle (Hinton 1986). Throughout exponential growth, the total Pel activity was approximately proportional to bacterial cell density, in agreement with other data for *Ecc* (Moran and Starr 1969, Zucker and Hankin 1970), but as the cells entered

Figure 3.2.1a

The Growth Curve of HC131

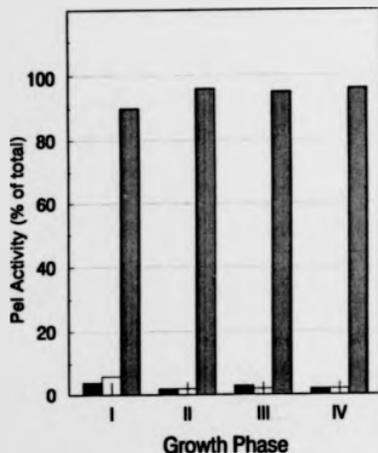


Time points at which samples were removed for fractionation and enzyme assays, (indicated by) were at I: early, II: mid and III: late exponential, and IV: stationary phases of growth.

The sampling time points for all the enzyme assay data presented in this chapter, refer to those shown in the above figure.

Figure 3.2.1b

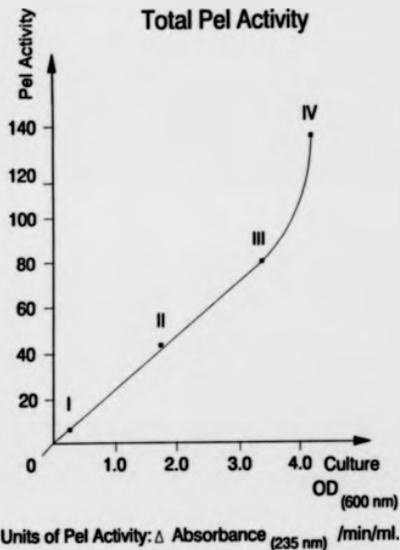
The Localization of Pel Activity



■ Periplasm □ Sonicate ▒ Supernatant

Figure 3.2.1c

Total Pel Activity



Units of Pel Activity: Δ Absorbance_(235 nm) /min/ml.

I: early exponential, II: mid exponential, III: late exponential, IV: stationary growth phase (see figure 3.2.1a).

stationary phase activity increased. It has been reported that for *Ech* grown in liquid culture, Pel synthesis is dependent on growth phase. Pel activity was scarcely detected until the end of exponential growth, when it increased by twenty fold (Collmer and Bateman 1982). It was proposed that a mechanism exists in *Ech* which allows cells to synthesize Pel when growth conditions become poor. Mutants (*gpi*: growth phase independent) were isolated in which Pel production followed the growth curve (Hugouvieux-Cotte-Pattat *et al* 1986). It was concluded that *Ecc* lacks the growth-phase dependent regulatory mechanism found in *Ech*, behaving more like *gpi* mutants.

From figure 3.2.1b it can be seen that for every sample taken, over 90% of the total Pel activity was localized in the culture supernatant, with no evidence for an intracellular build-up at the time points sampled. However, the low total activity early in the growth phase might have made localization data inaccurate.

3.3 MARKER ENZYMES

3.3.1 THE USE OF MARKER ENZYMES.

Marker enzymes were used to determine the purity of the different subcellular fractions and to show that Pel was actively secreted by a specific mechanism, and did not enter the culture supernatant accompanied by other cellular components, as would occur due to cell lysis.

The periplasmic marker enzyme used was β -lactamase (Bla: E.C.3.5.2.6). This was found to be predominantly localized within the periplasmic fraction, irrespective of the stage of growth, as indicated in figure 3.3.1a. The small amount of leakage into the supernatant fraction observed in the stationary phase may be explained by cell lysis. As stated for Pel (3.2), localization data may have been inaccurate for samples taken early in the growth cycle.

The localization of β -galactosidase (LacZ: E.C.3.2.1.23), chosen as a cytoplasmic marker, was found to be greatly affected by growth phase. Apparent leakage into the periplasmic fraction was observed in samples taken throughout the exponential phase,

Figure 3.3.1a

The Localization of Bla Activity

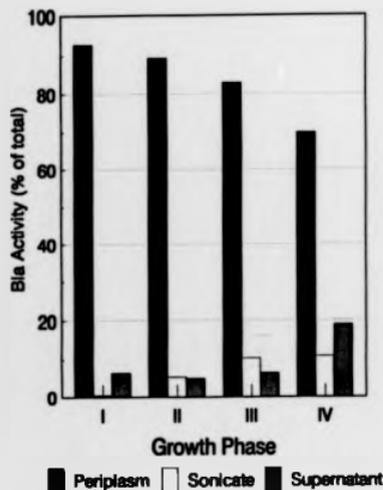
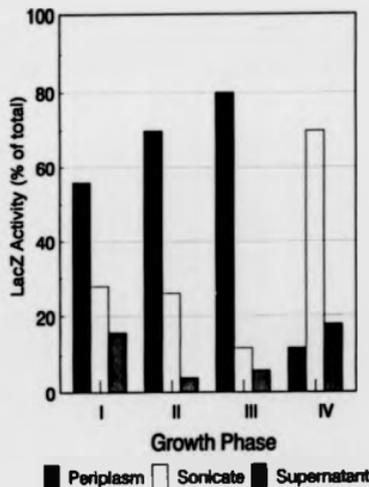


Figure 3.3.1b

The Localization of LacZ Activity



I: early exponential, II: mid exponential, III: late exponential, IV: stationary growth phase (see figure 3.2.1a)

but by the stationary phase, the enzyme was predominantly located in the cytoplasmic fraction (figure 3.3.1b). It was necessary to determine whether the leakage of LacZ was a genuine phenomenon, or artifactual, caused by the fractionation or assay procedures.

The total activities of the marker enzymes Bla and LacZ, when plotted against bacterial density, produced graphs similar to that showing total Pel activity throughout the growth curve (data not shown). This supported the proposal that there is no specific, growth-phase dependent mechanism for the regulation of Pel synthesis in *Ecc*.

3.3.2 THE EFFECT OF CULTURE AND FRACTIONATION CONDITIONS ON THE LOCALIZATION OF LACZ.

The effect of growth media on enzyme localization was investigated by growing 5 ml cultures in LB or PM to comparable cell densities ($OD_{600} = 1.5$). After fractionation, samples were assayed for the activities of Bla and LacZ. The results showed that growth in LB rather than PM resulted in a higher total activity of LacZ and reduced leakage into the periplasm. However, the results of the Bla assays showed an increase (5% approx.) in the leakage of the periplasmic enzyme into the supernatant, when the cells were grown in LB (data not shown).

It seemed that the activities and apparent localizations of the marker enzymes were affected by growth media, and that the suitability of a particular medium varied, depending on the enzyme being assayed (data not shown). Since the experiments were designed to study Pel secretion, and Pel synthesis is induced by the PGA in PM, it was necessary to continue to use this medium.

In addition to studying the effect on localization of medium alteration, the osmotic cold shock fractionation procedure, described earlier (2.3.2), was also varied.

The solution used to resuspend the cell pellet after the removal of the supernatant fraction was changed from 40 to 50% sucrose. If the apparent leakage of cytoplasmic

components into the periplasmic fraction was due to cell lysis at this stage, it seemed possible that by increasing the osmotic pressure of the solution, lysis, and hence leakage might be reduced. The length of incubation on ice at this stage was also altered. Instead of 10 min, samples were left for a range of times between 2 and 8 min, to reduce the chance of enzyme degradation.

The cold-shock stage was similarly varied: replacing the cold water with 5 or 10% sucrose solution and varying incubation time from 2 to 10 min.

Most of these modifications had no marked effect on the total activity of LacZ, although longer incubation times during the cold-shock gave lower activity, suggesting that LacZ was unstable on ice.

An alternative fractionation procedure, involving the production of shockates (2.3.3) was used and samples were assayed for the activities of Bla and LacZ. For Bla, the activity and localization were comparable to those obtained after cold-shock fractionation. The results for LacZ were highly variable and it was concluded that the shockate method of fractionation did not improve the marker enzyme localization data.

3.3.3 OPTIMIZATION OF THE LACZ ASSAY.

Tests showed that the LacZ assay reaction was not substrate limited. No significant increase in activity was observed by using different reaction temperatures in the range: 28-37°C.

The absorbance values obtained at 420 nm were not entirely due to o-nitrophenol, but to a combination of this and the scattering of light by cell debris (Miller 1972). Although this effect was likely to be minimal, as sub-cellular fractions should not contain much cell debris, it was eliminated by taking an additional optical density reading at 550 nm for each sample. At this wavelength, values were solely due to light scattering. Since the light scattering at 420 nm was proportional to that at 550 nm:

$$OD_{420}(\text{light scattering}) = 1.75 \times OD_{550}$$

corrections could be made so that the absorbance values were true reflections of the amounts of o-nitrophenol present.

As expected, the error introduced by light scattering was found to be very small. For all the samples tested, there was most light scattering in the supernatant fractions, meaning that the corrections reduced the 'apparent' leakage of LacZ from the cytoplasm. The improvement was, however, minimal (data not shown).

As discussed previously, the localization of LacZ was most aberrant in samples taken early in the growth cycle, i.e. in those with the lowest total activity. The LacZ assay was performed by incubating the reaction tubes until the samples turned yellow, when the reactions were stopped by the addition of Na_2CO_3 . Samples with low activity were incubated for long periods (often overnight): a method used routinely (Koop and Bourgeois pers. comm.). In order to confirm that the colour change (due to the breakdown of ONPG to o-nitrophenol) remained linear with respect to time over such long periods, batches of identical LacZ reaction mixes were prepared. The reactions were stopped at different time points, the absorbances (420 nm) were read, and graphs were plotted to show the change in optical density against reaction time at 37°C. The graph obtained for a typical set of subcellular fractions of HC131 is shown in figure 3.3.3a.

The results showed that the increase in optical density was not proportional to incubation time over long periods. Interestingly, the degree of deviation from the straight line graph was highly dependent on the subcellular fraction assayed: being greatest for the cytoplasmic fraction and least for the periplasm. This would account, in part, for the apparent leakage of LacZ from the cytoplasmic, to the periplasmic fraction. The continued activity of LacZ over long periods for the periplasmic fraction confirmed previous findings that substrate was not limiting. It appears that either LacZ, or the reaction product: o-nitrophenol, was less stable in the cytoplasmic fraction and supernatant, than in the periplasmic fraction. The most likely explanation would seem to be degradation of LacZ by proteases.

These findings suggested that LacZ reactions should be stopped after 1 hr, long before any visible colour change had occurred, although the low absorbance (420 nm) of these samples would inevitably introduce other errors.

Figure 3.3.3a

Apparent LacZ Activities of Subcellular Fractions after Varying Reaction Times.

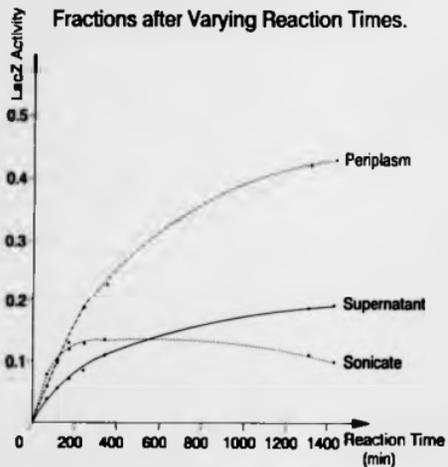
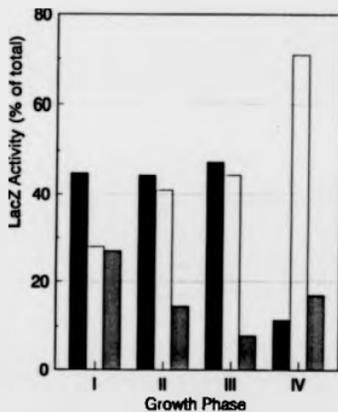


Figure 3.3.3b

The Localization of LacZ Activity (using optimized assay conditions)



■ Periplasm □ Sonicate ■ Supernatant

I: early exponential, II: mid exponential, III: late exponential, IV: stationary growth phase (see figure 3.2.1a)

Figure 3.3.3b shows the localization of LacZ activity in samples taken throughout the growth curve, assayed using the modifications to the standard procedure described above.

It is possible that LacZ must be induced in *Ecc*, as in *E.coli*, although other experiments have not involved the addition of lactose to the growth media (Hinton and Salmond 1987). Lack of induction would reduce the total activity, but would not affect localization data.

The total activity obtained for stationary phase cultures was comparable to data presented previously (Hinton and Salmond 1987), suggesting that the assay was being performed correctly. This is supported by the close resemblance of the graph showing total LacZ activity with bacterial density, and the same graph for Bla (data not shown). It was therefore concluded that while the fractionation and assay procedures were suitable for the determination of LacZ localization in stationary phase cells, no reproducibly meaningful data could be obtained earlier in the growth cycle.

3.4 STUDIES ON VARIOUS 'CYTOPLASMIC' ENZYMES.

It has been shown that there were problems, inherent in the LacZ assay, which could not easily be overcome. An alternative approach was to use another cytoplasmic marker enzyme.

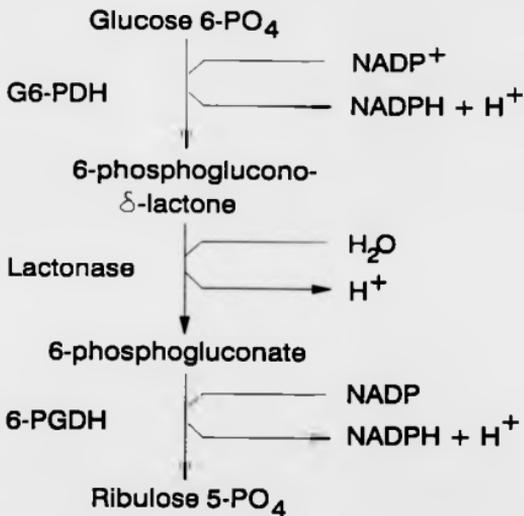
3.4.1 ACTIVITY AND LOCALIZATION OF 6-PHOSPHOGLUCONATE DEHYDROGENASE (6-PGDH).

6-PGDH (EC 1.1.1.44) is involved in the pentose phosphate pathway. It catalyses the formation of ribulose 5-phosphate by the oxidative decarboxylation of 6-phosphogluconate: produced by the hydrolysis of the G6-PDG reaction product (3.4.2). The series of reactions is summarized in figure 3.4.1a.

Samples of HC131 subcellular fractions, taken from various stages of the growth cycle were assayed for 6-PGDH activity as described previously (2.5.4).

Figure 3.4.1a:

The Pentose Phosphate Pathway
(Oxidative Branch)



Reference: Stryer (1981)

All the samples exhibited such low activity that it was necessary to optimise the assay procedure.

The possibility that the reaction was limited by the concentration 6-phosphogluconate or NADP^+ was eliminated (data not shown). No significant increase in 6-PGDH activity was observed when the pH of the assay reaction mixture was varied in the range: pH 7.4-7.8 (data not shown).

Raising the reaction temperature from 30°C to 37°C led to a 10 fold (approx.) increase in activity (data not shown). All subsequent 6-PGDH assays were performed at the higher temperature.

6-PGDH is activated by MgCl_2 : up to 0.03M, above which concentration inhibition occurs (Pontremoli and Grazi 1966). By doubling the concentration of MgCl_2 (from 0.013 to 0.026 M) in the reaction mixture, a two fold (approx.) increase in activity was achieved (data not shown). This modification to the procedure was adopted routinely.

In order to confirm that the (still low) rate of NADP^+ reduction observed was due to enzymic activity, samples, pre-boiled for 2 min were assayed. For the supernatant and periplasmic fractions, the apparent 'activities' were similar for the boiled and fresh samples, while for the sonicate, boiling significantly reduced the activity.

Tests were carried out on pure 6-PGDH (yeast: crystalline form, $\leq 0.1\%$ G6-PDH, Sigma). The activities of varying amounts of the enzyme, either fresh, or pre-boiled for 2, 10 or 60 min, were determined. The results are summarized in figure 3.4.1b. The fact that the rate of NADP^+ reduction was proportional (approx.) to enzyme concentration, confirmed that the enzyme was active. The low residual rate observed in the absence of 6-PGDH, or after boiling the enzyme for anything from 2 to 60 min (i.e. background: not due to 6-PGDH activity), was similar to the rate observed for supernatant or periplasmic samples. The data suggest that boiling for 2 min was sufficient to eliminate 6-PGDH activity.

Reactions were set up using both fresh and boiled (2 min) samples of each of the subcellular fractions. The change in OD(340 nm) was calculated at 1 min intervals, over 20 min for each sample. By subtracting the values obtained with those with the

Figure 3.4.1b

Activity of Pure 6-PGDH

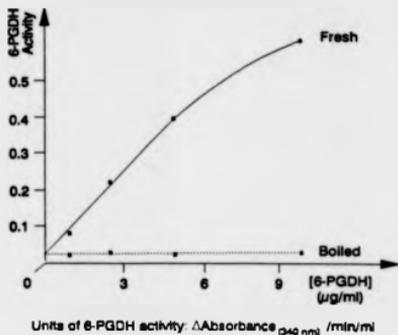
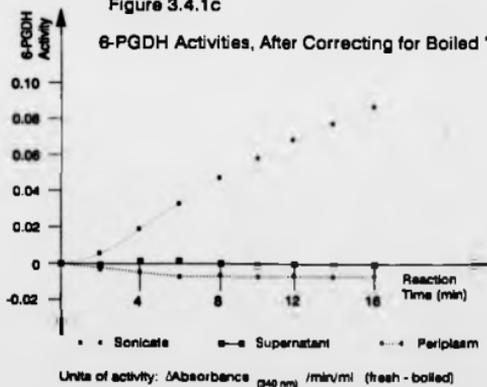


Figure 3.4.1c

6-PGDH Activities, After Correcting for Boiled 'Activity'.



boiled samples from the corresponding fresh samples at given time points, it was possible to plot graphs showing the change in OD (340 nm) due to 6-PGDH activity, against reaction time. The graph obtained for stationary phase samples is shown in figure 3.4.1c.

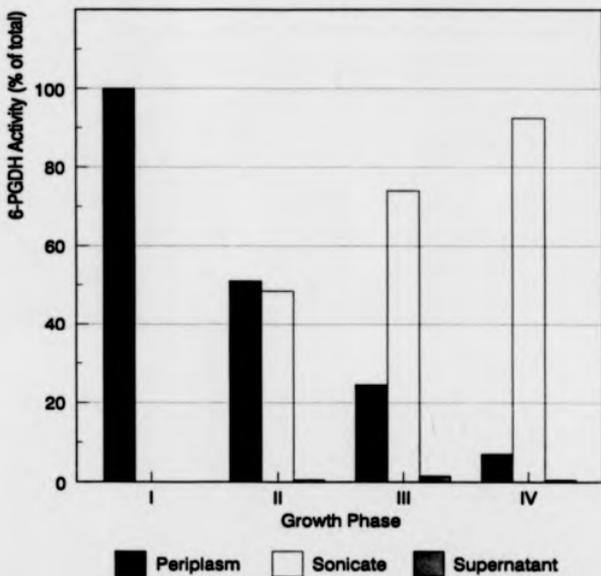
This method of correcting for non-enzymic activity produced graphs which were linear for reaction times of 2 to 9 min. Using this procedure, no 6-PGDH activity was detected in the supernatant. The decrease in OD (340 nm) observed with the periplasmic sample may indicate the presence of an NADPH-oxidizing enzyme in that fraction. This raises the question of NADPH-utilizing enzymes which, if present in an extract could lead to artificially low 6-PGDH activity values. Had this been thought likely to affect the results significantly, reactions could have been set up, adding NADPH to the assay mixture, instead of NADP^+ to determine the decrease in OD(340 nm), so that corrections could be made.

In summary, the results discussed above (3.4.1) confirmed that, within a given range, the reaction followed by the 6-PGDH assay was linear with respect to both time and enzyme concentration. In addition, it was shown that activity was abolished (in most cases) by heat-denaturation; proving that the observed reaction was due to enzymic activity.

The activity, and localization, of 6-PGDH was determined by assaying samples taken throughout the growth cycle; correcting for non-enzymic activity to obtain linear reaction rates in each case.

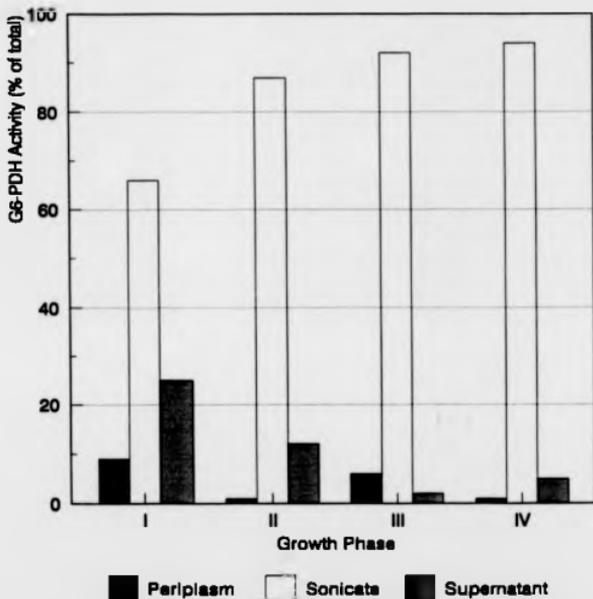
The total activity of 6-PGDH (as for the enzymes discussed above), was dependent on growth phase (data not shown). Figure 3.4.1d shows the localization of the enzyme activity throughout the growth curve. As with LacZ, the proportion of 6-PGDH activity apparently located in the cytoplasm was low during the early stages of growth.

Figure 3.4.1d
The Localization of 6-PGDH Activity



I: early exponential, II: mid exponential, III: late exponential
IV: stationary growth phase (see figure 3.2.1a)

Figure 3.4.2a
The Localization of G6-PDH Activity



I: early exponential, II: mid exponential, III: late exponential,
IV: stationary growth phase (see figure 3.2.1a).

3.4.2 ACTIVITY AND LOCALIZATION OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE (G6-PDH).

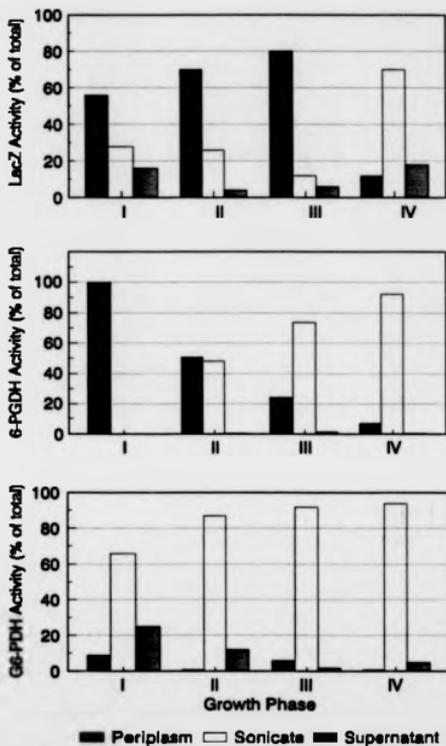
G6-PDH (EC 1.1.1.49) catalyses the initial step in the pentose phosphate pathway: the dehydrogenation of glucose 6-phosphogluconate, to yield 6-phosphoglucono- δ -lactone (as shown in figure 3.4.1a). G6-PDH is highly specific for NADP⁺ as the electron acceptor, with K_M for NAD⁺ being about 1000 times that for NADP⁺ (Stryer 1981). Enzymic activity was assayed as described previously (2.5.3).

Samples of the subcellular fractions of HC131, obtained at various stages of the growth cycle were assayed for G6-PDH activity. Total activity was found to be dependent on growth phase: increasing approximately in line with the culture cell density, as for the enzymes discussed above (data not shown). The localization of G6-PDH, shown in figure 3.4.2a, was less dependent on growth phase than that of LacZ. The proportion of activity found in the cytoplasmic fraction was consistently higher for G6-PDH than for LacZ or 6-PGDH, suggesting greater suitability as a cytoplasmic marker.

3.5 COMPARISON OF THE SUITABILITY OF PUTATIVE CYTOPLASMIC MARKER ENZYMES.

The localizations (% activities) of the enzymes: LacZ, G6-PDH and 6-PGDH, from samples taken at different stages of the growth cycle are summarized in figure 3.5.1a. As discussed previously (3.3), there was apparently a leakage of LacZ activity into the periplasm during the early stages of the growth cycle, as determined by the crude data. The localization of 6-PGDH was also highly dependent on growth phase, with leakage into the periplasm occurring during early and mid exponential phases. The state of growth had far less effect on the localization of G6-PDH activity, although leakage from the cytoplasm was still greatest in samples taken from early in the growth phase. In contrast to the results from the other 'cytoplasmic' enzymes, G6-PDH leaked into the supernatant.

Figure 3.5.1a The Localizations of 'Cytoplasmic Enzymes'.



I: early exponential, II: mid exponential, III: late exponential, IV: stationary growth phase (see figure 3.2.1a)

3.6 CONCLUSIONS / DISCUSSION.

The aim of the work described above was to use spectrophotometric enzyme assays to study the synthesis and localization of Pel throughout the growth cycle, looking in particular, for the intracellular build-up reported previously (Hinton 1986, Collmer and Bateman 1982). Marker enzymes were used to determine the purity of the subcellular fractions: supernatant, periplasm and cytoplasm (sonicate).

Bla activity was located predominantly in the periplasmic fraction throughout the growth cycle, confirming the suitability of this enzyme as a periplasmic marker. For those enzymes assumed to be cytoplasmic (LacZ, G6-PDH and 6-PGDH), apparent localization was highly variable, depending on the particular enzyme studied and on the stage of growth.

Variation in the physiological state of the cells throughout the growth cycle might cause them to fractionate differently. Although this could lead to differential leakage from the cytoplasm for different samples, it would not explain why the extent of leakage varied for the different enzymes studied, or why some entered the periplasmic fraction while others were detected in the supernatant. It should be considered that rather than leakage from the cytoplasm occurring in samples fractionated early in the growth cycle, it is possible that cold-shock treatment of these samples did not yield true periplasmic and cytoplasmic fractions. Although not definite, the former explanation seems more likely in view of the fact that the enzymes were not affected equally.

The experiments described here did not provide any evidence for the previously reported intracellular build-up of Pel, at any of the stages of the growth cycle tested. In the earlier experiments, the combined cytoplasmic and periplasmic fractions were assayed for Pel activity. The separation of these fractions for this study, might have brought the level of activity in individual samples below that which may be accurately assayed. In addition to this, the longer fractionation procedure might itself have caused a decrease in enzyme activity, due to degradation, although as stated previously (3.3.3), the total LacZ activity in stationary phase samples was as expected. The ability

of workers to detect an accumulation of Pel within the cells of *Ech* (Collmer and Bateman 1982) might be similarly explained. In addition, since the total activities of Pel and Cel are considerably higher in *Ech* than in *Ecc* (Starr *et al* 1977, Chatterjee *et al* 1979) assays should be more reliable and it should be easier to detect an intracellular build-up of Pel in *Ech*, although there is variation between strains.

The data presented here are extremely crude. Samples were taken from very few time points, making it possible that a transient intracellular build-up of Pel was missed. The differential leakage of cytoplasmic components into the other subcellular fractions raised doubts as to the validity of experiments to determine the localizations of extracellular enzymes at different stages of growth. Even if sampling at more frequent time points allowed the detection of an intracellular accumulation of Pel, it would not be possible to characterise this further, as the fractionation and assay procedures described could not distinguish between accumulation in the periplasm, and accumulation in the cytoplasm, combined with leakage into the periplasm during fractionation.

Some improvements to the assay data might have been possible. It is customary to express enzyme activity per mg protein. This is more important in the comparison of total activity in different samples than when the data are relative. Indeed, expression of activity/mg protein would have distorted the results discussed here, since the activity in the supernatant would be greatly increased by the correction for total protein. The assay data for samples taken early in the growth cycle might have been made more reliable by concentrating the samples, and so increasing the total enzyme activities. However, the concentration process is time consuming and so could actually cause a decrease in enzyme activity due to degradation.

More recent work has concentrated on alternative fractionation procedures and it has been shown that sphaeroplasting (Osborn and Munson, 1974) was more reliable than either of the methods described here, with over 90% of the total LacZ activity being reproducibly located in the sonicate (Reeves pers. comm.). However, this work was based on samples from the stationary phase of growth, rather than the earlier stages.

when the problems described here were encountered. In addition to this, the longer protocol for the generation of sphaeroplasts meant that this would be impractical for use on samples taken at frequent intervals.

Current experiments involve radiolabelling and SDS-PAGE to visualise proteins from the different subcellular fractions (Housby pers. comm.) and it is hoped that antibodies will be available for the study of extracellular enzyme localization in the near future. Although both of these methods require a reliable fractionation procedure, they have an advantage in that they do not rely on enzyme activity, which might vary between the intracellular and secreted forms.

The work discussed in this chapter reinforced the reliability of the fractionation and assay procedures for stationary phase cultures, while highlighting the problems of studying enzyme localization: a vital part of the analysis of Out^- mutants. It was concluded that, in order to compare mutants with the wild-type strain, samples must be taken from the same stage of the growth cycle: preferably the stationary phase, when enzyme localization data were most reliable.

By comparing the total activities of Pel and the various marker enzymes tested, at different bacterial densities, it was noted that all the enzymes showed a similar dependence on growth phase. It appeared that the growth phase dependent regulatory mechanism for the production of Pel in *Ech* was absent in *Ecc*.

The work was technically far more difficult than had been envisaged. The aims of finding whether secretion is regulated at, or after the export step, and of determining the point in the growth cycle at which the secretory apparatus is switched on, no longer seemed feasible. It was decided instead to concentrate on studying the secretory genes themselves.

CHAPTER FOUR.

CHARACTERIZATION OF THE *OUT* GENE CLUSTER OF *ECC*.

4.1. INTRODUCTION.

Out⁻ mutants (1.1.5, 1.7.1) were generated either chemically, with EMS, or by insertional mutagenesis using transposable elements (Hinton and Salmond 1987, Gibson *et al* 1988). These mutants were transduced with a wild-type *Ecc*, pH79 cosmid library; testing Tc^R transductants for complementation to the Out⁺ phenotype by screening on Pel and Cel assay plates. ComPLEMENTING cosmids were shown to carry overlapping restriction fragments from a 12 Kb (approx.) region of the genome: thought to define the region encoding the *Ecc* secretory apparatus. Since Out⁻ mutants could be divided into several distinct complementation groups, it was proposed that the 12 Kb region comprised a cluster of *out* genes (Gibson *et al* 1988). An approximate restriction map of the region (Whitcombe pers. comm.) is shown in figure 4.1a, which also shows some of the complementing cosmid inserts and the mutants complemented (Reeves pers. comm.).

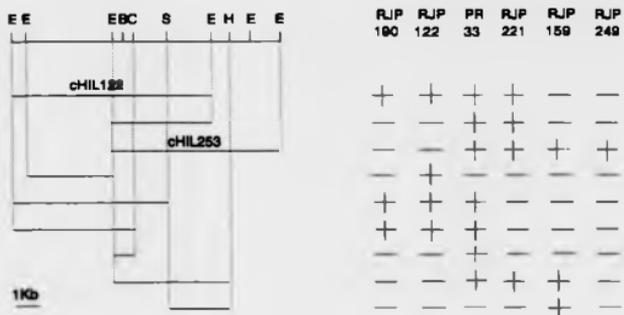
The mutant RJP208 could not be fully complemented, but a partially restored Pel⁺Cel⁺ phenotype was observed after transduction with the cosmid cHIL208: containing an insert of 17 Kb (approx.). Preliminary results suggested that other Out⁻ mutants were also partially complemented by this cosmid (Reeves pers. comm.). It was not known whether cHIL208 included the entire 12 Kb *out* region previously identified, but it was proposed that if this was the case, cHIL208 might encode a regulatory protein.

Simultaneous, but independent work also identified an *out* gene cluster in *Ecc* (*Ecc* 71); cloned into pDSK519 on a 15.7 Kb *Sall* fragment and named pAKC601 (Murata *et al* 1990). This plasmid was obtained, in order to compare it with cHIL208.

The principal aim of this part of the project was to characterize cHIL208 to determine whether it included the entire 12 Kb *out* gene cluster previously identified. If this was shown to be the case, it was planned to analyse the region thought to be involved in regulation of the *out* gene cluster. In addition, comparisons were to be

Figure 4.1a

Cosmid Complementation of *Out* Mutants.



Cosmids defining the 12 Kb *out* region are shown, together with their abilities to complement a selection of *Out* mutants.

Work performed by P. Reeves.

made between cHIL208 and pAKC601, to establish whether these carried inserts from the same chromosomal locus.

4.2. *OUT* CLUSTER RESTRICTION ANALYSIS.

The cosmids chosen for restriction analysis were cHIL122 and cHIL253 (which together delineate the 12 Kb *our* gene cluster identified originally: see figure 4.1a), along with cHIL208 and pAKC601.

Restriction digests and agarose gel electrophoresis were performed as described previously (2.13, 2.18). A typical gel photograph is shown in figure 4.2a. Since restriction analysis was used extensively throughout this project, the logic of restriction mapping is described in some detail for this first example.

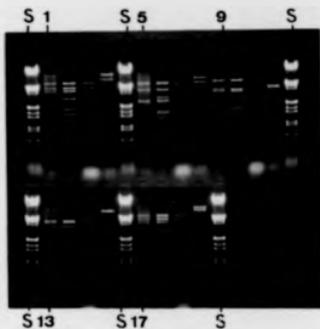
The size markers were used to generate standard curves by plotting the log of distance (mm) travelled, against fragment size (Kb). These curves were used to determine the size of each restriction fragment.

The principle of restriction mapping is the comparison of fragments generated by a series of single and double digests. By seeing which of a set of fragments, resulting from cleavage with one enzyme (A) is (are) lost upon cleavage with a second enzyme (B), and *vice versa*, it is possible to determine the relative positions of the A and B sites.

The mapping of the *our* cosmids was facilitated by the existence of a partial restriction map (Whitcombe pers. comm. See figure 4.1a), which was used as a starting point for further mapping. The logic followed for restriction mapping is outlined, taking *EcoRI* and *SalI* digests as examples. The fragment sizes generated when these enzymes were used to cleave cHIL208 are listed in table 4.2a. The total size obtained for each digest was calculated to check that no doublets had been missed, and to show the likely accuracy of the mapping. Figure 4.2b shows the *EcoRI* sites mapped previously (Whitcombe pers. comm.).

FIGURE 4.2a: RESTRICTION DIGESTS OF *OUT* PLASMIDS.

The photograph shows an agarose gel, following electrophoresis of the *out* plasmid digests listed below.



KEY TO TRACKS: (*out* plasmid and restriction endonuclease used)

S Standard: λ HindIII/EcoRI

- | | |
|-------------------------|------------------------|
| 1 cHIL122: EcoRI | 13 cHIL122: EcoRI/SphI |
| 2 cHIL208: EcoRI | 14 cHIL208: EcoRI/SphI |
| 3 cHIL253: EcoRI | 15 cHIL253: EcoRI/SphI |
| 4 pAKC601: SalI | 16 pAKC601: SalI/SphI |
| 5 cHIL122: EcoRI/SalI | 17 cHIL122: EcoRI/PstI |
| 6 cHIL208: EcoRI/SalI | 18 cHIL208: EcoRI/PstI |
| 7 cHIL253: EcoRI/SalI | 19 cHIL253: EcoRI/PstI |
| 8 pAKC601: SalI/EcoRI | 20 pAKC601: SalI/PstI |
| 9 cHIL122: EcoRI/EcoRV | |
| 10 cHIL208: EcoRI/EcoRV | |
| 11 cHIL253: EcoRI/EcoRV | |
| 12 pAKC601: SalI/EcoRV | |

TABLE 4.2.a

THE SIZES OF RESTRICTION FRAGMENTS OBTAINED BY
DIGESTION OF cHIL208.

ENZYME(S)	<i>EcoRI</i>	<i>SalI</i>	<i>EcoRI + SalI</i>	
FRAGMENT SIZES (Kb)	6.4	14.3	5.7	
	4.8	8.2	3.7	
	3.7	0.9	3.0	
	3.0		2.4	
	1.8		2.4	Doublet
	1.6		1.6	Doublet
	1.3		1.6	
	0.7		1.3	
			0.7	
			0.7	Doublet
TOTAL SIZE (Kb)	23.3	23.4	23.2	

The restriction fragment sizes shown above were calculated from standard curves, as described in the text. The *EcoRI* and *EcoRI/SalI* digests are shown in figure 4.2.1a. The *SalI* digests are not shown. Some of the restriction fragments appeared as doublets, as indicated above.

Figure 4.2b: *Eco*RI Sites in the 12 Kb *out* Region,
Showing the Orientation of the Inserts in pHC79

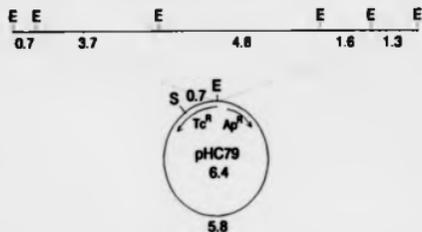
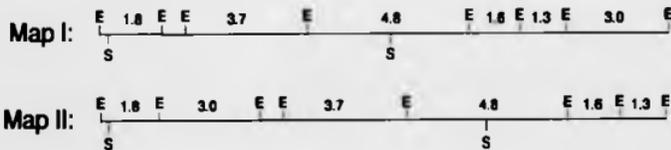


Figure 4.2c: Possible Restriction Maps of cHIL208.



Both figures show fragment sizes in Kb

The region mapped previously accounted for all except the 3.0 Kb and 1.8 Kb *EcoRI* fragments, which were therefore located at one or both end(s) of the insert.

When cHIL208 was digested with *SalI* in addition to *EcoRI*, the 4.8 Kb and 1.8 Kb *EcoRI* fragments were lost, suggesting that *SalI* cut within each of these. The single *SalI* digest produced three fragments, showing that the DNA contained three *SalI* sites. Since one of these is in the vector, it was concluded that there was one *SalI* site in both the 4.8 Kb and 1.8 Kb *EcoRI* fragments.

The 0.9 Kb *SalI* fragment showed that there was a *SalI* site 0.2 Kb (approx.) from one end of the insert. This had to be in the 1.8 Kb *EcoRI* fragment: confirmed by the 1.6 Kb fragment (seen as a doublet) in the *EcoRI/SalI* digest.

The replacement of the 4.8 Kb *EcoRI* fragment with two fragments (seen as a doublet), each of 2.4 Kb (approx.) *EcoRI/SalI* fragments suggested that there was a *SalI* site near the centre of the 4.8 Kb *EcoRI* fragment. The information discussed so far is summarized in figure 4.2c. At this stage there were two possible maps (I and II in the figure). If map I was correct, a *SalI* digest would yield fragments of 0.9 Kb, 8.4 Kb and 14.1 Kb, while if map II was correct, the expected *SalI* fragments would be 0.9 Kb, 11.2 Kb and 11.5 Kb. By comparing these predicted fragment sizes with the observed values (table 4.2a), it was concluded that map I (figure 4.2c) most accurately represented cHIL208.

Restriction mapping for other sites was performed in exactly the same way as that described above, using various double digest combinations to build up the map data. The shape of the standard curves obtained for restriction mapping meant that it was not possible to accurately predict the sizes of larger fragments. Different enzyme combinations were therefore needed to adjust the proposed positions of certain sites.

The restriction data showed that cHIL208 included all the DNA in cHIL122 and cHIL253, as predicted, and extended the original 12 Kb *owt* cluster in both directions, as indicated in figure 4.2d. The insert in cHIL208 was in the opposite orientation relative to that in cHIL122, cHIL253 and all the other *owt* cosmids studied previously (Reeves pers. comm.)

Figure 4.2d

Restriction Map of the cHIL208 Insert.

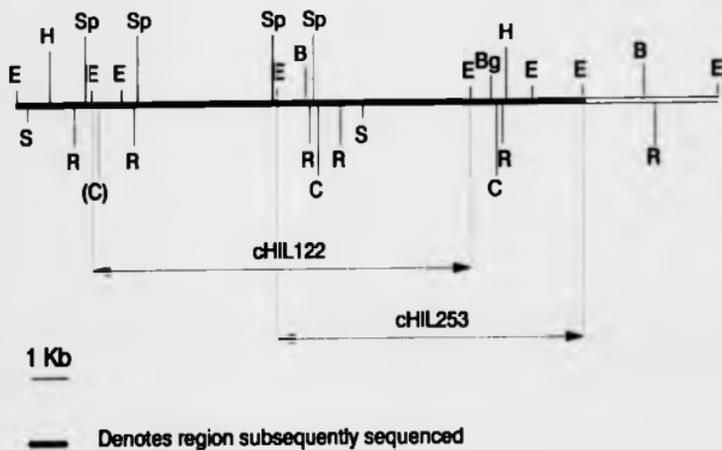
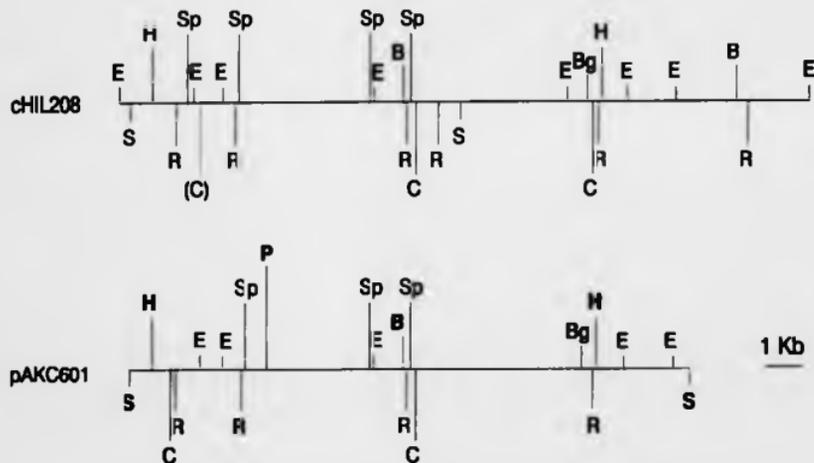


Figure 4.2e

Comparison of cHIL208 and pAKC601



Comparison of the restriction maps of the *ouf* regions cloned in cHIL208 and pAKC601. Restriction sites shown in bold are those mapped previously (Murata *et al.*, 1990).

Restriction sites common to cHIL208 and pAKC601 suggested that the two independently isolated *out* clusters were from the same chromosomal locus. There were however some differences, as shown in figure 4.2e which compares the restriction maps obtained for cHIL208 and pAKC601.

Subsequent DNA sequence analysis of the *out* cluster (Reeves *et al* in press; this study (6.2.1)) confirmed the positions of most of the restriction sites identified by mapping. The only discrepancy appeared to be a *Cla*I site (dotted in on figure 4.2e) which was not predicted by restriction mapping. Closer inspection of the DNA sequence: ..TATCGATC.. showed that it included the *dam* methylation site: GATC, and so would not be cut with *Cla*I.

4.3. OUT CLUSTER ANALYSIS USING SOUTHERN HYBRIDIZATIONS.

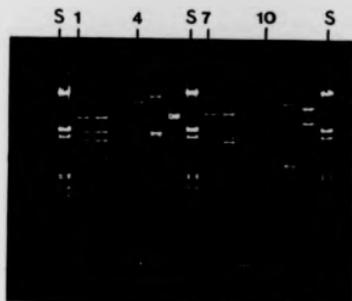
Studies were carried out on those plasmids previously compared by restriction analysis (4.2), in addition to pMG54 and cMG54. pMG54 contains a region of *out* DNA (2.5 Kb approx.), obtained by cloning *TnphaA* and its flanking sequence from a *TnphaA* insertional *Out*⁻ mutant: PR54. By probing a pHCT9 cosmid gene library, the wild-type *out* gene was obtained: in cMG54 (Gibson *et al* 1988).

All plasmids (cHIL122, cHIL253, cHIL208, pAKC601, cMG54 and pMG54) were digested with *Eco*RI and *Eco*RV. The resulting fragments were separated by agarose gel electrophoresis and blotted on to a nylon filter as described previously (2.20.1).

Two fragments of DNA were used as probes: the 0.7 Kb *Eco*RI fragment of cHIL208 and an *Eco*RV fragment of pMG54, containing *TnphaA* and 1.3 Kb (approx.) of flanking DNA. This was thought to correspond to the 1.3 Kb *Eco*RV fragment of cHIL208 which includes the 0.7 Kb *Eco*RI (probe) fragment. Each fragment, obtained by agarose gel electrophoresis and elution into a trough (2.19), was labelled with α^{32} P-dCTP by random hexamer priming. The technique for this, in addition to those for

FIGURE 4.3a: RESTRICTION DIGESTS OF *OUT* PLASMIDS.

The photograph shows an agarose gel following electrophoresis of *out* plasmid digests.



The agarose gel shown was used for Southern hybridization analysis (see figure 4.3b)

KEY TO TRACKS: (*out* plasmid and restriction endonuclease used)

S Standard: λ *Hind*III/*Eco*RI

1 cHIL122: *Eco*RI

7 cHIL122: *Eco*RV

2 cHIL208: *Eco*RI

8 cHIL208: *Eco*RV

3 cHIL253: *Eco*RI

9 cHIL253: *Eco*RV

4 pAKC601: *Eco*RI

10 pAKC601: *Eco*RV

5 cMG54: *Eco*RI

11 cMG54: *Eco*RV

6 pMG54: *Eco*RI

12 pMG54: *Eco*RV

FIGURE 4.3b

SOUTHERN BLOT: *OUT* PLASMIDS PROBED WITH DNA FROM pMG54.

The photograph shows an autoradiograph (16 hr exposure), giving the result of hybridization analysis, using the agarose gel shown in figure 4.3a.



hybridization and washing the filter have been described previously (2.20). Hybridizing bands were identified by autoradiography.

The two gels used, one of which is shown in figure 4.3a, were identical. Figure 4.3b shows the autoradiograph obtained using the pMG54 *EcoRV* fragment probe. Both probes gave similar results, suggesting that the fragments used were from the same locus, as predicted, and hence that the *TnphoA* insert in the mutant PR54, was located within the 0.7 Kb *EcoRI* fragment of the *out* cluster.

Hybridization occurred with all plasmids except cHIL253: in agreement with the relative positions of the cosmid inserts predicted by restriction analysis. The hybridization studies proved that the *out* DNA in cMG54 and pMG54 mapped to the same region of the genome as that defined by the *out* cosmid cHIL208. It was also confirmed that the *out* cluster identified in *Ecc* 71 (Murata *et al* 1990) included the same DNA as cHIL208, although the bands seen with pAKC601 were of a lower intensity than the others, suggesting that the DNA was not identical.

The *EcoRV* probe fragment might have been expected to hybridize with three *EcoRI* fragments (of 3.7 Kb and 1.8 Kb, in addition to 0.7 Kb). In fact, only the 0.7 Kb bands were visible on the autoradiographs, presumably because of the high stringency of the washes used.

4.4. COMPLEMENTATION OF *OUT* MUTANTS.

The aim was to compare the abilities of cHIL208 and pAKC601 to complement a range of *Out*⁻ mutants, mapping to various regions of the *out* gene cluster.

4.4.1. INTRODUCTION OF THE *OUT* PLASMIDS INTO MUTANTS.

The mobilizing strain GJ342 was transformed with cHIL208 and pAKC601, plating on to NBA containing Ap or Kn respectively.

Out⁻ mutants were chosen which had been shown, by previous complementation tests (Reeves pers. comm.) and transposon linkage mapping (Housby pers. comm.), to

map to a range of loci across the *out* cluster. The mutants used: RJP122, RJP159, RJP190, RJP208, RJP220, RJP221, RJP249 and RJP251 had all been derived from HC131, and so were Ap^R due to pHCP2. In order to use Ap^R selection for cHIL208, each mutant was cured of pHCP2 by repeated sub-culture in nutrient broth lacking Ap, followed by screening for sensitivity to Ap. Prospective cured mutants were shown to be Pel⁻Cel⁻Prt⁺ (using enzyme assay plates (2.1)) and sensitive to Φ KP, confirming their identity as *Ecc*.

Each cured mutant was patch mated (2.10.2) with GJ342(cHIL208) and GJ342(pAKC601). Colonies were streaked on to minimal sucrose medium (MMS), to counter select the *E. coli* donor, containing Ap or Kn as appropriate, to select for *Ecc* transformants.

Also studied was the mutant AC4000, which differed from the others listed in that it was generated by insertional mutagenesis using Tn10 (Connolly pers. comm.). It was also of particular interest because it had never been complemented previously. AC4000 was transconjugated with pAKC601 as described above, but no colonies carrying cHIL208 were obtained.

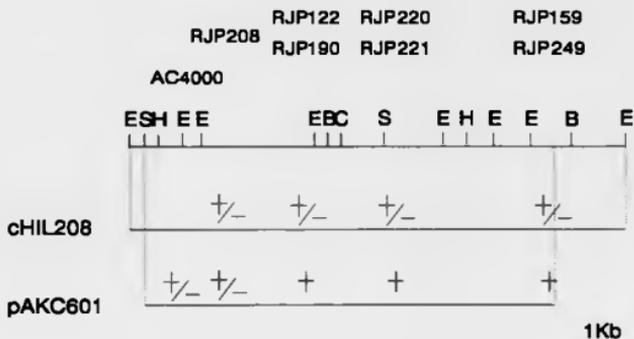
4.4.2. COMPLEMENTATION ABILITIES OF cHIL208 AND pAKC601.

For each strain generated, as described above, twenty colonies were picked on to Pel and Cel assay plates. Colonies of HC131 and the relevant cured Out⁻ mutant were picked as positive and negative controls respectively.

pAKC601 fully complemented all the mutants tested, except RJP208 and AC4000 which were both partially complemented. cHIL208 could not fully complement any of the mutants tested, as predicted from previous experiments (Reeves pers. comm.). Although the expected partial complementation was often achieved, the phenotypes were variable. For any particular strain, there was a range from full (rare), through intermediate, to extremely poor complementation. No such variability was observed with pAKC601, even with the mutants which were only partially complemented to Out⁺.

Figure 4.4.2a

The Complementation of Out Mutants
by cHIL208 and pAKC601



The approximate relative map positions of some Out mutants are shown, together with the abilities of cHIL208 and pAKC601 to cause complementation. Full complementation is indicated by +, and complementation to an intermediate Out phenotype, by +/-.

Figure 4.4.2a shows the relative map positions of the *Out*⁻ mutants within the *ou* cluster of *Ecc*, together with the results of complementation tests with cHIL208 and pAKC601. It should be noted that the positions of the *Out*⁻ mutants are approximate. Although most were located by complementation analysis (Reeves pers. comm.), RJP208 and AC4000 were only positioned using transposon linkage (Housby pers. comm.), which is often inaccurate.

The experiment demonstrated clear differences between the abilities of the plasmids cHIL208 and pAKC601 to complement *Out*⁻ mutants. It was interesting that cHIL208, carrying a larger insert, extending beyond that in pAKC601 in both directions, gave poorer complementation of the mutants. This strongly suggested that the expression of the *ou* genes was different in the two plasmids and implied that cHIL208 carried DNA with a regulatory function, which was not found in pAKC601.

The inability of pAKC601 to fully complement RJP208 and AC4000 suggested that it did not include the entire *ou* cluster, or that the *ou* clusters of *Ecc* HC131 and *Ecc* 71 were not completely interchangeable. The most likely explanation for the complementation pattern observed with pAKC601 was that the *ou* cluster is comprised of a number of different transcriptional units, with an initiation site lying downstream of the mutation in RJP208, i.e. between the map positions of RJP208 and RJP122/RJP190.

4.5. CONCLUSIONS/FINAL DISCUSSION.

Restriction analysis and Southern hybridizations showed that all the plasmids studied: cHIL122, cHIL253, cHIL208, cMG54, pMG54 and pAKC601 carried overlapping fragments of DNA which therefore came from the same region of the genome. This is in agreement with other evidence suggesting that the *Ecc ou* genes are clustered in a single chromosomal locus (Gibson *et al* 1988, Murata *et al* 1990). It was shown that cHIL208 encompassed all that DNA included in the original 12 Kb *ou*

cluster, suggesting that its ability to cause only partial complementation might be due to a regulator, encoded by the extra 5 Kb.

cHIL208 and pAKC601 differed in their abilities to complement a range of Out⁻ mutants. cHIL208 produced a generally intermediate, but highly variable Out⁺ phenotype, while pAKC601 restored the phenotype to Out⁺ for all except two mutants: RJP208 and AC4000. The data implied that DNA present in cHIL208, but not in pAKC601, i.e. located at one end of the cHIL208 insert, encoded a regulatory protein, although other possibilities must be considered.

Complementation depends on transcriptional units and whether a promoter is present. The inability of pAKC601 to complement RJP208 and AC4000 suggested that it did not include the entire *ou* gene cluster (although the polar nature of the transposon mutation in AC4000 might have affected the complementation of this strain). The data indicated that the *ou* cluster has at least two transcriptional units, one of which is initiated downstream of the RJP208 mutation, between this and the positions of the mutations in RJP122 and RJP190. Weak complementation may be achieved in the absence of a promoter by read through from the vector. Although this could explain the intermediate phenotypes of RJP208 and AC4000 with pAKC601, it would not account for the intermediate complementation of all mutants by cHIL208 if, as proposed the cluster consists of more than one transcriptional unit.

Restriction analysis showed that the insert in cHIL208 was in the opposite orientation to those in the other *ou* cosmids. This might cause a different amount of read through from the vector, but would not account for the variability in complementation.

There are various ways in which multi-copy inhibition of gene expression may occur, such as the titration of a regulatory protein. Alternatively a multi-copy plasmid could encode a truncated protein with partial activity, such as an activator protein, capable of binding, but not of causing activation.

Although these explanations might (at least in part) account for the intermediate complementation observed using cHIL208, they would not explain the variability of the observed phenotypes. It therefore seemed most likely that the unusual complementation patterns seen using cHIL208 were due to DNA which was not included in pAKC601. It was proposed that this encoded a regulatory protein, and for this reason the DNA of cHIL208 was analysed in greater detail.

CHAPTER FIVE.

**INSERTIONAL MUTAGENESIS OF THE *OUT* GENE CLUSTER
USING TRANSPOSONS.**

5.1 INTRODUCTION.

Transposons (mobile genetic elements) are segments of DNA which can move to new locations, without using homologous recombination. Transposons are important genetic tools: used originally only in *E. coli*, but more recently in other Gram-negatives, Gram-positives and eukaryotes. The many uses of transposons, including mutagenesis, cloning non-selectable genes and the analysis of operon organization and gene expression have been reviewed previously (Berg and Berg 1983, de Bruijn and Lupski 1984).

There are several advantages in using transposons to study pathogenic bacteria (Daniels 1984, Salmund *et al* 1985). The antibiotic markers 'tag' pathogenicity genes, to assist in genetic mapping, and flanking sequences may be cloned, using restriction enzymes known not to cut within the transposon (de Bruijn and Lupski 1984).

Transposon mutagenesis is only possible in bacteria other than *E. coli* if a suitable delivery vehicle (unable to replicate in the recipient) is available. These 'suicide vectors' include phage P1 (Kuner and Kaiser 1981, Quinto and Bender 1984), narrow host-range plasmids (Simon *et al* 1983, Ely 1985) and phage λ , carrying a mutation for DNA replication (Palva and Liljstrom 1981, de Vries *et al* 1984).

Phage λ can normally only attach to *E. coli* K12 as it requires the *lamB* product: a membrane-bound receptor protein (Randall-Hazelbauer and Schwartz 1973, Schwartz and Le Minor 1975). Other strains may be made λ sensitive by the introduction of *lamB*, cloned in a multi-copy vector, which allows the phage to adsorb and inject DNA. Lytic growth does not occur due to blocked replication, transcription or morphogenesis (Schwartz and Le Minor 1975, de Vries *et al* 1984, Harkki and Palva 1985). SCRI193 was transformed with pHCP2: a pBR322 derivative carrying the *E. coli lamB* gene (Clement *et al* 1982), to generate HC131 which was successfully mutagenized by λ ::TnJ (Salmund *et al* 1986).

A wide range of transposon-carrying λ derivatives are available, providing a choice of transposons, including ones carrying reporter genes (Kleckner *et al* 1977, Berg and Berg 1983, de Bruijn and Lupski 1984).

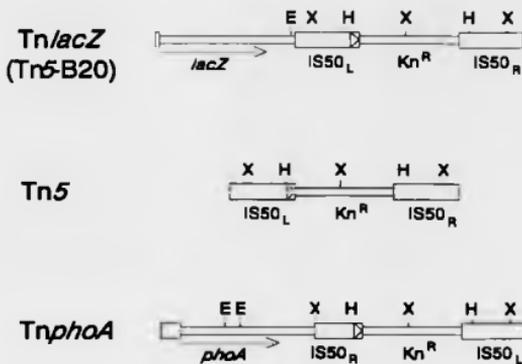
One of the best characterized transposons is Tn5 (Berg *et al* 1975, Berg and Berg 1983, de Bruijn and Lupski 1984). Tn5 has been mapped (Jorgensen *et al* 1979), its genes characterized (Mazodier *et al* 1985) and a mechanism has been proposed for its transposition (Berg 1977, 1983). Tn5 carries genes for resistance to kanamycin (and neomycin), bleomycin and streptomycin (cryptic in *E. coli*, but expressed in non-enteric bacteria). Advantages of Tn5 include its low specificity of insertion (Berg 1977, Shaw and Berg 1979, Berg *et al* 1980, Miller *et al* 1980), the generation of relatively stable inserts, with a low probability of genomic rearrangements (Berg and Berg 1983, Berg *et al* 1983) and the fact that mutations are usually completely polar (Berg *et al* 1980). Although Tn5 is not suitable for use in all Gram-negative bacteria (its transposition frequency and insertional specificity being species, and even strain dependent), it has been found to have a high transposition frequency (5×10^{-5} Kn^R colonies per plaque forming unit of $\lambda::\text{Tn5}$) and low insertional specificity in all enteric bacteria studied so far, and is a valuable tool for the study of *Erwinia* spp.

Systems have been developed in which Tn5 carries a reporter gene, the expression of which accurately reflects that of the gene containing the insert. Such derivatives of Tn5 include TnlacZ and TnphaA. TnlacZ (Kroos and Kaiser 1984) carries the *E. coli* lac operon, lacking the promoter. The expression of lacZ can be determined using the chromogenic substrate X-Gal. A series of improved derivatives of TnlacZ have been constructed, including Tn5-B20 (Simon *et al* 1989), developed to eliminate non specific lacZ expression. A simplified map of Tn5-B20 is shown in figure 5.1.1a.

TnlacZ is often unsuitable for studying exported proteins. LacZ, normally located in the cytoplasm, is unable to cross the bacterial inner membrane, even when fused to the amino terminal signal sequence of an exported protein: probably because it folds into a transport-incompetent form within the cytoplasm (Bassford *et al* 1979). Fusion proteins are often localized in the membrane where LacZ activity is low. Since the

Figure 5.1.1a

Tn5 Derivatives: Tn*lacZ* and Tn*phoA*



KEY:  *nip*-promoter

Not drawn to scale

block in export is often lethal to the cell, a reporter enzyme which may be secreted by the signal sequences of other proteins is required.

TnphoA is a derivative of *Tn5* which carries a truncated copy of the *E. coli* alkaline phosphatase gene: lacking the promoter, translational start point and signal sequence-encoding region (Manoil and Beckwith 1985). *PhoA* activity is easily determined, either on plates containing XP, or spectrophotometrically. The enzyme is normally periplasmic and is therefore able to cross the cytoplasmic membrane. In fact, activity actually depends on export, probably because the reducing environment of the cytoplasm prevents disulphide bond formation, so that the correct protein conformation cannot be achieved (Hoffman and Wright 1985). Hence, *TnphoA* inserts only produce alkaline phosphatase activity if in the correct orientation and reading frame in a gene which is expressed and encodes an exported protein. The secretion of *PhoA* by other signal sequences makes this an extremely useful system for studying protein secretion and the production of exported proteins. *TnphoA* has been used successfully to enrich for extracellular enzyme mutants in *Ecc* (Hinton and Salmond 1987). A simplified map of *TnphoA* is shown in figure 5.1.1a.

The aim of the work described in this chapter was to use transposon mutagenesis in the study of the *ouf* gene cluster.

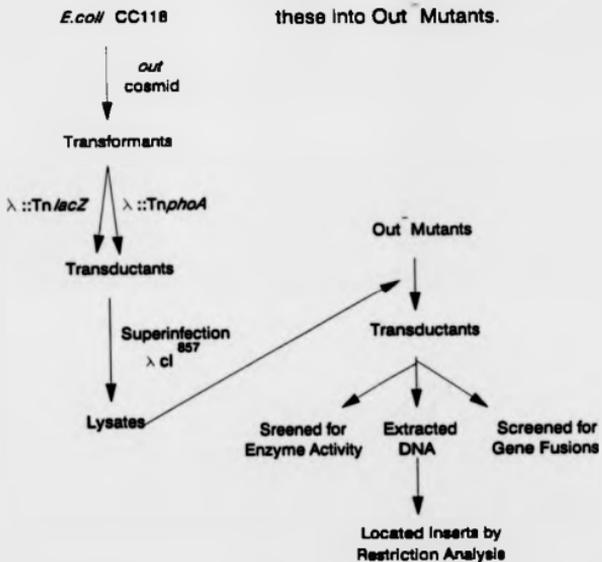
5.2 INSERTIONAL MUTAGENESIS OF *OUT* COSMIDS.

5.2.1 INTRODUCTION.

The complementation data discussed previously (4.4) suggested the presence of a regulatory element in the *ouf* cluster. It was proposed that the creation of inserts in *cHIL208*, by random transposon mutagenesis might allow the cosmid to complement *Out⁻* mutants fully. The presence of a negative regulator would hence be confirmed, and its position within the *ouf* cluster could be determined. In addition, it was hoped that information on the transcriptional units of the *ouf* cluster might be obtained, because of the polarity of mutations caused by *Tn5* and its derivatives.

Figure 5.2.2a

Protocol for the Insertional
Mutagenesis of *out* Cosmids
and the Introduction of
these Into *Out* Mutants.



5.2.2 THE GENERATION OF RANDOM INSERTS IN *OUT* COSMIDS.

The *out* cosmids: cHIL122, cHIL253 and cHIL208 were mutagenized with *Tn*lacZ (*Tn*5-*B20*: Simon *et al* 1989) and *Tn*phoA (Manoil and Beckwith 1985), using λ as the delivery vehicle. The method, essentially that described previously (de Bruijn and Lupski 1984) is outlined in figure 5.2.2a.

Each cosmid was transformed into CC118, with selection on Ap. The resulting colonies were harvested and infected with λ ::*Tn*5-*B20* or λ ::*Tn*phoA, selecting for transductants on $\text{Kn}(300)\text{Ap}(100)\text{Tc}(50)$. Selection on Ap and Tc increased the probability of the transposon being inserted into the *out* DNA, rather than the vector. The high concentration of Kn was used to enrich for cells carrying transposon inserts in the multi-copy cosmid, rather than in the host chromosome (Berg *et al* 1983).

Random colonies (six of each type) were picked and used for small-scale DNA preparations, by the boiling method (2.8.1). Restriction digests with *Eco*RI, followed by agarose gel electrophoresis showed that, in every case, there was a transposon insert within the cosmid. The smaller cosmids: cHIL122 and cHIL253 (with 8.8 Kb and 7.7 Kb (approx.) inserts respectively) were always isolated as cointegrates. Assuming that only one copy of the insert carried a particular mutation, any effect would not be seen as it would be masked by the other, normal copy. The larger cosmid: cHIL208 (17 Kb (approx.) insert) did not form dimers, presumably because it was already large enough to be packaged efficiently. The size selection imposed by packaging, cleavage of *cos* sequences and transduction means that with pHC79, inserts of 40 Kb (approx.) are expected (Hohn and Collins 1980). Although much smaller inserts are commonly cloned, no explanation for this has been found. Since the cosmid of principal interest was cHIL208, it was decided to concentrate on this, so avoiding the complication caused by dimerization.

5.2.3 THE INTRODUCTION OF MUTATED cHIL208 INTO *ECC*.

Transductant colonies of CC118 (carrying cHIL208, mutated with Tn5-B20 or TnphoA) were pool harvested and used to generate heterogeneous lysates by superinfection with λ cl⁸⁵⁷ (2.6.2).

Each lysate was used to transduce various Out⁻ mutants (RJP122, RJP159, RJP190, RJP208, RJP220, RJP221, RJP249 and RJP251), selecting on Kn(300)Ap(100)Tc(50).

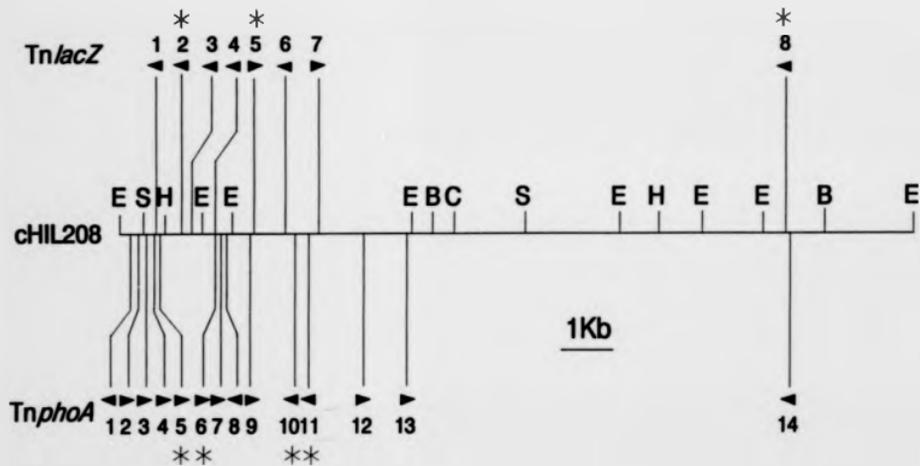
5.2.4 THE PHENOTYPIC EFFECT OF INSERTIONAL MUTATIONS IN cHIL208.

Since the principal aims of this experiment were to test the hypothesis that there was a regulatory region in cHIL208 and map its position, it was hoped that some mutated cosmids would restore secretion by Out⁻ mutants and that they would carry inserts mapping to a particular region of the cluster.

Transduced mutant colonies were screened on Pel and Cel assay plates, with HC131, the relevant mutant and this mutant transduced with cHIL208, as positive, negative and intermediate controls respectively. Colonies of each transduced strain showed variable phenotypes: ranging from Out⁻, through intermediate, to Out⁺. Colonies of each Out phenotype were picked and the DNA was isolated by the boiling method (2.8.1). By digestion with *Eco*RI, followed by agarose gel electrophoresis (2.18), the position of each insert within cHIL208 was determined, confirming the results using *Hind*III digests. Since both enzymes cleave Tn5-B20 and TnphoA asymmetrically, the orientations of the inserts were also found, using logic identical to that previously described for restriction mapping (4.2). The inserts generated by Tn5-B20 and TnphoA are shown in figure 5.2.4a. For future reference, cosmid derivatives carrying TnlacZ inserts will be referred to as cHIL208/L (1-8) and those carrying TnphoA inserts as cHIL208/P (1-14).

The number of different mutations generated was small, as the transductions gave rise to large numbers of sibling colonies carrying identical cosmids. This was unusual for transposon mutagenesis using λ as the delivery vehicle and could not be explained.

Figure 5.2.4a Transposon Inserts in cHIL208



Positions of the transposon inserts in cHIL208. Direction of transcription of the *lacZ* and *phoA* genes are indicated by \blacktriangleright . Inserts screened for gene fusions are marked: *.

The insertions did not show the random distribution expected for Tn5-derivatives. The low specificity of Tn5 makes it capable of insertion into many different sites within a genome and within a single gene (Berg 1977, Shaw and Berg 1979, Berg *et al* 1980, Miller *et al* 1980).

Tn5 has insertional 'hot spots' in pBR322. The principal hot spot in pBR322 lies in the -10 region of the Tc promoter and is also near a second promoter in the opposite orientation (Stueber and Bujard 1981). There is evidence for a correlation between the transcriptional activity of nearby promoters and transposition into a hot spot (Sasakawa *et al* 1982, McKinnon *et al* 1985, Lodge *et al* 1988): an increase in the amount of transcription running into a transposon from flanking DNA increases the efficiency of transposition.

The clustering of transposon insertions at one end of the cHIL208 insert might be indicative of promoter activity in that region. Subsequent work has demonstrated that cHIL208 contains genes arranged into a putative operon (Reeves *et al*, in press). The DNA found to be the major target site for insertion was thought to correspond to the upstream region of the operon. Although this might have been due to *ow* promoter activity, it must be considered that this region of DNA was located close to the Tc promoter of pHC79.

There was no evidence to suggest that inserts in certain regions of cHIL208 led to particular complementation patterns, although, as stated previously, the number and distribution of insertions generated were limited. Restriction mapping showed that in many cases, transposition had been accompanied by gross deletions or rearrangements, although less than 1% of Tn5 insertions normally cause DNA rearrangements (de Bruijn and Lupski 1984). These insertions (not included in figure 5.2.4a) could not be used for further experiments.

To test the abilities of particular insert-containing derivatives of cHIL208 to complement different *Out*⁻ mutants, homogeneous lysates were generated. CC118 was transformed with 7 cHIL208 derivatives (3 carrying *TnlacZ*; 4 carrying *Tnp_{hA}1*: marked * in figure 5.2.4a.) and the strains produced were superinfected with λ c1⁸⁵⁷.

Each lysate was used to transduce the following mutants: AC4000, RJP122, RJP159, RJP190, RJP208, RJP220, RJP249 and RJP251, selecting on NBA Kn, Ap. The colonies were screened on Pel and Cel assay plates. Each mutant, transduced with any of the homogeneous lysates produced three types of colony, with Out⁺, intermediate and Out⁻ phenotypes, although there was some variation between the relative frequencies of the different colony types.

If cHIL208 encoded a repressor, insertional inactivation of that region might allow cHIL208 to fully complement Out⁻ mutants. Colonies with an intermediate phenotype could have occurred due to loss of the insert by precise excision (antibiotic selection was not used on assay plates to ensure good growth), while Out⁻ colonies could have occurred due to deletions. This could not fully explain the observed phenotypes, as inserts in different regions of the *out* cluster would not all be expected to inactivate a repressor.

5.2.5 ANALYSIS OF THE VARIABLE COMPLEMENTATION CAUSED BY MUTATED cHIL208.

Transduced mutant colonies of each phenotype were picked and the cosmid DNA was extracted. Restriction analysis using *EcoRI* and *HindIII* as before (5.2.4) showed that each cosmid still carried a transposon insert in its original position, and that gross rearrangements or deletions had not occurred. This seemed to disprove the proposed explanation for the phenotypic variation (5.2.4). Although small deletions, not detected by agarose gel electrophoresis could have occurred, it has been demonstrated previously by sequence analysis that small DNA rearrangements at the point of insertion of Tn5 are extremely rare (Lupski *et al* 1983).

Various tests were carried out to determine the cause of the variable phenotypes, using RJP190 as a representative mutant.

To eliminate the possibility that Out⁺ colonies were due to spontaneous reversion, 100 colonies of RJP190 were screened on Pel and Cel assay plates. No phenotypic variation was observed, with all colonies being Out⁻.

It was confirmed that the variable phenotype was due to the insert-carrying cosmid by curing RJP190 (cHIL208/L2). This was achieved by repeated subculturing without antibiotic selection, followed by screening, as described previously (2.11), resulting in 20% (approx.) curing. Twenty cured colonies were screened on Pel, Cel and Prt plates, and tested for Kn resistance, and sensitivity to Φ KP. The Prt and Φ KP tests confirmed that the colonies were *Ecc*. Only one colony was Kn^R, in agreement with previous findings that secondary transposition into the chromosome was not a common event (de Bruijn and Lupski 1984). All the colonies tested were found to be Pel⁻Cel⁻, showing that the cosmid had been responsible for the phenotypic variation.

Cosmid DNA was extracted from one Out⁺ and one Out⁻ colony of RJP190(cHIL208/L2) and used to transform *E. coli* CC118. 'Out⁺' and 'Out⁻' lysates were generated by superinfection with λ cI⁸⁵⁷ and used to transduce RJP190. Screening on Pel and Cel assay plates showed that the 'Out⁺' lysate produced colonies of each Out phenotype (+, - and intermediate), but, as before, these carried cosmid DNA which appeared identical after restriction analysis. Interestingly, although the 'Out⁻' lysate also gave rise to colonies with variable phenotypes, only Out⁻ and intermediate colonies were found in this case. Cosmid DNA from an Out⁻ colony could not restore the Out⁺ phenotype, suggesting that some irreversible change, not detectable by restriction analysis, had occurred.

5.2.6 DISCUSSION.

The effect of transposon inserts, on the ability of cHIL208 to complement Out⁻ mutants was found to be extremely complex. The apparent variable effect of the mutations, combined with the variability of complementation by cHIL208 itself (4.4.2) meant that no information on the existence and possible location of a repressor could be obtained. It is possible that limitations were imposed on the experiment by the use of λ . In retrospect, a more conventional technique might have proved more successful.

5.3 SCREENING FOR GENE FUSIONS.

The seven transposon inserts chosen for complementation analysis (5.2.4) were to be screened for the formation of gene fusions in the *Ecc* strains: GS2001 and GS4000 (PhoA⁻ and LacZ⁻ respectively, due to Tn10 insertions). The control strains GS2001(cHIL208) and GS4000(cHIL208) were required, but could not be generated directly as the recipients and cosmid had the same resistance markers: Ap and Tc. GS2001 and GS4000 were cured of Tn10 using fusaric acid selection (2.12). Two rounds of growth on fusaric acid resulted in 100% loss of Tn10. Screening for PhoA and LacZ activity (on XP and XGal plates: 2.2), showed that the activity of each enzyme had been lost. Previous work showed that approximately half of excision events involving Tn10 caused alterations in the chromosome very close to the original insertion site. Other events caused more extensive alterations, mainly inversions, with 10% of all excisions creating extensive deletions (Bochner *et al* 1980). The PhoA⁻ and LacZ⁻ colonies obtained from GS2001 and GS4000 were therefore likely to be due to a variety of inversion and deletion events.

GS2001(Δ phoA) and GS4000(Δ lacZ) were transduced with cHIL208 and the 7 Tn-insertion derivatives (5.2.4), selecting for Tc resistance. Colonies were screened for PhoA and LacZ activity on the relevant chromogenic substrates (2.2), using HC131 as a positive control for both enzymes. Both GS2001(cHIL208) and GS4000(cHIL208) produced only white colonies, as expected for the negative controls.

All the chosen insert-containing derivatives of cHIL208, when transduced into GS2001 or GS4000 produced occasional blue colonies in a white background, rather than the blue only colonies expected if functional gene fusions had been formed. It was possible that gene fusions had been formed, which were extremely unstable, but it seemed more likely that secondary transposition had created rare fusions with chromosomal genes. This hypothesis was tested by the extraction, and restriction analysis of cosmid DNA from blue colonies. Since the mechanism of TnJ transposition is conservative rather than replicative (Berg 1977, Berg 1983), secondary transposition

into the chromosome should be characterized by the loss of the transposon from the cosmid. Cosmids extracted from blue colonies were found to contain the original insertions and there was no evidence for a mixed population of cosmids, only some of which carried transposons. However, further evidence for secondary transposition was obtained. White colonies (taken from mixed plates), were streaked out to give single colonies: some of which were blue. It was concluded that none of the transposon inserts tested had generated useful protein fusions with enzyme activity.

TnlacZ, used to generate transcriptional fusions, must be inserted in the correct orientation, within an expressed gene, if *LacZ* activity is to be detected. Although sequence data (Reeves *et al* in press) suggested that the insertion in cHIL208/L5 was in the correct orientation relative to the direction of transcription of the *ou*t cluster, it might have been in a non-coding or poorly expressed region of DNA. Alternatively, the insert could have formed a fusion with an exported or trans-membrane protein. Such fusions tend not to be functional, as discussed previously (5.1). Work done subsequently (discussed earlier: 1.7, 1.9) suggested that the *ou*t cluster encodes predominantly periplasmic and/ or membrane-associated proteins. Considering this, functional *LacZ* fusions would not be expected.

To yield a functional *PhoA* fusion, an insertion must be in the correct reading frame of an expressed gene, since these fusions are translational. It is possible that of the three insertions (cHIL208/P5, P6 and P12) which were apparently correctly orientated relative to the direction of transcription, none were in the correct reading frame of transcribed regions. Since *PhoA* is only active when transported to the periplasm or beyond (5.1), and the *ou*t cluster is thought to encode periplasmic and/ or membrane-associated proteins, functional *PhoA* fusions would be expected. It is possible that in-frame fusions were formed within cytoplasmic or trans-membrane domains of integral membrane proteins, making them inactive for *PhoA*.

Further information on the locations of the *Tn* inserts could have been obtained by DNA sequence analysis of the junction sites. Since the inserts did not yield either functional gene fusions, or information on the putative repressor, no such analysis was

performed, although information on the precise location of the insertions might have been useful for the prediction of open reading frames and the organization of protein domains across the cytoplasmic membrane.

It might have been possible to identify gene fusions by performing the same analysis on each of the other insert-containing derivatives of cHIL208 which were shown by sequence analysis to be in the correct orientation relative to the direction of transcription of the *out* cluster. Generating a homogeneous lysate for each insert, transductions and screening for fusions would have been very laborious: work which did not seem justified. It was clear that no useful information on the ability of each cHIL208 derivative to complement *Out*⁻ mutants would be obtained, due to variable phenotypes (5.2.5). Although information on the topology of transmembrane proteins might have been obtained, far more efficient methods are available for this (Broome-Smith and Spratt 1986), and are currently being used to study the *Out* proteins (Reeves pers. comm.).

5.4 GENE FUSIONS IN THE STUDY OF GENE EXPRESSION.

5.4.1 INTRODUCTION.

It was hoped that the work described above (5.2-5.3) would generate gene fusions, in which reporter enzymes (LacZ or PhoA) were produced under the control of *out* gene promoters. The intention was to use these fusions to investigate the factors responsible for the regulation of particular *out* genes. Since the expression of a reporter gene in a multi-copy plasmid may be different from that of the same insertion in the chromosome (reporter activity being affected by the vector promoter and copy number), it was proposed to introduce the fusions into the *Ecc* chromosome by reverse genetics. Since this technique was causing problems at the time (Kell pers. comm.) and no gene fusions were identified (5.3), an alternative strategy was found.

Gene fusions had been generated previously by λ ::*TnphaA* mutagenesis of GS2001 (Hinton and Salmond 1987). Among the strains generated were two *PeI*⁻*Cel*⁻ mutants:

PR33 and PR54 (Hinton and Salmond 1987, Gibson *et al* 1988). DNA sequence analysis later showed that these contained inserts in *owG* and *owD* respectively (Reeves *et al*, unpublished work).

The chromosomal fusions between *phoA* and genes of the *ow* cluster in PR33 and PR54 were used to investigate whether the *ow* cluster is regulated. The factors chosen were temperature and growth substrate, as these are easy to vary *in vitro* and are both thought to affect Pel synthesis and the plant/ pathogen interaction (Perombelon and Kelman 1980, Collmer *et al* 1982).

As discussed previously (3.6), the state of growth of a culture can have a great effect on enzyme activity and even on the assays themselves. For this reason, the growth curve was followed for each strain, under the different culture conditions, and doubling times were calculated. These, together with final OD (600 nm) readings, indicated whether comparisons of enzyme activities in different cultures were valid. Such checks were particularly important, since the experiment aimed to compare the total enzyme activities in different samples, rather than the relative activities of different fractions from a particular sample, as described previously (3). Samples were fractionated in stationary phase, following the findings (3.6) that enzyme activities are highest, and assays most reliable in samples from this phase in the growth cycle.

Each sample was assayed for the total activities of PhoA, Pel and Bla. The activity of PhoA would provide information on the expression of the *ow* genes, involved in the secretion of Pel. By comparing the changes in the activity of Pel with those of PhoA, the factors affecting Pel synthesis and secretion could be compared. Finally, Bla was chosen as the control enzyme as it was easily assayed, and was not expected to be regulated along with Pel synthesis and/ or secretion.

Previous experiments had shown that Pel synthesis by SCRI193 was switched off at 37°C (Hinton and Salmond 1987) and it was suggested that secretion of Pel and Cel was also less efficient at the higher temperature. There have also been reports that Pel synthesis by *Ech* is reduced (8 fold) at 37°C, relative to 30°C (Favey *et al* 1992), while Pel synthesis by *Eca* is reduced 3-6 fold at 30.5°C compared to 27°C (Lanham *et*

al 1991). It was therefore decided to compare the levels of Pel synthesis and *owt* gene expression in cultures grown at 30°C and 37°C.

In addition, the activities of the three enzymes were to be compared in cultures grown in the presence and absence of PGA: thought to have a role in the induction of Pel synthesis. The true inducer of Pel synthesis is thought not to be PGA itself, but breakdown products of this, including digalacturonic acid: DGA (Tsuyumu 1977, Collmer and Bateman 1981, Chatterjee *et al* 1985, Condemine *et al* 1986, Yang *et al* 1992). Since the strains of interest were *Out*⁻ mutants, thought to be incapable of degrading PGA, the inducing molecule DGA might not be produced. This problem was tackled in two ways. Firstly, complemented *Out*⁺ versions of PR33 and PR54 were produced and used in the experiments, and secondly, cultures were grown in the presence of DGA rather than PGA.

5.4.2 PREPARATION OF SAMPLES FOR ENZYME ASSAYS.

In addition to the strains HC131, PR33 and PR54, complemented versions of the two *Out*⁻ mutants were used. Cosmid cHIL208/5 carries a 30 Kb (approx.) insert: including the entire *owt* cluster previously identified, cloned into pSF6. (Reeves pers. comm.). cHIL208/5 was isolated for its ability to fully restore the *Out*⁺ phenotype to all *Out*⁻ mutants tested, including RJP208 (see 7.2). The cosmid was introduced into PR33 and PR54 by electroporation (2.10.3), selecting on NBA Sp. Restoration of the *Out*⁺ phenotype was confirmed by picking colonies on to Pel and Cel assay plates.

Each strain was grown under a variety of conditions in 25 ml side arm flasks. Liquid media (2 ml), containing appropriate antibiotic(s), were inoculated with 80 µl of a fresh overnight culture (grown in MM at 30°C). The cultures were incubated and their cell densities followed, so that their doubling times (min) could be determined.

The growth conditions were varied as follows. All strains were grown in MM, PM and DM (MM supplemented with 0.5% (w/v) Digalacturonic acid), at both 30°C and 37°C.

After 24 hr (approx.) incubation, when the cells were in stationary phase, the cultures were sonicated (2.3.2), cell debris was removed and the samples were frozen in aliquots at -20°C .

The samples were assayed (in triplicate) for the activities of PhoA (2.5.7), Pel (2.5.5) and Bla (2.5.1) and the protein contents (mg/ml) were determined (2.4).

5.4.3 THE EFFECT OF GROWTH CONDITIONS ON ENZYME ACTIVITY.

The doubling times (DT) in min, and the final cell densities (600 nm) of the cultures (listed in table 5.4.3a), were usually similar for a particular strain grown under various conditions, suggesting that any comparisons between enzyme activities in the different samples were valid. There were however, differences between the strains, with PR33 growing relatively slowly. PR54 carrying cHIL208/5 grew more slowly, and to a slightly lower final OD(600 nm) than the parent strain, presumably due to the constraints imposed by the need to replicate such a large plasmid. This effect was so marked in the already slow growing PR33, that preliminary data (not listed) showed DTs of up to 3 hours and final OD(600 nm) readings as low as 0.7, suggesting that assay data from this strain would not be valid.

The total activities of Pel, PhoA and Bla under the various culture conditions are shown in figures 5.4.3a, 5.4.3b and 5.4.3c respectively. Activities are given as the change in absorbance (at the particular wavelength) per min per mg protein (as determined from the protein assays).

The Pel assays gave a measure of the amount of Pel synthesized, but did not provide any information on secretion since only total activities, rather than activities and localization were determined. The PhoA assays measured the independent expression of the secretory apparatus genes: *omfD* and *omfG*.

The data in figure 5.4.3a show that regardless of growth media, Pel synthesis was reduced at 37°C , compared to that at 30°C . This is in agreement with previous findings that Pel synthesis is switched off at 37°C (Hinton and Salmond 1987).

TABLE 5.4.3a

THE DOUBLING TIMES (MIN) AND FINAL CELL DENSITIES (OD 600 nm)
OF STRAINS GROWN UNDER VARIOUS CULTURE CONDITIONS.

STRAIN	MEDIA	TEMP. (°C)	DT. (MIN)	FINAL OD(600nm)
HC131	MM	37	57	2.20
	MM	30	63	2.17
	PM	37	57	2.14
	PM	30	68	2.07
	DM	37	68	2.35
	DM	30	66	2.28
PR33	MM	37	90	2.19
	MM	30	84	2.14
	PM	37	87	2.15
	PM	30	87	2.04
	DM	37	81	2.26
	DM	30	96	2.31
PR54	MM	37	54	2.18
	MM	30	56	2.05
	PM	37	72	2.08
	PM	30	48	1.99
	DM	37	52	2.24
	DM	30	64	2.15
PR54(cHIL208)	MM	37	130	1.84
	MM	30	100	1.81
	PM	37	114	1.73
	PM	30	85	1.70
	DM	37	120	2.27
	DM	30	115	2.10

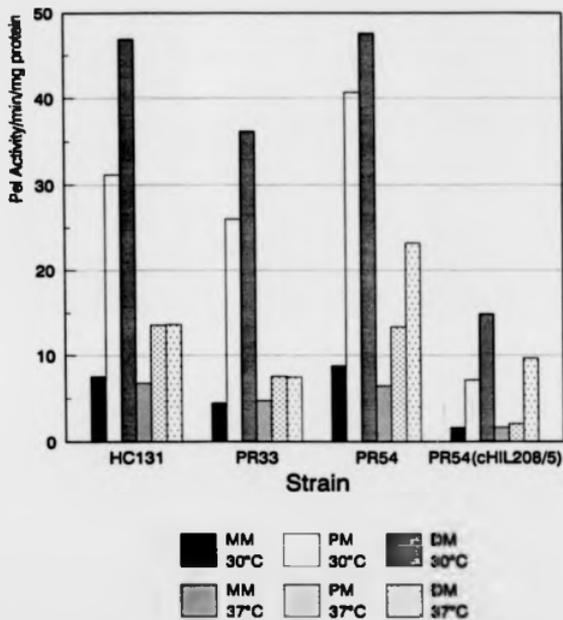
KEY: DT = Doubling Time

MM = Minimal Medium

PM = Pel Minimal Medium

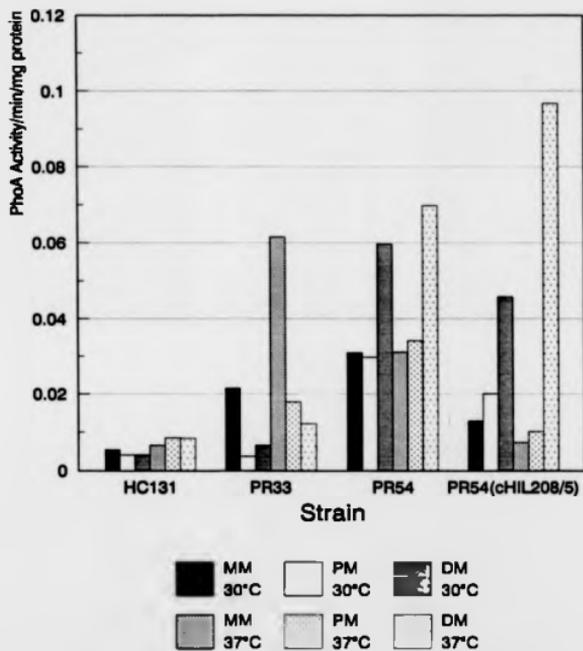
DM = Minimal Medium, supplemented with 0.5 % DGA

Figure 5.4.3a
Effect of Growth Conditions on Pel Activity



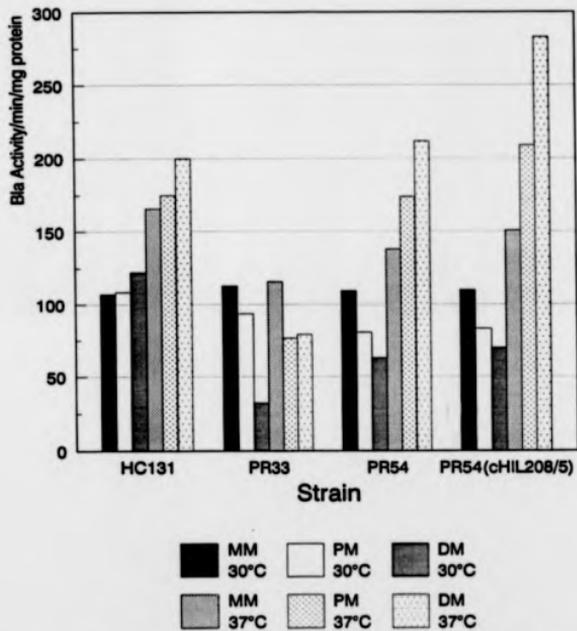
Units of Pel activity: Δ Absorbance (235 nm) /min/mg protein

Figure 5.4.3b
Effect of Growth Conditions on PhoA Activity



Unit of PhoA activity: Absorbance_(420 nm) /min/mg protein

Figure 5.4.3c
Effect of Growth Conditions on Bla Activity



Units of Bla activity: Δ Absorbance (500 nm) /min/mg protein

Both PGA and DGA induced Pel synthesis, with DGA having a greater effect. Surprisingly, the inducing effect of PGA was not diminished in the *Out⁻* strains. This might suggest that PGA is able to induce Pel synthesis almost as well as DGA, contradicting previous evidence which showed that in *Ech*, DGA was ten times as effective at inducing Pel synthesis as PGA (Collmer *et al* 1982). It is possible that the PGA used actually contained a high proportion of DGA, or that much of the polymer was hydrolysed during the preparation of PM. Spectroscopic analysis would be needed to confirm these theories. Another possibility is that *Out⁻* mutants of *Ecc* are capable of degrading PGA to DGA. This theory is favoured in view of the findings discussed later (7).

The inducing molecules raised the levels of Pel synthesis, even at 37°C (although they were less effective than at 30°C). This suggests that the switching off of Pel synthesis at higher temperatures is not absolute, and may be overcome (at least partially) by other factors.

The wild type strain HC131 and the *Out⁻* mutant PR54 showed similar levels of Pel synthesis, in agreement with previous reports that *Out⁻* mutants are affected only in the secretion and not production of extracellular enzymes (Andro *et al* 1984). It is possible that the lower levels of Pel activity in PR33 were connected with the poor growth of this strain, a factor which might also explain the low Pel activity in PR54(cHIL208/5). The complementation of PR54 to *Out⁺* did not, as might have been expected, lead to increased induction by PGA. It is possible that low levels of Pel (due to poor growth of the strain) meant that PGA was not readily broken down to DGA. Alternative explanations have been discussed above.

Figure 5.4.3b shows the effect of various growth conditions on the activity of PhoA. In the HC131 control, the level of PhoA activity was low, and was hardly affected by growth substrate, but activity was increased slightly by growth at the higher temperature. PhoA was generally more active in cultures grown at 37°C rather than at 30°C, suggesting that genes of the *out* cluster were not down-regulated at the higher temperature, along with the *pel* structural genes. This contradicted previous claims that

Pel, Cel and Peh are not secreted at 37°C (Hinton and Salmond 1987): based on the fact that the enzymes could not be detected in the S/Ns of cultures grown at the higher temperature. The decreased level of total activity at 37°C might have meant that these assays were inaccurate.

The PhoA activities of PR33 and PR54 were differently affected by PGA and DGA. Both these substrates reduced the activity of PhoA in PR33 (i.e. inhibited the expression of *owG*). PhoA activity in PR54 (*oud* expression) was not affected by PGA, but was increased by the breakdown product DGA. The patterns of PhoA activity in PR54(cHIL208/5) were similar to those in PR54, except that at 30°C there seemed to be some induction by PGA, possibly because the complemented strain was able to degrade PGA to DGA. At 37°C (when Pel synthesis would have been switched off), PGA no longer caused an increase in PhoA activity.

The activity of a gene fusion depends on several factors, in addition to the level of expression of the gene carrying the insert. Sensitivity to proteolytic degradation can make a fusion unstable. PhoA is inactive until it has crossed the cytoplasmic membrane and so its activity is dependent on transport. Preprotein leader sequences may vary in their abilities to direct the transport of PhoA. For fusions to transmembrane proteins, the site of transposon insertion is important, as PhoA activity varies, depending on whether exposure to the periplasm is optimal (Gentschev and Goebel 1992). It is clear then, that different PhoA fusions will yield very different enzymic activities, meaning that no conclusions could be drawn about the relative levels of expression of *oud* and *owG*.

From figure 5.4.3c it can be seen that, as reported previously (Hinton and Salmond 1987), Bla activity was generally higher in cultures grown at 37°C than at 30°C. This confirms that the apparent switching off of Pel synthesis was a genuine and specific phenomenon, and not due to a general reduction of gene expression at the higher temperature.

Surprisingly, both PGA and DGA affected the activity of Bla, with DGA having a more marked effect than PGA. The two molecules appeared to cause induction in HC131 and in PR54 (Out⁻ and complemented version) at 37°C, while causing a reduction in activity in PR33 and in PR54 at the lower temperature. No explanation could be found for these results, or for the fact that while Pel and PhoA activities were low in the poorly growing PR54(cHIL208/5), the Bla activities in this strain were anomalously high.

The unexpected results of the Bla assays raised doubts as to the validity of the PhoA assay results discussed above. It seems reasonable to conclude that the Pel assay data are genuine, but similarities between the induction/ inhibition patterns of PhoA and Bla, particularly by DGA caused concern.

5.4.4 DISCUSSION.

There are several problems associated with the work described above and improvements could have been made. The samples could have been fractionated, so that the supernatant, periplasm and sonicate activities could have been determined separately. Information on the localization of Pel activity should have confirmed the results of the PhoA assays, indicating the expression of the *ouf* genes. However, the experiment aimed to look at *ouf* gene expression, independent of Pel synthesis. Data showing enzyme localization would have been complicated and would probably have yielded little extra information. Localization data for PhoA activity would have been meaningless, since the enzyme is only active once it has been transported across the cytoplasmic membrane. In addition to this, as discussed previously (3.6), enzyme assays may be less accurate after cell fractionation, either because activity is reduced by the fractionation process, or because of the relatively low activity of individual samples.

The use of gene fusions is based on the assumption that the hybrid proteins are produced at the rate of the normal gene products, and have the same stabilities. This could have been tested using specific antibodies.

There were further problems, associated with the comparisons of the relative inducing properties of PGA and DGA. The two molecules were added to the media at the same % (w/v), but possible differences in the intracellular concentrations should be considered. Although there was concern about the effect of the small size of DGA molecules compared to those of PGA, previous findings showed that whether the dimer and polymer were added in molar, or weight equivalents, the dimer caused higher levels of induction of Pel synthesis (Collmer and Bateman 1982).

Despite the crude nature of the experiment, some conclusions could be drawn from it. It was confirmed that Pel synthesis in SCRI193 is switched off at 37°C. It is interesting to note that *pel* genes from *Ech*, cloned into *E. coli* are equally expressed (and cause soft-rot) at 37°C and at 30°C (Keen *et al* 1984), suggesting that the temperature switching is specific to *Erwinia*. Pel synthesis was shown to be induced by PGA and DGA, although for the reasons discussed above, conclusions could not be drawn as to the relative effects of these substrates. Levels of induction were relatively low compared to the twenty fold increase observed in *Ech*, upon addition of 0.5% PGA (Collmer and Bateman 1982), which could account for the high levels of Pel activity reported for *Ech*, compared to *Ecc*. The abilities of inducers to raise Pel activity at 37°C (although by less than at 30°C), suggested that the temperature switch was not absolute.

The genes of the *out* cluster were not co-ordinately regulated with Pel synthesis. *OutD* and *outG* were not switched off at 37°C, despite the fact that in the homologous cluster of genes, involved in the secretion of pullulanase by *Klebsiella* sp. (1.8), expression is reduced at 37°C (Pugsley and Reys 1990).

Although it was difficult to compare the effects of PGA and DGA on *out* gene expression relative to Pel synthesis (5.4.3), there was evidence to suggest the involvement, of DGA in particular, in the regulation of the *out* cluster. Interestingly, *outD* and *outG* appeared to be regulated differently, with DGA having an opposite effect on the expression of the two genes. This was quite possible, as sequence analysis has suggested that *outD* and *outG* are not translationally coupled.

Various additional experiments could have been tried. There is evidence for catabolite repression of Pel synthesis by glucose (Tsuyumu 1979, Collmer and Bateman 1981, Condemine *et al* 1986), so it might have been useful to compare Pel production and *out* gene expression in glucose-grown cultures. It has also been shown that the inclusion of cAMP in inducing media, increases the level of induction (Chatterjee *et al* 1981). Although it would have been interesting to see the effects of glucose, and of cAMP, these experiments were beyond the scope of the project.

5.5 CONCLUSIONS/ FINAL DISCUSSION.

Transposon mutagenesis was used to generate inserts in the *out* cosmid cHIL208. Although in many cases insertion was accompanied by deletions and/ or rearrangements, eight TnlacZ and fourteen TnphoA inserts were successfully mapped. These inserts were not randomly arranged, possibly due to clustering in a region of the *out* cluster with promoter activity. The region favouring transposition was shown subsequently to be at the upstream end of the *out* cluster (Reeves *et al* in press).

DNA sequence analysis showed that the central region of cHIL208 includes several open reading frames, all running in the same direction, forming part of a putative operon. Tn5 and its derivatives cause polar mutations in operons, as transcription from the operon's promoter is terminated within the element, irrespective of the orientation of the transposon insertion (Berg *et al* 1980). Such polar mutations would have interfered with complementation analysis. A Tn5 insertion does not always inactivate all the distal genes in an operon, as transcription may be re-initiated, either by a second promoter within the operon, or by a hybrid promoter: formed by Tn5 and its target sequence (Berg *et al* 1980). Ideally then, by looking at the abilities of various insert-containing derivatives of cHIL208 to complement *Out*⁻ mutants mapping to different regions of the *out* cluster, it should have been possible to confirm the existence of an operon and its direction of transcription. In addition to this, it might have been possible

to obtain information on the number and location of transcriptional units, while confirming the relative map positions of the *Out*⁻ mutants.

The transposon inserts had a very complex effect on the ability of cHIL208 to complement *Out*⁻ mutants. The phenotypes of 'complemented' strains were both variable and unstable, so that no evidence for the existence and location of the putative repressor, or the organization of an operon, could be obtained.

DNA sequence analysis (Reeves *et al* in press) suggested that the direction of transcription, relative to the approximate map of cHIL208 (eg figure 5.2.4a) was from left to right. Although sequence data had been obtained up to and including the 0.7 Kb *EcoRI* fragment, no upstream promoter of the *out* gene cluster had been identified. This was in agreement with the complementation data obtained using pAKC601 (4.4.2), which suggested that this plasmid did not include the upstream region of the *out* cluster. This, together with the relatively high frequency of transposition into the upstream region of the cHIL208 insert, suggested that the 1.8 Kb *EcoRI* fragment from this region might include the promoter. Sequence analysis was required to confirm this (6.2, 7.4)

Of the inserts generated in cHIL208, three *TnlacZ* and four *TnphoA* were screened for the formation of functional gene fusions. None were identified and this part of the work was not pursued further.

The *Out*⁻ mutants PR33 and PR54, containing chromosomal *PhoA* fusions in genes of the *out* cluster were used to study the expression of individual *out* genes, independent of *Pel* synthesis.

The activities of *Pel*, *PhoA* and *Bla* were assayed in cultures grown at 30°C or 37°C, in the presence or absence of PGA or DGA. The results confirmed that *Pel* synthesis in SCRI193 is induced by PGA and particularly DGA, and switched off at 37°C. It seemed that *out* genes were also regulated, but not in the same way as *Pel* synthesis, as they were not switched off at 37°C. The effect of substrate was not clear,

with DGA seeming to have the opposite effect on expression of different *ou* genes. Surprisingly, the activity of Bla (constitutively expressed) was also affected by the growth substrate. This observation could not be explained, and raised serious doubts as to the validity of any data showing the effect of substrate on *ou* gene expression.

It seemed that the best way to obtain further information on the regulation of the *ou* genes was to analyse the DNA sequence in the upstream region of the cluster.

CHAPTER SIX.**ANALYSIS OF THE UPSTREAM REGION OF CHIL208.**

6.1 INTRODUCTION.

As stated previously (1.7.2), sequence analysis (Reeves *et al* in press) showed the *our* cluster to contain several genes, all transcribed in the same direction. No typical promoter sequence was identified, suggesting that the sequence data did not cover the entire *our* gene cluster. It was therefore decided to sequence that region of cHIL208, shown by restriction analysis (4.2) to extend upstream of the sequenced region. Since transposon insertional mutagenesis (5.2.4) had indicated possible promoter activity in the upstream region of the cHIL208 insert (by the increased frequency of transposition into that region), it was hoped to identify the start of the *our* gene cluster. The region was also of interest because of the unusual complementation patterns it seemed to cause (4.4.2). It was hoped that sequence analysis would explain the apparent repression of *our* gene expression in cHIL208. The DNA chosen for sequencing was the 1.8 Kb *EcoRI* fragment of cHIL208.

6.2 SEQUENCE ANALYSIS.

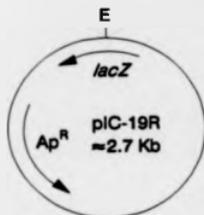
6.2.1 SEQUENCE ANALYSIS OF THE 1.8 Kb *EcoRI* FRAGMENT OF cHIL208.

The required 1.8 Kb *EcoRI* fragment was obtained by digestion of cHIL208 with *EcoRI*, followed by agarose gel electrophoresis and elution into a trough (2.19). The resulting DNA was ligated (2.17) into *EcoRI*-cut, phosphatased pIC-19R, and transformed into TG1, plating on to NBA, XGal, IPTG, Ap. DNA was extracted from individual white colonies and checked by restriction analysis as described previously (4.2). The construct obtained, named pSW1, is shown in figure 6.2.1a.

Large scale samples of pSW1 were prepared and purified using CsCl gradients (2.8.3). *EcoRI* digests of pSW1 were electrophoresed and the 1.8 Kb fragment was eluted. A combination of random and directed sequencing techniques was used, as set out in figure 6.2.1b. DNA was prepared for random sequencing as described

Figure 6.2.1a

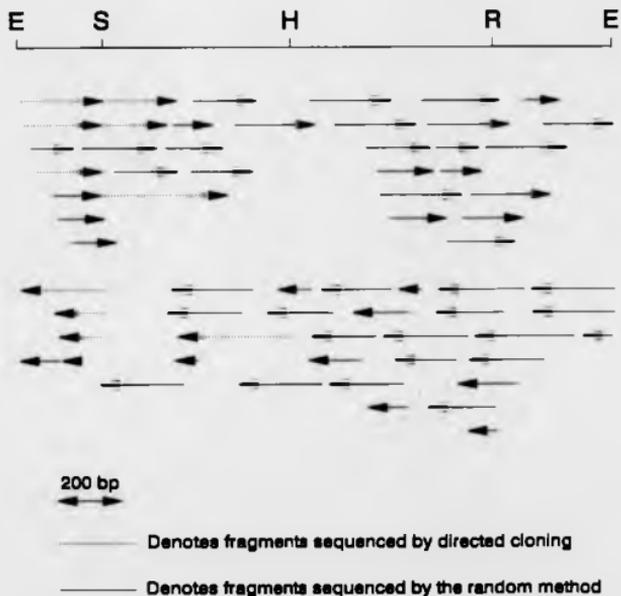
pSW1



Approximate restriction map of pSW2: constructed by cloning a 1.8 Kb *Eco* RI fragment from the upstream region of the *old* cluster, into pIC-19R, as shown. Vector and insert DNA are not drawn to scale.

Figure 6.2.1b

Strategy for Sequencing the pSW1 Insert



previously (2.21), using 25 μg (approx.) insert DNA for self-ligation and sonication. After end repair, fragments of 0.4-1 Kb (approx.) were ligated into *Sma*I-cut, phosphatased M13mp18 and transformed into TG1, plating on to NBA, XGal, IPTG. White plaques were harvested, template DNA was prepared, and sequencing was performed as described earlier (2.21), using extension, taqenase or dITP reactions as required.

The project was completed by directed sequencing of the 260 bp *Eco*RI/*Sal*I and the 560 bp *Sal*I/*Hind*III fragments of pSW1, which were shotgun cloned into M13 in both orientations, (using M13 mp18 and M13 mp19). In order to obtain sequence information from as near the primer sites as possible, manganese buffer was added to the reactions (2.21.4.5). By the end of the project, all the DNA had been sequenced at least once (and usually several times) on each strand.

The region was found to include one complete gene (*ouc*) and two partially sequenced ORFs. Figure 6.2.1c shows the DNA sequence of the 1.8 Kb *Eco*RI fragment, together with the protein sequences encoded by the predicted ORFs. The likely positions of ribosome binding sites are also shown.

Ouc was predicted to encode a protein composed of 286 amino acid residues, with a molecular weight of 31,033 Da and an average pI value of 5.97. The *ouc* start codon was preceded by a likely ribosome binding site (Gold and Stormo 1987), but no consensus promoter sequence was identified. The G/C content of *ouc* was found to be 51.6%, close to the typical *Ecc* value of 52.1%.

Hydrophilicity analysis, performed using the Microgenie (Beckman) package, showed that OutC includes a single hydrophobic stretch of 19 amino acids: residues 28-46. This is located near the N-terminus and is followed by a possible signal cleavage site, suggesting that the protein might be exported to the periplasm and post-translationally processed (a hypothesis discussed in greater detail later: 6.4.3).

The predicted structure of OutC is shown by the 'squiggle plot' representation in figure 6.2.1d. This was obtained using the University of Wisconsin genetics computer group 'Proteinstructure' programme (Devereux *et al* 1984), which uses the algorithms

FIGURE 6.2.1c

SEQUENCE OF THE 1.8 Kb *ECORI* FRAGMENT OF THE *OUT* CLUSTER

10 20 30 40 50 60
GAATTCGGCAACAGCCAGAACGTCATGGTATTCAATAACTTTTGGACACGGGTGATGAC
 ..E F G N S Q N V M V F N N F F D T G D D
 OutX'
 70 80 90 100 110 120
AGTGTAAACTTTGCGGCGGATACGGTGCAGAGTCCGACGCTGGGGCAAAAAGCGCAG
 S V N F A A G Y G A E V A T L G Q K A Q
 130 140 150 160 170 180
AGTGGTCCCTGGATCTTCAATAACTACTTCGCGCGTGGGATGGTGGCGGTGTCACGGT
 S G A W I F N N Y F R R Q H G A V V T G
 190 200 210 220 230 240
AGCCATACCGGTGCTGATTGAAAAATTGTCTGAAGACAACGTAATGAATAAGACG
 S H T G A W I E K I V A E D N V M N K T
 250 260 270 280 290 300
GATGTTGGCCTGCGCATGAAGAGTGGACCCCTATTATGGTGGCGGCTCGCGGTGACGTGGTA
 D V G L R M K S R P Y Y G G S R D V V
 310 320 330 340 350 360
TTCCGTAATAACCGGATCGGTGATATTGTCAACGAGCCGTTGTGTTACAGGATTAATAT
 F R N N A M R D I V N E P F V F T I K Y
 370 380 390 400 410 420
AAAGCGGACGTGAACGATACCCAGCCTGCGCTGAGCCTGCCAGTCCCGTATGATGAGCC
 K A D V N D T Q P A A E P A Q F R D V T
 430 440 450 460 470 480
GTTTCAAAGCTCACGGTTGATGGTACAGCGAAGAAAAACAGTATCATGGTCGATGGGATG
 V S N V T V D G T A K K N S I M V D G M
 490 500 510 520 530 540
ACCGTGGCTGAAATGGCTGATGCGTATAAGTCTCTTTTGGACGTGATGCGCTATCACCAA
 T V A E M A D A Y K F S F G R D A Y H Q
 550 560 570 580 590 600
GGCTTGCAATTTGAGAATGTAATAATCAAAAAATGTAAGGCGACGGACATTACGTTCTGT
 G L H F E N V K F K N V K A T D I T F L
 610 620 630 640 650 660
ANGAACAGTGAATTTAAAAATGTCATTTTGGAGAATGTACCGAGAGCCTGGAATTTGGT
 K N S E F K N V I F E N V P R A W N F G
 670 680 690 700 710 720
CATATTGAAAAATACAGGCTCGAAGACCGTGTAAATAAGGATGCGCGCTGACGACCGAC
 H I E N I R L E D R V N K D A A L T T S

Figure 6.2.1c continued

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730          740          750          760          770          780
GGTGTGAAACCATCACCCGGGAAGCGGCAACGGGAATAAGGCATTTCCCGCACGGCAATC
G D E T I T R E A A T E                               Inverted repeat

790          800          810          820          830          840
GGCGTGGGGAGTATTATGGATGATTAATCTATGGCAGGATTGCAAGCTTCAAAGATCCGT
RBS      OutC: M A R L Q A F K D P

850          860          870          880          890          900
CTTTTCATTGCGTTGGTGGCCACTTTTCGGTCACTCCGCGTGAATCCGCGCCTTTTGTTGG
S F H S L V A T F R S L P L I R R F V L

910          920          930          940          950          960
GGCTGATTTTGCTGCTGATTTGTGTCAGCAGCTGGCCGTATTGACCTGGCGCTTTCCTCTAC
S L I I I I I I C G Q L A V L T M R F L L
Hydrophobic sequence

970          980          990          1000          1010          1020
CTGAAGACTCACGCATTTGCGGTGTCCGTCACGCGTCCCAAGCGAAAGAAAAGCCCG
P E D S R I V G V S V T P A Q A K E K P

1030          1040          1050          1060          1070          1080
CAACGCCGGGTGATTTTACCTTATTGGCCATGCCCCCGATGCAGATGCCCTACGGTTA
A T P G D F T L F G H A P D A D A S T V

1090          1100          1110          1120          1130          1140
ACGACCGCGCATTATCCGGCGATATCCGCTAACCTCGTTAAATATCAGCTGACTGGGG
N D A A L S G D I P L T S L N I S L T G

1150          1160          1170          1180          1190          1200
TGTGGCAGTGAGAGACGCCAAGCGTTCCGATTGCCATTATCGCTAAAGATAGTCAGCAAT
V L A S G D A K R S I A I I A K D S Q Q

1210          1220          1230          1240          1250          1260
ACAGTCGCAATGTCGGCGATGCCATTCCGGGCTATGAGCCAAAATGTGACTATCTCG
Y S R N V G D A I P G Y E A K I V T I S

1270          1280          1290          1300          1310          1320
CCGATCGTGTCTGCTCCAGTATCAGGGGCGCTATGAGGCGCTGCATTTGTATCAGGAAG
A D R V V L Q Y Q G R Y E A L H L Y Q E

1330          1340          1350          1360          1370          1380
AAGAAAGGACTGGCCACCGTCTGCTGCGCATTAATCAGGTGAAGATGAGATAC
E E A T G A P S S S G A F N Q V K D E I

1390          1400          1410          1420          1430          1440
AAAAGATCCCTTCTCGGGCAGGACTACCTGACCATTTCTCCCGTACCGAAGAAGAAG
Q K D P F S A Q D Y L T I S P V T R E E

1450          1460          1470          1480          1490          1500
TCCTGAAAGGATATCAGTTAAACCCCGGCAAAAATCCCGATCTTTTCTACCGCGCAGGCT
V L K G Y Q L N P G K N P D L F Y R A G

```

Figure 6.2.1c continued

```

1510      1520      1530      1540      1550      1560
TGCAGGATAACGATCTGCCCGTGCATTAAATGGCGATGGATTTACGTGATGCCGGATCAGG
L Q D N D L A V S L N G M D L R D A D Q

1570      1580      1590      1600      1610      1620
CACAGCAGGCGATGGCGCAACTGGCAGGGATGAGCAATTTAATTTGACCGTCGAGCGTG
A Q Q A M A Q L A G M S K F N L T V E R

1630      1640      1650      1660      1670      1680
ATGGTCAACAGCAGGATATATATCTGGCACTGGATGGAGACCACTAATTTGTTAGCAAG
D G Q Q Q Q D I Y L A L D G D H
          RBS           OutD': M E T T N L F S K

1690      1700      1710      1720      1730      1740
GGACAGGGATTTTTAAACGTCAGGTTTTTTCGAAGAATAAAAAACCAATGGCTTGGTCAG
G Q G F F K R Q V F S K N K N Q W L G Q

1750      1760      1770      1780      1790      1800
GTACGCCGCAAGAGCATGCTATTGCTCAGCGGGAGTGTTCTGCTGATGCCGTCATCATTO
V R R K S H L L L S G S V L L H A S S L

1810
GCGTGGAGCGCTGAATC
A W S A E P .....

```

Nucleotide sequence of 1.8 Kb *Ecc* DNA (noncoding strand) including *oucC* and the partially sequenced genes: *oufX* and *oufD*. The positions of putative ribosome binding sites and an inverted repeat sequence are shown, and are discussed in the text. The predicted amino acid sequences encoded by the ORFs are given, and the possible signal sequence and cleavage site within *OutC* is shown (†).

FIGURE 6.2.1d

REPRESENTATION OF THE STRUCTURE OF OUTC

Figure 6.2.1d (opposite) shows a plot of the predicted secondary structure of OutC: obtained using the UWGCG programmes 'Proteinstructure' and 'Plotstructure' (Devereux *et al* 1984).

The programmes predict secondary structure, using the algorithms of Chou and Fasman (1978) and hydrophobicity, using the algorithms of Kyte and Doolittle (1982), with a window of 20 residues.

Key to symbols:

α -helix: 

random coil: 

β -pleated sheet: 

β -turn region: 

Regions of hydrophobicity are represented by red diamonds and regions of hydrophilicity by blue ovals.

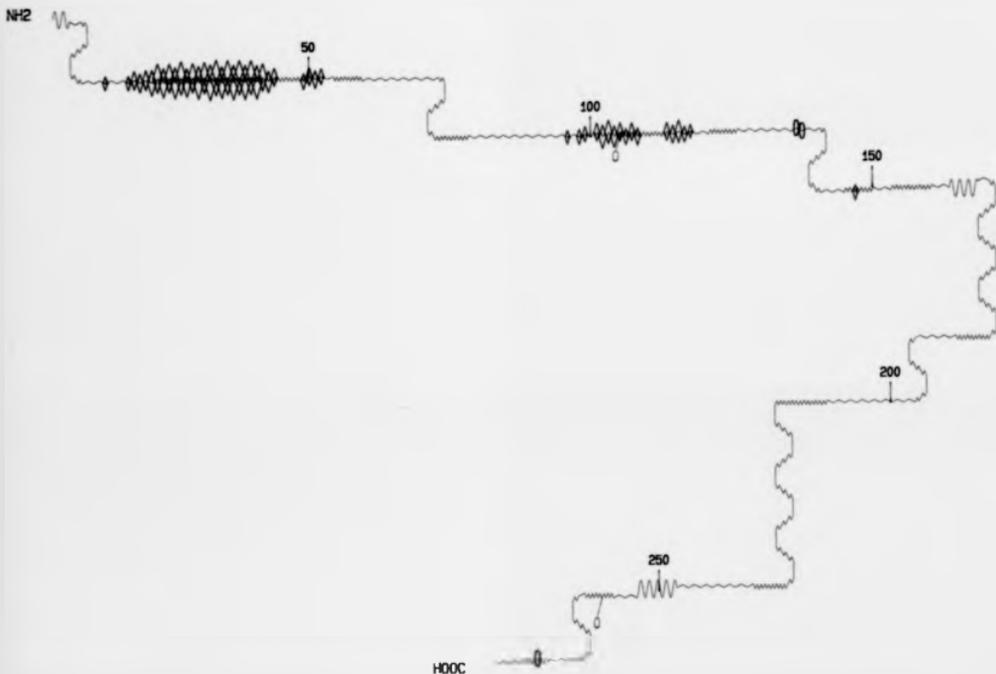
The potential signal sequence of OutC is shown by the large number of red diamonds (indicating a highly hydrophobic region) near the N-terminus of the protein.

PLOTSTRUCTURE of: outc1.gcg ck: 168

User: [Sketch.Wedge.Outc]Outc1.Txt:2 => OUTC1

Chou-Fasman Prediction
May 17, 1981 11:36

○ KD Hydrophilicity ≥ 1.3
◊ KD Hydrophobicity ≥ 1.3



of Chou and Fasman (1978) and Kyte and Doolittle (1982) to predict secondary structure and hydrophathy.

The ORF lying downstream of *ouc*, encoding the start of OutD, included several possible initiation sites. Only the one with the best consensus ribosome binding site, thought most likely to be the start point of *outD*, is shown in figure 6.2.1c. This would suggest a 5 codon overlap between *ouc* and *outD*. Such overlaps are quite common among genes in an operon which are co-ordinately regulated by transcriptional coupling. It should be noted that it is not possible to assign ORFs and, in particular initiation sites, with any certainty from DNA sequence data alone. Confirmation of the predictions would require N-terminal amino acid sequence data.

An ORF (ORFX) was identified upstream of *ouc* and running in the same direction, which extended beyond the region sequenced. Between this and *ouc* there was an inverted repeat sequence (shown by arrows in figure 6.2.1c), predicted to cause a stem loop with $\Delta G = -32.6$ Kcal, in the mRNA transcript.

6.2.2 SEQUENCE HOMOLOGUES OF OUTC AND OUTX.

The Seqnet system was used to search for homologues of the predicted protein products of *ouc* and *outX*.

The only protein found to show significant homology to OutC was PulC: a 30,963 Da protein required for the secretion of pullulanase (Pul) by *Klebsiella oxytoca* (d'Enfert *et al* 1989). An alignment of the two proteins is shown in figure 6.2.2a. Over its entire length, OutC showed 40% amino acid identity to PulC. The percentage homology was actually higher than this throughout most of the length of the proteins, but decreased towards the N-termini.

The region of *outX* sequenced encoded part of a protein showing homologies to various Peh enzymes. The analysis of *outX* and its product are discussed in greater detail later (7).

6.2.3 DISCUSSION.

DNA sequence analysis of the 1.8 Kb *EcoRI* fragment from the upstream region of the cHIL208 insert resulted in the identification of a gene which was named *ouC*. The predicted product of this showed strong sequence homology to PulC: a protein involved in pullulanase secretion. The secretion of Pul by *Klebsiella* sp. has been intensively studied (reviewed: Pugsley *et al* 1990) and was discussed earlier (1.8). PulC has been shown to be essential for Pul secretion, by Tn5 mutagenesis (d'Enfert *et al* 1987b) and by using a reconstitution system in which genes downstream of a polar mutation in *pulC* were under *lac* promoter control (d'Enfert *et al* 1989). Such mutations abolished the cell surface exposition and secretion of Pul. Results obtained with Tn*phoA* fusions indicated that PulC is an envelope protein, since in frame fusions were generated, which produced blue colonies on XP media (d'Enfert *et al* 1989). Confirmation of this was achieved by cell fractionation, followed by immunoblotting with anti-PhoA serum, which showed that most of the PulC-PhoA hybrid protein was located in fractions enriched for inner membrane vesicles (d'Enfert *et al* 1989).

As discussed previously (1.7.2), during the course of this project, there was mounting evidence of extensive similarities between the *Klebsiella* sp. *pul* gene cluster and the *ou* cluster of *Erwinia* spp. In the light of these findings, the homology between PulC and OutC was exactly as predicted. Homologies were also being found with proteins involved in the transport of various macromolecules across the bacterial cell wall. The family of 'traffic wardens': proteins involved in the trafficking of molecules across biological membranes has been described earlier (1.9). Although a large number of proteins homologous to the PulC-O proteins have been identified, there is only one report of homology to proteins encoded by genes lying upstream of *pulC* (Condemine *et al* 1992). This region of the *ou* cluster was of particular interest as it was thought likely to hold the key to both the regulation and the specificity of the *Ecc* secretory apparatus.

The sequence data presented above suggested that in the position occupied by *pulA* (the structural gene for Pul) in the *pul* cluster, there was also a structural gene in the

out cluster. This gene, named *outX* was transcribed in the same direction as *outC* (in contrast to the organization of genes in the upstream region of the *pul* cluster) and appeared to encode a polygalacturonase.

From the DNA sequence data, there was no sign of a typical *E. coli* consensus promoter region, or of the MalT controlled type of promoter found in the *pul* cluster, upstream of *pulC* (as described previously: 1.8.4). Clearly then, the point at which the *pul* and *out* cluster homologies ceased, had been identified.

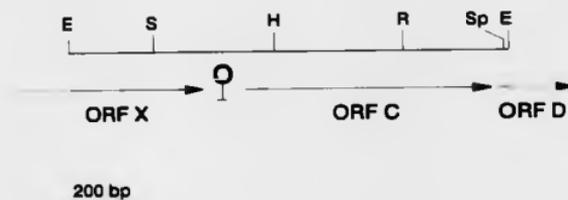
6.3 COMPLEMENTATION TESTS USING THE REGION ENCODING OUTC.

pSW1, and various derivatives of this were tested for their abilities to complement a range of Out^- mutants, thought to map towards the upstream region of the *out* gene cluster. The mutants used included the chemically induced RJP190, RJP208, RJP251 (Reeves) and HJN1000 (Housby), in addition to the insertional mutants PR54 (*TnphaA*, Reeves) and AC4000 (*Tn10*, Connolly). Each strain was cured of pHCP2 as described previously (2.11) and the phenotypes: $\text{Pel}^- \text{Cel}^- \text{Prt}^+ \Phi \text{KP}^S$ were checked, to confirm the identity of *Ecc*.

A simplified map of the insert carried by pSW1, together with the ORFs identified by sequence analysis (6.2) is shown in figure 6.3a. The entire insert and various sub-fragments were cloned into pUC vectors in both orientations. Since the 1.8 Kb *EcoRI* fragment was always found in the same orientation after random cloning, a *SphI* digestion was carried out to allow force cloning. The 0.26 Kb and 1.55 Kb *EcoRI/SalI*, and the 0.82 Kb and 0.99 Kb *EcoRI/HindIII* sub-fragments were also used. All the fragments were shotgun cloned into pUC18 and pUC19, using restriction analysis of plasmid DNA extracted from CC118 transformants, to identify the required constructs. Fragments from the left hand end of the insert (as shown in figure 6.3a) were extremely difficult to clone, with the orientation in the vector proving important. Although all the required constructs were obtained, it is possible that some of the

Figure 6.3a

The pSW1 Insert



A simplified restriction map of the pSW1 Insert is shown, together with the predicted ORFs of this region of the *out* cluster. The location of the putative stem-loop forming region is indicated by: $\text{\textcircled{O}}$.

inserts contained mutations (not detectable by restriction analysis), which allowed cloning. Each construct was transformed into GJ342, plating on to NBA Ap,Cm,Tc and patch mated into the cured *Out⁻* mutants listed above. Single *Ecc* transconjugant colonies were picked on to Pel and Cel assay plates, with HC131 and the relevant cured mutant as positive and negative controls respectively. None of the clones complemented any of the mutants.

In an attempt to see whether the high copy number (≈ 200) of the pUC vectors caused problems, the 1.8 Kb *EcoRI* fragment was cloned into the lower copy number (30-50) pBR322. As before, the fragment could only be easily inserted in one orientation, possibly indicating that insertion in the other orientation lead to lethal over-expression. When the clone obtained in pBR322 was introduced into the *Out⁻* mutants, no complementation was observed. It is possible that, if the 1.8 Kb *EcoRI* fragment could not be cloned in one orientation due to a lethal effect of over-expression, in the opposite, easily obtained orientation, there would be no gene expression and hence no complementation. This explanation would rely on the fact that expression required read-through from the vector, due to the absence of a promoter in the insert. The lack of complementation might therefore be seen to confirm the DNA sequence data, suggesting that no promoter was present in the 1.8 Kb *EcoRI* fragment from the upstream region of the *owt* gene cluster.

It is possible that, as proposed above, those constructs which could be obtained only rarely, carried a mutation. Such mutations might prevent the complementation of *Out⁻* mutants. Another possible explanation for the lack of complementation is that none of the mutations tested mapped within *owC*, or that in the case of the PR54 and AC4000, complementation was not possible due to the polar nature of the transposon mutations.

The cloning could have been repeated, using a much lower copy number plasmid (eg pSF6: copy number ≈ 4) to overcome the possible lethal effects of multi-copies of the insert. Complementation would probably not have been achieved if an *ow*

promoter was not present. It was decided instead to study the product of *outC*, using gene expression experiments.

6.4 EXPRESSION OF *OUTC*

6.4.1 INTRODUCTION.

Attempts to study *out* gene expression using the Zubay system had previously proved unsuccessful (Reeves per. comm.). Zubay expression experiments were tried on pSW1, using the method described earlier (2.22.3), but no protein bands specific to the insert were observed after SDS-PAGE and autoradiography (data not shown). This seems to confirm that no *out* promoter was included in the construct. The T7 RNA polymerase system was used to express genes from the middle of the *out* cluster (Reeves 1991). It was decided to use this method to study *OutC*.

The T7 RNA polymerase/ promoter system for the controlled expression of specific genes (Tabor and Richardson 1985) requires that the gene(s) of interest is (are) cloned into a vector (pT7-5 or pT7-6) such that expression is under the control of the T7 polymerase promoter. The expression of this promoter depends on a second vector: pGPI-2, which contains the gene for T7 RNA polymerase (gene *I* of phage T7), under the control of the inducible λP_L promoter and λCl^{857} (the heat-sensitive repressor gene). Exclusive expression of the gene(s) of interest requires the addition of rifampicin (Rif), after heat induction at 42°C. Rif specifically inhibits DNA-dependent RNA polymerase, by the formation of a very stable, inactive complex with the enzyme (Wehrli *et al* 1968a,b). Transcription of host genes is prevented, but the resistance of T7 RNA polymerase to Rif means that the genes of interest are expressed. The protein products may be labelled with ^{35}S -Met, separated by SDS-PAGE and visualized by autoradiography, as described previously (2.23).

6.4.2 EXPRESSION OF *OUTC* USING THE T7 RNA POLYMERASE/ PROMOTER SYSTEM.

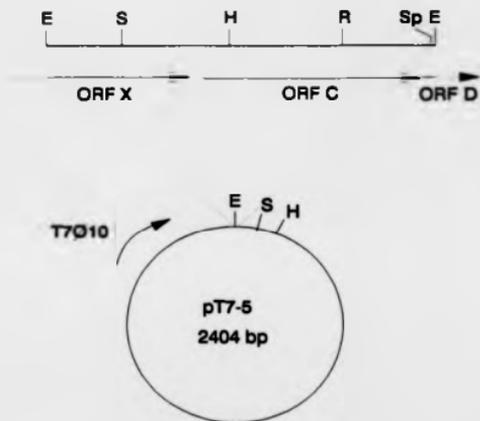
pT7-5 DNA was prepared by the large scale alkaline lysis method (Maniatis *et al* 1982). This was preferred to the use of CsCl gradients which produced very low yields of the pT7 vectors.

The 1.8 Kb *EcoRI*, *OutC* encoding fragment was ligated into *EcoRI*-cut, phosphatased pT7-5. DNA was extracted by boiling (2.8.1) from Ap^R DH1 transformants and checked by restriction analysis, to identify a plasmid carrying the insert in the correct orientation relative to the T7 RNA polymerase promoter. This plasmid, named pT7-5SW100 is shown in figure 6.4.2a.

pT7-5SW100 was introduced into K38(pGP1-2) by electroporation (2.10.3), plating on to NBA Kn, Ap. *E. coli* K38 was chosen as the host strain, due to its great sensitivity to Rif (Tabor pers. comm.). Control electroporations were performed with pT7-5 to see whether the insert was toxic. There are three levels of toxicity for genes cloned into pT7 vectors, as discussed below. A few genes are so toxic that cloning them into multi-copy plasmids, even in the absence of an *E. coli* promoter is very difficult. Such genes would probably not have been cloned into pT7-5. Rather less toxic genes cannot be cloned in multi-copy if they are under the control of a relatively strong *E. coli* promoter. Such genes (approx. 5% of those studied: Tabor pers. comm.) are toxic in pT7-5, if the gene for T7 RNA polymerase (expressed at a low level by pGP1-2, even before induction) is also present. pT7-5 carrying a gene of this toxicity level, while transforming most cells normally, would not stably transform K38(pGP1-2). The frequency of Kn^R Ap^R transformants would be reduced (approx. 50 fold, relative to that with the pT7-5 control), and those colonies obtained would be because of mutations (usually deletions) in one of the plasmids. Hence, a marked reduction in the frequency of transformation, relative to that with the control, would suggest that the desired gene product could not be produced. Many other genes are mildly toxic. Colonies of K38(pGP1-2), transformed with pT7 carrying one of these genes, would not produce the desired protein if they were stored for long periods before use. To

Figure 6.4.2a

pT7-5SW100

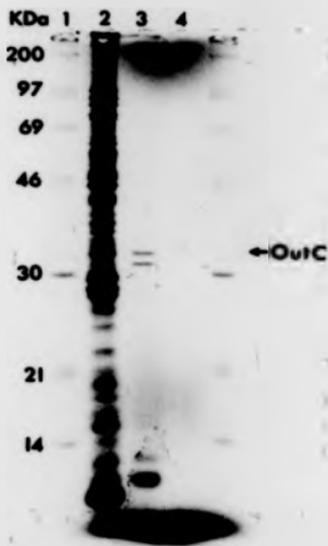


Vector and insert DNA not drawn to scale

FIGURE 6.4.2b

EXPRESSION OF *OUTC* USING THE T7 RNA
POLYMERASE/PROMOTER SYSTEM.

The photograph shows an autoradiograph (24 hr exposure), giving the result of an experiment to express *outC*, using the construct pT7-5SW100.



KEY TO TRACKS: construct and growth conditions used.

- 1 Molecular weight standards (sizes given in KDa).
- 2 pT7-5SW100, induced, - Rif
- 3 pT7-5SW100, induced, + Rif
- 4 pT7-5, induced, + Rif

eliminate this problem, transformations were normally carried out only 1-2 days before the induction experiment.

Single transformant colonies were grown up, with colonies of K38(pGP1-2, pT7-5) used as a control. The cultures were used for T7 RNA polymerase/ promoter expression, as described previously (2.22.1). In addition to the use of pT7-5 carrying no insert, a further control was carried out in which the normal protocol was followed, except that no Rif was added, so that any specifically expressed protein(s) could be compared with normal host proteins.

The result of the expression experiment using pT7-5SW100 is shown in figure 6.4.2b, where the proteins produced by pT7-5SW100 may be compared with those produced by pT7-5, and by the host. A standard curve (not shown) was obtained by plotting the log of distance (cm) moved, against size (KDa) of the markers (listed: table 2e). From this, it was estimated that the proteins produced by the specific expression of the genes on pT7-5SW100 were sized: 32, 31, 13 and 11 KDa (approx.).

6.4.3 DISCUSSION.

From the sequence data (6.2.1), *outC* was predicted to encode a 31 KDa protein. It was clear then that OutC had been produced by the T7 RNA polymerase expression of pT7-5SW100. The protein gel (figure 6.4.2b) suggested that OutC existed in two forms. There are several possible explanations for this, including processing of the protein to remove a signal sequence. Other possibilities are that another proteolytic cleavage event occurred, the protein was initiated at an alternative start site, or that the doublet was an artefact. Each of these explanations is considered below.

It seems unlikely that such a distinct doublet could be an artefact. Theoretically, a sub-population of protein molecules could migrate aberrantly if they were not denatured. For example, a disulphide bond might not be reduced by β -mercaptoethanol. This was not possible for OutC which includes only one Cys residue.

Under the control of a promoter as strong as that of the T7 RNA polymerase system, translation might be initiated at a start codon not normally used. The sequence data showed that initiation could not occur upstream of that site predicted for the start of *OutC*, due to the presence of stop codons. The upper of the two protein bands (32 KDa) was therefore likely to be due to *OutC*. There are no other possible ATG initiation codons near the start of *outC*, but there are two GTG codons (encoding Val: at positions 29 and 59). These both have a possible ribosome binding site, and would give rise to proteins of 28 and 24 KDa respectively. No other ORFs in the *outC* region are large enough to encode a protein of around 30 KDa and the truncated *outX* cloned in pT7-5SW100 did not include a possible initiation site.

Proteolytic cleavage could have resulted in the observed band pattern, depending on the signal specificity of proteases present in the *E. coli* host.

Doublets on a protein gel normally indicate post-translational processing: the cleavage of an N-terminal signal sequence. Leader sequences are typically 15-30 amino acids long, and composed of mainly hydrophobic residues. Such a sequence (19 residues) does exist near the N-terminus of *OutC*. Signal peptidases (1.5.2.5) are divided into two classes, one type being specific for the processing of lipoproteins, the other cleaving many different exported proteins. The cleavage site of the broad specificity enzyme has certain characteristics. There are normally 6 or 7 amino acids between the hydrophobic leader sequence and the cleavage site. The residue adjacent to the cleavage site, to the N-terminal side (position -1) is Ala, Gly or Ser, while residue -3 is Gly, Ala, Val, Leu, Ile or Ser. In addition, Pro or Gly residues are normally located between positions -4 and -6 (von Heijne 1983).

From the translation of the DNA sequence data, there is a possible cleavage site after residue 58 (Gly). Processing here would result in exactly the same protein molecule as that formed by initiation at the second GTG codon, with a size of 24 KDa: probably too small to account for the band seen on the protein gel.

The protein PulC (homologous to *OutC*), has a hydrophobic stretch of amino acids close to its N-terminus, very similar to that found in *OutC*. Studies of the localization

of PulC-PhoA hybrids have suggested that PulC is bound to the inner membrane (d'Enfert *et al* 1989). This idea was supported by sequence data, showing that the hydrophobic region was preceded by a strongly positively charged stretch of amino acids, thought to remain in the cytoplasm (von Heijne 1986a), anchoring the protein into the membrane (d'Enfert *et al* 1989). OutC also has a stretch of positively charged residues at its N-terminus. Considering the homology between OutC and PulC, and hence the probable similarities in function of the two proteins, it seems likely that OutC is located predominantly in the periplasm, anchored to the inner membrane by its uncleaved signal sequence.

Although it remains a possibility that protein processing gave rise to the second band on the protein gel (figure 6.4.2b), it seems more likely that initiation at an alternative site (GTG: Val) had occurred.

N-terminal amino acid sequence analysis should be used to confirm the translational start points predicted from DNA sequence data. This might also identify the protein causing the lower band on the gel, although the proteins generated by signal processing and initiation at the second GTG (Val) codon would be identical.

Various experiments could be performed to determine whether or not OutC is processed. Possible processing could have been eliminated by mutating the putative cleavage site. Alternatively, pulse-chase labelling could be used. If OutC was processed, the upper band would gradually be replaced by the lower band, whereas if an alternative initiation site was used, both proteins would be produced together. The findings could be confirmed by mutating the alternative initiation codons, or their putative ribosome binding sites.

From the information on PulC, and the predicted sizes of proteins generated by OutC processing or alternative initiation, it seems most likely that OutC is not processed, but that under the conditions of the T7 RNA polymerase expression experiment, a protein was initiated at Val (residue 29 of OutC). It would be interesting to repeat the expression experiment, separating the induced, ³⁵S-Met labelled culture into the different subcellular fractions, to see whether OutC was

membrane associated. A protein initiated at the first GTG (Val) codon would have an almost entire signal sequence, but no anchor region. If the protein causing the lower band on the gel was due to this product, it should therefore be localized in the periplasm. This experiment would need to be carried out in an *Ecc* host to ensure correct processing and targeting. Since the work described here, the T7 RNA polymerase expression system has successfully been used in *Ecc* (Douglas pers. comm.).

The other bands on the protein gel (figure 6.4.2b) corresponded to predicted 13 and 11 KDa proteins. pT7-55W100 included a truncated *outD*. Sequence analysis identified several possible initiation codons in this ORF, although only one of these had a likely ribosome binding site. The predicted OutD truncate would be 6 KDa. Such small proteins could not be accurately sized by SDS-PAGE using the mid-range size markers. The protein band still unaccounted for might have been due to the production of a truncated protein from one of the ORFs, or might have been an artefact of the system.

6.5 CONCLUSIONS/ FINAL DISCUSSION.

The 1.8 Kb *EcoRI* fragment of DNA, thought to map to the upstream region of the *ow* cluster was sequenced. A gene was identified (*owC*) which was predicted to encode a 31 KDa protein. *OutC* was successfully expressed using the T7 RNA polymerase/promoter system. The size of the protein observed after SDS-PAGE was in close agreement with that predicted from translation of the DNA sequence.

OutC was found to be homologous to PulC: a protein essential for the secretion of Pul by *Klebsiella* sp. This was as expected, due to the homologies previously discovered, of proteins encoded by the downstream genes (*D-O*) of the two clusters. OutC was found to possess a hydrophobic leader sequence and possible signal cleavage site. However, evidence from the work on PulC, together with the presence of a strongly positive sequence upstream of the OutC signal sequence suggested that post-translational processing did not occur. It was proposed that OutC is predominantly

located in the periplasm, with its hydrophobic leader sequence being anchored in the inner membrane by positive residues remaining in the cytoplasm.

Analysis of the DNA sequence revealed that homology with the *pul* cluster did not extend upstream of *outC*. There was no DNA sequence homology with the *mal* promoters of the *pul* cluster, and no other promoter sequence was found in the region. Instead, part of a gene (*outX*) was identified.

The sequence data obtained for *outC* and the truncate of *outX* did not provide any explanation for the apparent regulation of the *out* gene cluster by this region, or for the difficulty of cloning it. There are no reports of a regulatory effect of PulC.

Located between ORFX and the start of *outC*, is a G/C rich inverted repeat, likely to create a stem loop structure in the mRNA transcript. Such features, normally associated with transcription termination, may control gene expression by modulating the frequency of transcription. Stem loop structures have different functions, depending on their position. When present at the end of operons, they normally act to prevent further transcription. Sometimes stem loops occur within genes, where they can lead to polarity: decreasing the expression of downstream genes. In biosynthetic operons, such as that for tryptophan, stem loops, situated between the first and second genes, act as attenuators. In other cases, the inverted repeats are positioned between genes of an operon, where they allow the regulation of expression of downstream genes, without affecting those genes nearer the promoter (Galloway and Platt 1986).

A G/C rich inverted repeat is normally followed by a stretch of about six Ts (to the 3' end), meaning that the stem loop of the mRNA is followed by six Us. Termination occurs when RNA polymerase pauses at the stem loop and is then released from the transcript due to weak U-A base pairing. If the U bases are lost (due to a mutation), the stem loop still allows the RNA polymerase to pause, but does not cause termination (Farnham and Platt 1981). It has been suggested that pausing may help to couple

transcription and translation (Yanofsky *et al* 1983). The inverted repeat found upstream of *ouxC* was not followed by a series of Ts.

Another type of inverted repeat sequence has been studied (Higgins and Smith 1986). These REP (repetitive extragenic palindromic) sequences are found in the intergenic regions of bacterial operons (Higgins *et al* 1982). REP sequences are thought to create stem loop structures in transcripts, which stabilise upstream mRNA. It has been proposed that REP sequences are involved in the regulation of gene expression, since they are often located between two genes which are differentially expressed (Higgins *et al* 1982), although their exact role and mode of action are not known. REP sequences are long and highly conserved. The consensus sequence, together with the inverted repeat sequence from the *ouxC* cluster, is shown below.

```

      (T)  (A)  (A)(T)  (A)
REP  GCGGATG.CCGCGCwwwwwwGGCCTTATCCGGCCTAC
OUI  TCCCCAGCCwwwwwwGGCGTGGGA
  
```

The inverted repeat from the *ouxC* cluster is not a typical REP sequence, although it may affect gene expression since it has been established that secondary structures in mRNA can prevent 3'→5' exonuclease attack (Gilson *et al* 1987).

Although no conclusions could be drawn as to the exact function of the inverted repeat between ORFX and *ouxC*, it seems possible that its presence was the cause of the unusual complementation patterns observed with cHIL208. Interestingly, REP sequences may only function under certain conditions (Higgins and Smith 1986), since mRNA stability varies greatly depending on growth rate. The presence of an inverted repeat in the upstream region of cHIL208 might explain the variability of complementation, although the problems associated with cloning DNA fragments from the upstream region of cHIL208 could not be similarly explained.

The partially sequenced gene *ouyX*, which lies upstream of *ouxC* and is transcribed in the same direction, appeared to be a structural gene for Peh. Although *ouyX* is in the position occupied by *pulA* (the structural gene for Pul) in the *pul* cluster, the two genes

are in opposite orientations. The apparent homology between *OutX* and *Peh* proteins was surprising since a *peh* gene had already been identified, mapping to a different region of the chromosome and thought to be the only gene in *Ecc* to encode *Peh* (Plastow *et al* 1986).

Clearly, it was necessary to find out whether *outX* really is a gene for *Peh* and if so, whether it is expressed to produce an active enzyme. It was hoped that by completing the DNA sequence analysis of *outX*, more information on the identity and possible function of the product might be obtained, and that this in turn, might provide clues as to the mechanisms of regulation and specificity of the *Ecc out* gene cluster.

CHAPTER SEVEN.

ANALYSIS OF THE *OUT* GENE CLUSTER UPSTREAM OF *OUTC*.

7.1 INTRODUCTION.

As discussed previously (6.2.1), an open reading frame (ORFX) was discovered upstream of *ouxC* and running in the same direction. This was in contrast to the gene organization of the *pul* cluster, in which *pulA* (the structural gene for pullulanase) lies upstream of *pulC*: the two genes being divergently transcribed. This was the first major difference found between the two clusters. Interestingly, the predicted protein product of *ouX* was homologous to the enzyme Peh. Before any conclusions could be drawn as to the possible function of OutX, it was necessary to complete the sequence analysis of the gene. It was hoped that the *ou* gene promoter would also be identified.

The sequenced 1.8 Kb *EcoRI* fragment (6.2) extended to the end of the cHIL208 insert. Before sequencing could be continued, the required DNA had to be isolated, as described below.

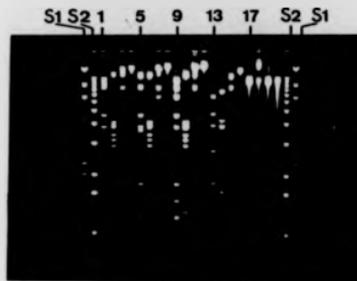
7.2 THE ISOLATION OF *OUTX* CONTAINING DNA.

While the sequencing of *ouxC* was in progress, a new *Ecc* genomic library (partial *Sau3A* digests, cloned into the *BamHI* site of pSF6) was produced (Reeves pers. comm.). Tests showed that some of the cosmids were able to complement Out⁻ mutants, including ones thought to map to the upstream region of the *ou* cluster, which had not been fully complemented previously (Reeves pers. comm.). These cosmids included cHIL208/5 (fully complemented RJP208), cHIL208/4 (partially complemented RJP208) and cHIL251/1 (complemented RJP251). The cosmids cHIL208/5, cHIL208/4 and cHIL251/1 (thought likely to contain the entire *ou* gene cluster), were digested with *EcoRI*, *EcoRV*, *SalI* and *PstI*, as were cHIL208 and pSF6 for comparison. Agarose gel electrophoresis and Southern hybridizations were performed as described previously, probing with template DNA from the 260 bp *EcoRI/SalI* region of *ouX*. The agarose gel and the autoradiograph obtained by Southern blotting are shown in figures 7.2a and 7.2b respectively. The cosmids had many fragments in common,

FIGURE 7.2a

RESTRICTION DIGESTS OF *OUT* PLASMIDS: FOR THE IDENTIFICATION OF *OUTX* DNA.

The photograph shows an agarose gel, following electrophoresis of the *out* plasmid digests listed below. The gel was used for Southern hybridization analysis (see figure 7.2b)



KEY TO TRACKS: figures 7.2a and 7.2b (*out* plasmid and restriction endonuclease used).

S1 Standard (λ HindIII)

1 cHIL208/5: *EcoRI*

2 cHIL208/5: *EcoRV*

3 cHIL208/5: *Sall*

4 cHIL208/5: *PstI*

5 cHIL208/4: *EcoRI*

6 cHIL208/4: *EcoRV*

7 cHIL208/4: *Sall*

8 cHIL208/4: *PstI*

9 cHIL251/1: *EcoRI*

10 cHIL251/1: *EcoRV*

11 cHIL251/1: *Sall*

12 cHIL251/1: *PstI*

S2 Standard (1 Kb ladder)

13 cHIL208: *EcoRI*

14 cHIL208: *EcoRV*

15 cHIL208: *Sall*

16 cHIL208: *PstI*

(17 pSF6: *EcoRI*)

(18 pSF6: *EcoRV*)

(19 pSF6: *Sall*)

(20 pSF6: *PstI*)

S1, S2 and tracks 17-20 refer only to figure 7.2a.

FIGURE 7.2b

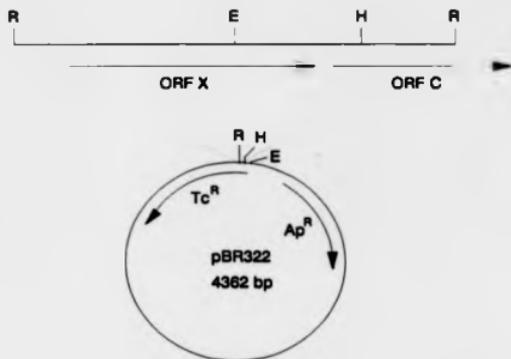
SOUTHERN BLOT: FOR THE IDENTIFICATION OF *OUTX* DNA.

The photograph shows an autoradiograph (16 hr exposure), giving the result of hybridization analysis. The agarose gel shown in figure 7.2a was probed with sequencing template DNA from the 260 bp *EcoRI/SalI* fragment of *outX*. See figure 7.2a for the key to the tracks.



Figure 7.2c

pSW2



Approximate restriction map of pSW2: constructed by cloning a 3.6 Kb *EcoRV* fragment (including ORF X) into pBR322, as shown. Vector and insert DNA are not drawn to scale.

including the sequenced 1.8 Kb *EcoRI* fragment which hybridized with the *ouX* probe. Also common to the new cosmids (but not found in cHIL208), was a 3.5 Kb (approx.) *EcoRV* hybridizing fragment.

The 3.5 Kb *EcoRV* fragment, obtained from a digest of cHIL208/5 by elution from an agarose gel (2.19), was ligated into *EcoRV*-cut, phosphatased pBR322 and transformed into DH1, plating on to NBA Ap. When plasmid DNA was extracted and checked by restriction analysis (as described earlier: 4.2) it was found that the insert was almost always in the same orientation, (with more than a twenty fold difference between the frequencies of the two possible constructs), suggesting that cloning difficulties arose with DNA from the upstream region of the *ou* cluster (see 6.3). It was possible that the rarely obtained construct included a mutation which was not detectable by restriction analysis. The frequently generated construct, named pSW2, is shown in figure 7.2c. pSW2B carried the 3.5 Kb *EcoRV* insert in the opposite orientation.

Samples of pSW2 DNA, prepared using CsCl gradient purification, were used for Peh assays (7.3), sequence analysis (7.4.1) and for the generation of constructs for gene expression experiments (7.5, 7.6). Samples of pSW2B were also prepared, for use in Peh assays. This was so that, if the 3.5 Kb insert did not include a promoter, *ouX* would be expressed from the Tc promoter of pBR322: assuming that pSW2B did not contain a mutation.

7.3 ASSAYING FOR PEH ACTIVITY.

It was important to determine whether the protein encoded by *ouX* showed Peh activity. Both spectrophotometric and plate assays were used, as described below.

7.3.1 SPECTROPHOTOMETRIC DETERMINATION OF PEH ACTIVITY.

Assays were performed as described previously (2.5.6), using a method derived from that of Collmer *et al* (1988).

The Peh assay relies on the fact that cleavage of the glycosidic bonds of PGA leads to an increase in the number of reducing groups: detected using arsenomolybdate reagents (Nelson 1944, Somogyi 1952). Any Pel activity must be abolished by the addition of EDTA to the assay buffer. There are two classes of Peh: endo-Peh, which cleaves internal glycosidic bonds of PGA to yield progressively shorter chains of PGA, and exo-Peh, which attacks chain termini to release DGA (Nasuno and Starr 1966, Collmer *et al* 1982). Since the latter enzyme causes a slower breakdown of PGA, with the production of fewer reducing groups, a lower activity would be detected. As the sequence analysis (6.2.1) was incomplete, it was not known which type of Peh (if any) was encoded by *outX*.

The strains assayed for Peh activity were as follows: HC131, DH1, DH1(pSW2B), DH1(cHIL208/5), DH1(pE4) and RJP249. cHIL208/5 was used in case pSW2B contained a mutation (7.2), while pE4, carrying the gene for an endo-Peh from *Ecc* (Plastow *et al* 1986) was used as a positive control. For preliminary experiments, strains were grown to stationary phase in 10 ml LB (with antibiotic selection). Induction with PGA was thought to be unnecessary, due to previous reports that Peh is expressed constitutively in *Ecc* (Chatterjee *et al* 1981). The cultures were fractionated by osmotic cold shock to yield supernatant, periplasmic and cytoplasmic samples (2.3.2), which were assayed for Peh activity. As the rates obtained were low (data not shown) and thought to be inaccurate, various modifications were made to the protocol.

As discussed earlier (3.6), the process of cell fractionation might decrease the level of enzyme activity detected. Peh was assumed not to be secreted by *E. coli* and the supernatants were discarded. The cell pellets were resuspended and sonicated directly, to yield a combined periplasmic/ cytoplasmic sample: referred to as the 'cellular' fraction. A two fold concentration of this sample was achieved by resuspending the cell pellet in half the original culture volume, prior to sonication. In order to treat the *Ecc* control strain in the same way, an *Out⁻* mutant was used instead of HC131. Since Peh is secreted by the same pathway as Pel and Cel (Thurn and Chatterjee 1985), it would be localized in the periplasm of RJP249 (chosen as a representative *Out⁻* mutant). The

assumption that RJP249 would produce near wild type levels of Peh, was based on the reports of constitutive production of the enzyme.

It has been reported previously, that measuring the OD of the assay reaction mixture at 500 nm does not produce optimum readings, but that this wavelength was chosen to minimise the effect of variation due to reagents (Nelson 1944). It was decided to investigate the claim (Nelson 1944) that a four fold increase in sensitivity could be achieved by taking readings at 660, rather than 500 nm.

Reaction mixtures were prepared, containing various concentrations of glucose (used to provide reducing groups). The OD of each sample was measured (Philips PU 8720 UV/vis scanning spectrophotometer), in the range: 340-700 nm and graphs were plotted, showing OD against glucose concentration at given wavelengths, as shown in figure 7.3.1a. The linearity of the graphs was not affected by wavelength, over the range of reducing sugar concentrations likely to be encountered in the Peh assays. Sensitivity however, was greatly increased by the use of longer wavelengths. Since the experimental conditions were exactly as they would be for Peh assays, it was concluded that a longer wavelength could be used. Assays for Cel activity use similar reagents and a wavelength of 623 nm. It was decided to take readings at 623 nm for future Peh assays.

Cellular samples (concentrated two fold) were assayed for Peh activity (taking OD readings at 623 nm). Protein concentrations were determined using the Bio-Rad procedure described previously (2.4). The Peh activities of the different strains, calculated as $\Delta OD(623 \text{ nm})/\text{min}/\text{mg}$ protein are given in table 7.3.1a.

The assays appeared unreliable: producing low and variable values for Peh activity. There was however evidence of some Peh activity encoded by *oufX*, with the plasmid-carrying derivatives of DH1 consistently showing more activity than DH1.

It was impossible to draw any conclusions from the relative activities found in the different samples. The use of different cloning vectors (pBR322 for pSW2B, pSF6 for cHIL208/5 and pUC8 for pE4) would lead to variations in copy number and vector promoter activity.

Figure 7.3.1a

'Peh Assay' Absorbances at Varying Glucose Concentrations and Wavelengths.

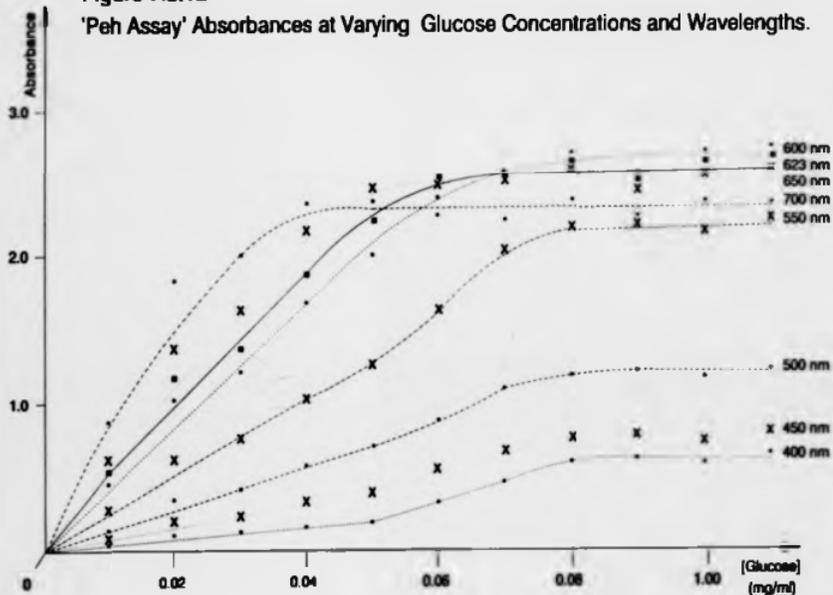


TABLE 7.3. 1a

PEH ACTIVITIES OF VARIOUS STRAINS:
DETERMINED SPECTROPHOTOMETRICALLY.

STRAIN	PEH ACTIVITY
RJP249	0.08 +/- 0.030
DHI(pE4)	0.04 +/- 0.020
DHI(pSW2B)	0.01 +/- 0.003
DHI(cHIL208/5)	0.05 +/- 0.020
DHI	ND

The peh assays were carried out as described in the text. Activities are shown as $\Delta OD(600 \text{ nm})/\text{min}/\text{mg}$ protein.

ND = Not Detected.

The spectrophotometric assays suggested that *ouyX* might encode an active Peh enzyme. In an attempt to confirm this, plate assays were used, as described below.

7.3.2 PLATE ASSAYS FOR PEH ACTIVITY.

As for the spectrophotometric assays (7.3.1), it was necessary to abolish any Peh activity by adding EDTA to the media. Since colonies were found to grow very poorly on such media, extracts were prepared from liquid cultures (grown in the absence of EDTA) and loaded into wells in the assay plates. Two types of plate assays were tried, as described below.

7.3.2.1 THE RUTHENIUM RED STAIN FOR PEH ACTIVITY

The use of ruthenium red to screen for Peh activity was developed by McKay (1988). The method was modified as described below. Media containing PGA (1.25% (w/v) final) and Peh assay buffer (50% (v/v) final), was solidified with agarose (0.5% (w/v) final). Agarose was used rather than agar, because of its ability to provide a relatively high gel strength at a low concentration (McKay 1988). Wells were made in the set media using the end of a Pasteur pipette, and filled with 28 μ l samples. For initial experiments, culture supernatants of HC131, grown in LB were used. The plates were incubated at 30°C overnight, before being flooded with ruthenium red solution (0.1% (w/v), freshly prepared in ELGA water) for 5 minutes, then rinsed with water.

The principle of this technique is that ruthenium red can only penetrate the medium where PGA has been degraded. Washing off unbound dye should leave red halos around samples with Peh activity. No such halos were observed.

Various alterations were made to the standard assay procedure. It was found that clearer halos were observed by increasing the agarose concentration to 0.6% (w/v) and by doubling the concentration of PGA in the medium. Further improvements were achieved by concentrating the samples and by destaining the plates with 1M NaCl. However, the contrast between the halos and the surrounding media remained too poor to allow photography.

7.3.2.2 THE COPPER ACETATE STAIN FOR PEH ACTIVITY.

This method was based on the plate assay for Pel activity, derived from that of Andro *et al* (1984). Assay medium was made as normal, except for the addition of EDTA (to 2 mM, final). Wells were made, samples loaded and the plates incubated as described above. Flooding the plates with copper acetate solution (7.5% w/v), caused a single halo to form around the HC131 supernatant sample (in contrast to the double halo formed on Pel plates).

The following strains were tested on the Peh assay plates: HC131, DH1, DH1(JS6161), DH1(JS6197), DH1(pE4) and DH1(cHIL208/5). JS6161 and JS6197 encode PelD and PelC respectively (Hinton *et al* 1989). Cultures were grown to stationary phase in LB (with antibiotic selection). Supernatant and cellular fractions were concentrated prior to use. Cellular samples were resuspended in a tenth the original culture volume before sonication, while Amicon centriprep tubes (30,000 MW cut off) were used to concentrate the supernatants ten fold (approx.). The concentrated samples were loaded on to the Peh assay plates and on to normal Pel assay plates for comparison. The results are shown in figure 7.3.2a. Looking at the samples from the control strains, it was clear that a simple and effective assay for Peh activity had been developed. Peh activity was found in both the supernatant and cellular fractions of HC131, but only in the cellular fractions of the plasmid-bearing DH1 strains, confirming that Pel and Peh are not secreted by *E. coli*. Although the assay plates detected Peh activity, there was no evidence of Peh being produced by cHIL208/5.

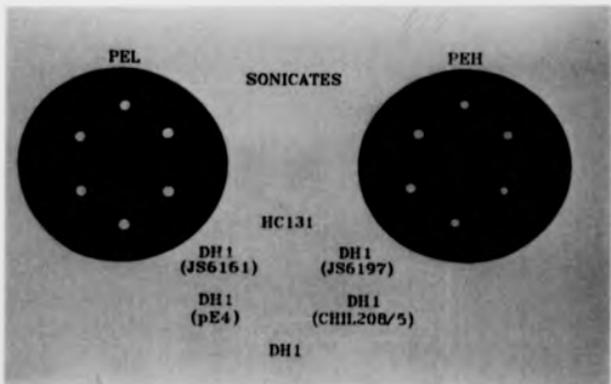
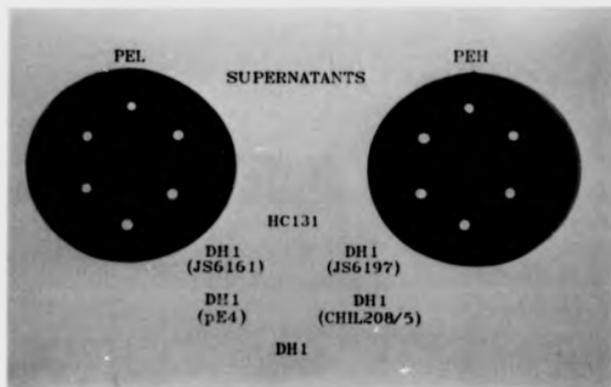
7.3.3 DISCUSSION.

Various assay procedures were used in an attempt to determine whether *ouyX* encoded an enzyme with Peh activity. Spectrophotometric assays, while producing low, and seemingly unreliable rates, did suggest that cHIL208/5 included a *peh* gene. Certainly, when DH1 carried an *ouyX* clone, the Peh activity was always greater than that for DH1 (not detected).

FIGURE 7.3.2a

ASSAY PLATES FOR THE DETECTION OF PEL AND PEH ACTIVITY.

Assay plates were prepared and used as described in the text. The photographs show the result of testing samples of *S/N* and sonicate (from the strains listed), for the activity of Pel and Peh. A halo surrounding the sample well indicates enzyme activity.



Assay plates stained with ruthenium red did not yield useful results. A plate assay derived from that for Peh was developed, using copper acetate as a stain. This gave distinct halos with extracts from HC131 and DH1 carrying a known *peh* clone, but not with DH1(cHIL208/5), even when the cellular fraction was concentrated ten fold.

These results might suggest that either the spectrophotometric, or the plate assay was unreliable. Alternatively, the two assays might differ in their abilities to detect the activities of different types of Peh enzyme. Spectrophotometric assays similar to that described here have been used previously for both (endo- and exo-) forms of Peh (Rexova-Benkova 1973, Collmer *et al* 1982). It is possible that the plate assays detected endo-, but not exo-Peh activity. This could have been confirmed if a clone encoding an exo-Peh had been available. Any assay would be more likely to detect the activity of endo-Peh, which causes a more rapid and extensive breakdown of PGA, than exo-Peh. Other workers have used the reducing sugar test for exo-Peh assays and different tests, such as viscosity assays for endo-Peh (Martinez *et al* 1991). Certainly, most assays would favour the detection of endo-Peh.

There are various methods available to determine whether a Peh enzyme exhibits endo- or exo- activity. The reaction products could be analysed using thin layer or paper chromatography (Brooks *et al* 1990, Collmer *et al* 1982), since exo-Peh activity is characterized by the release of a single product from PGA, rather than the series of oligomers generated by endo-Peh. Antibodies have been raised against purified exo-Peh from *Ech* CUCPB1273 (He and Collmer 1990). It might be possible to obtain a sample of this antibody and perform immunoblot analysis on the strains tested for Peh activity, looking for cross reactivity with the product of *ouX*.

It was stated earlier (7.3.1) that Peh is constitutively expressed in *Ecc* (Chatterjee *et al* 1981). It is possible however, that the gene was not expressed in the same way when cloned into *E. coli*, although multi-copies of a gene would normally lead to higher levels of the product being produced. It must be considered that previous reports claimed that there is only one *peh* gene (cloned in pE4) in *Ecc* (Plastow *et al* 1986), since only one band was seen on Peh IEF gels. This might suggest that *ouX* does not

encode an active Peh. Alternatively, the IEF gels might have failed to detect the less active exo-Peh. If *ouX* does encode a Peh, nothing is known about the expression of this, and induction might have been required.

It was not possible to draw any firm conclusions from the work described above. It is possible that *ouX* does not encode Peh, despite the homologies predicted from the partial sequence (6.2), or that it encodes a form of the enzyme which is inactive, or which was poorly expressed under the conditions of the experiment. Alternatively, *ouX* may encode an exo-Peh which was difficult to detect using the assay procedures described. Certainly, the possibility that *ouX* is a gene for Peh could not be ruled out.

It was concluded that if *ouX* does encode a Peh, this was likely to be of the exo class. This would be an interesting discovery, as only endo-Peh had previously been reported in *Ecc* (Nasuno and Starr 1966, Chatterjee *et al* 1981), exo-Peh being found in *Ech* (Collmer *et al* 1982). The experiments required to prove that *ouX* is a structural gene for exo-Peh (discussed above) were beyond the scope of this project. It was decided instead to concentrate on completing the DNA sequence analysis of *ouX*, in the hope that further information on its likely identity would be obtained.

7.4 SEQUENCE ANALYSIS.

7.4.1 DNA SEQUENCE ANALYSIS OF *OUTX*.

OuX was sequenced directly from pSW2 using the technique of double stranded sequencing described previously (2.21.4.1). The sequenced region of *ouX* was gradually extended, using oligonucleotides hybridizing to that DNA already sequenced, as primers for the sequencing reactions.

The sequencing strategy is outlined in figure 7.4.1a, which shows the positions of the oligo primers, the lengths of sequence obtained and the strand sequenced from each primer. The sequence of each oligo primer (chosen according to the factors described earlier: 2.21.4.3) is listed in table 7.4.1a, according to the numbering system used in figure 7.4.1a.

Figure 7.4.1a: Strategy for Sequencing *outX*.

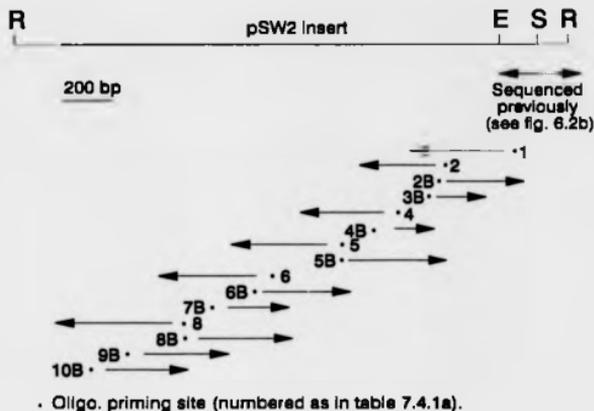


TABLE 7.4.1a

THE OLIGONUCLEOTIDES USED TO COMPLETE THE SEQUENCE ANALYSIS OF *OUTX*.

OLIGO. NAME	SEQUENCE (5'→3')
OLISW1	TTACACTGTCATCACCC
OLISW2	ATTCCCTCAGCAACGGC
OLISW2B	TGTTGGTAGTGACGGTA
OLISW3B	AGAATGAATTGCCGCTC
OLISW4	TTTTAAAGGTGCCTGCAT
OLISW4B	CGGGGCTACTATCTCTA
OLISW5	TCAACCACGACCTTACA
OLISW5B	TGTAAGGTCGTGGTTGA
OLISW6	GACATGCCAGTTATCGG
OLISW6B	TGGCGGAACCATTGAT
OLISW7B	TGTCGGTGTGATCGTT
OLISW8	GTCAGTCATAGCAITTC
OLISW8B	GAAATGCTATGACTGAC
OLISW9B	ATGGAGTCCCGCTTATG
OLISW10B	AACGGCTCCTGGTTCAA

The oligonucleotides, named as in figure 7.4.1a, are shown 5'→3'.

The complete DNA sequence of the *outX* region, including that shown previously (6.2.1), up to the start of *outC*, is shown in figure 7.4.1b, along with the predicted amino acid sequence of the gene product. Figure 7.4.1b also shows the likely positions of the ribosome binding site and promoter sequence, in addition to a proposed N-terminal signal sequence and cleavage site within the protein.

OutX contains only one plausible initiation site, which is preceded by a good consensus ribosome binding site. The gene encodes a protein of 660 amino acids, with a predicted molecular weight of 71,903 Da and an average pI value of 6.04. The G/C content of *outX* was found to be 50.7%, which is close to the *Ecc* average of 52.1%. Hydrophilicity analysis showed a single hydrophobic stretch of 15 amino acids (residues 10-24), near the N-terminus of the protein. This was followed by a likely signal sequence cleavage site (6.4.3) after the Gly residue at position 24. In the absence of a likely anchor region, it was proposed that OutX is exported to the periplasm and post-translationally processed, to give a protein with Cys at its N-terminus. This is a feature normally associated with lipoproteins, in which the Cys residue is modified by the addition of two fatty acyl groups through ester linkage (involving a glyceride residue), and a third fatty acyl group, via an amide group (Pugsley *et al* 1986). The signal peptide of a lipoprotein is cleaved by a specific peptidase: signal peptidase II. The sequence of amino acids around the signal peptidase II cleavage site (the recognition site for lipid modification enzymes) has been found to be highly conserved (Yamaguchi *et al* 1988). The predicted cleavage site in OutX is compared to those of various Gram-negative lipoproteins in table 7.4.1b, in which the sequences are aligned at the Cys residue (position +1 of the mature polypeptide). This Cys, and the four preceding residues make up the pentapeptide sequence thought to be involved in prolipoprotein processing. Clearly, OutX has a cleavage sequence homologous to those of known lipoproteins. It has been proposed that the amino acids at positions +2 and +3 of the mature protein play a crucial role in determining the final location of a lipoprotein (Yamaguchi *et al* 1988), with the presence or absence of negatively charged residues (underlined in table 7.4.1b) at these positions being particularly important. It

Figure 7.4.1b continued

```

1630      1640      1650      1660      1670      1680
TGGCGATGGTCCGGTGGTCCACGGGTAGCCATACCGGTGCCCTGGATTGAAAAAATTGTTGC
  G H G A V V T G S H T G A W I E K I V A

1690      1700      1710      1720      1730      1740
TGAAGCAACGTAATGAATAAAGACGGATTTGGCCCTGCCATGAAGAGTCGACCCCTATTA
  E D N V M N K T D V G L R M K S R P Y Y

1750      1760      1770      1780      1790      1800
TGGTGGCGGCTCGCGTGACGGTGGTATTCCGTAATAACCGGATGGTGGATTTGTC AACGA
  G G G S R D V V F R M N A M R D I V N E

1810      1820      1830      1840      1850      1860
GCGGTTTGTGTTACGATTAATAATAAAGCGGACGTTGAAAGATACCCAGCCTGCCGCTGA
  P F V F T I K Y K A D V N D T Q P A A E

1870      1880      1890      1900      1910      1920
GCCGTCGCCAGTTCCGTTGATGTGACCGTTTCCAAACGTCACCGGTTGATGGTACAGCGAAGAA
  P A Q F R D V T V S N V T V D G T A K K

1930      1940      1950      1960      1970      1980
AAACAGTATCATGGTGGATGGGATGACCGTGGCTGAAATGGCTGATGGGTATAAGTTCTC
  N S I M V D G M T V A E M A D A Y K F S

1990      2000      2010      2020      2030      2040
TTTTGGACGTGATGCCCTATCACCAAGGCTTGCATTTTGAGAATGTAAAAATCAAAAAATGT
  F G R D A Y H Q G L H F E N V K F K N V

2050      2060      2070      2080      2090      2100
AAAAGCGACGGACATTACGTTCCCTGAAGAACAGTGAATTTAAAAATGTCATTTTGAGAA
  K A T D I T F L K N S E F K N V I F E N

2110      2120      2130      2140      2150      2160
TGTACCGAGACCGCTGGAAATTTCCGTCATATTGAAAAATATCAGGCTCGAAGACCGTGTAAA
  V P R A W N F G H I E N I R L E D R V N

2170      2180      2190      2200      2210      2220
TAAGGATGCCGCGCTGACGACCAACGGGTGATGAAACCATCACCCGGGAAAGCCGCAACCGGA
  K D A A L T T S G D E T I T R E A A T E

2230      2240      2250      2260      2270      2280
ATAAGGCATTTTCCCGACCGCAATGCGCTGGGAGTATTATGGATGATTATCTATGGCA
  inverted repeat                               OutC: M A

2290      2300      2310      2320      2330      2340
CGATTGCAAGCTTCAAAAGATCCGTCCTTTTCATTGGTTGCCACTTTTCGGTCACTG
  R L Q A F K D P S F H S L V A T F R S L

```

Nucleotide sequence of 2.3 Kb *Ecc* DNA (noncoding strand) containing the *owx* gene and the start of *owc*. The positions of the putative promoter region (-35, -10), ribosome binding site (RBS) and inverted repeat sequence are shown and are discussed in the text. The predicted amino acid sequence of OutX is given, showing the possible signal sequence and cleavage site (↓).

TABLE 7.4.1b

AMINO ACID SEQUENCES OF SIGNAL PEPTIDES AND THEIR CLEAVAGE SITES, OF LIPOPROTEINS FROM GRAM NEGATIVE BACTERIA.

Taken from Yamaguchi *et al* (1988).

Lipoprotein-28 (<i>E.coli</i>)	MKLTTHHLRTGAALLLAGILLAG <u>CDQSSS</u> -
Major Lipoprotein (<i>E.coli</i>)	MKATKLVLGAVILGSTLLAG <u>CSSNAK</u> -
PAL (<i>E.coli</i>)	MQLNKVLKGLMIALPVMATAA <u>CSSNKN</u> -
36K Lipoprotein (<i>E.coli</i>)	MRKQWLGICIAAGMLAA <u>CTSDDG</u> -
19K Lipoprotein (<i>E.coli</i>)	MRYLATLLLSLAVLITAG <u>CGWHLR</u> -
TraT Protein (<i>E.coli</i>)	MGGKLLMMVALVSSTLALSG <u>CGAMST</u> -
Cytochrome subunit (<i>R.viridis</i>)	MKQLIVNSVATVALASLVAG <u>CFEPPP</u> -
17K Antigen (<i>R.rickettsii</i>)	MKLLSKIMIIALATSMLQA <u>CNGPGG</u> -
Chitinase (<i>V.harveyi</i>)	MLKHSLIAASVITTLAG <u>CSSLQS</u> -
Pcp Protein (<i>H.influenzae</i>)	MKKTNMALALLVAFSVTG <u>CANTDI</u> -
PAL Protein (<i>H.influenzae</i>)	MNKFVKSLLVAGSVAALAA <u>CSSSNN</u> -
Pullanase (<i>K.pneumoniae</i>)	MLRYTCNALFLGSLILLSG <u>CDNSSS</u> -
OutX Protein (<i>Ecc</i>)	MTDFLPRTRLTLCATLVLSGLIAG <u>CSQSPS</u> -

Negatively charged amino acid residues at the cleavage site are underlined. Signal peptide cleavage sites are shown by an arrow.

References: lipoprotein-28 (Yu *et al.*, 1986); major lipoprotein (Braun and Rehn, 1968); PAL of *E.coli* (Chen and Henning, 1987); 36K lipoprotein and 19K lipoprotein (Takase *et al.*, 1987); TraT protein (Perumal and Minkley, Jr., 1984); cytochrome subunit (Weyer *et al.*, 1987); 17K antigen (Anderson *et al.*, 1987); chitinase (Jannatipour *et al.*, 1987); Pcp protein and PAL of *H.influenzae* (Deich *et al.*, 1988); pullulanase (Chapon and Raibaud, 1985); OutX (this study).

FIGURE 7.4.1c

REPRESENTATION OF THE STRUCTURE OF OUTX

Figure 7.4.1c (opposite) shows a plot of the predicted secondary structure of OutX (originally named OutS, as in the figure): obtained using the UWGCG programmes 'Proteinstructure' and 'Plotstructure' (Devereux *et al* 1984).

The programmes predict secondary structure, using the algorithms of Chou and Fasman (1978) and hydrophobicity, using the algorithms of Kyte and Doolittle (1982), with a window of 20 residues.

Key to symbols:

α -helix: ~~~~~

random coil: - - - -

β -pleated sheet: ———

β -turn region: } }

Regions of hydrophobicity are represented by red diamonds and regions of hydrophilicity by blue ovals.

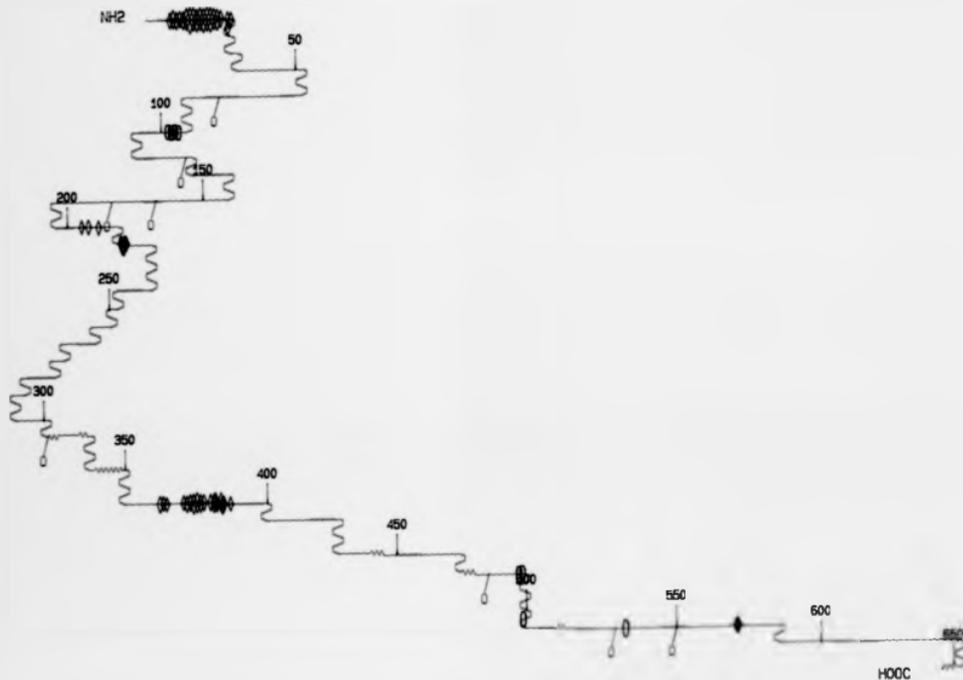
The potential signal sequence of OutX is shown by the large number of red diamonds (indicating a highly hydrophobic region) near the N-terminus of the protein.

The figure shows that there are a large number of regions in OutX which are predicted to form β -pleated sheet and β -turn structures

PLOTSTRUCTURE of: outs1.txt ck: 9067

Chou-Fasman Prediction
September 23, 1982 15:41

○ KD Hydrophilicity $\gamma=1.3$
◻ KD Hydrophobicity $\gamma=1.3$



is believed that an Asp residue at position +2 means that the lipid part of the lipoprotein stays in the inner membrane, while a Ser residue at that position leads to translocation to the outer membrane (Yamaguchi *et al* 1988). Sequence analysis therefore suggested that the *ouX* product was a lipoprotein, likely to be translocated to the outer membrane. The possible implications of this, together with the experiments required to confirm that OutX is a lipoprotein are discussed later (7.7).

A LUPES motif search was performed, but no consensus prokaryotic motifs were identified.

The predicted structure of OutX is shown by the 'squiggle plot' representation (Seqnet) in figure 7.4.1c.

The DNA lying upstream of the start of *ouX* was analysed, looking for a possible promoter sequence. A large number of *E. coli* RNA polymerase promoters have been studied (Hawley and McClure 1983), from which a consensus sequence has been derived. This consists of a -35 region (5'-TTGACA-) and a -10 (Pribnow box) region (5'-TATAAT-), with the transcription start being at position +1. A sequence showing a reasonable match with this was found (shown in figure 7.4.1b). The position of this putative promoter sequence would make the transcription start point 200 bp upstream of the *ouX* translation initiation site.

There was no evidence of a *kdgR* box (associated with other extracellular enzyme genes), or of an ORF encoding a PulS or PulB homologue in the region upstream of *ouX*.

7.4.2 SEQUENCE HOMOLOGUES OF OUTX.

OutX was found to be homologous to PehX: *exo-Peh* (EC 3.2.1.82) from *Ech* (He and Collmer 1990). The homology extended over the entire length of the protein, with 46.9% amino acid identity over a stretch of 400 residues. An alignment of the two proteins is shown in figure 7.4.2a. The high degree of similarity between the proteins suggested that OutX is an *exo-polygalacturonase*, as predicted from the enzyme assay data (7.3.3).

FIGURE 7.4.2a

SEQUENCE ALIGNMENT OF OUTX AND PEHX.

Gap penalty = 4 Cut-off = 10

Variable gap penalty = F Window = 10 Filtering = 2.5

* -> Exact match across all seqs; . -> Conservative matches across all.

```

OutX      1  NTFPLPSTRLLCATLVLSGLIAGCRSDSDTPDVGVDSSAOPMLKVPITLAYDSSVSLVA
PehX      1  -----MEVITFERRSALARI VATCLN--STPALAATAG-APKELGIPITLSTDDHSMVLY
          . . . . . * . . . . . * . . . . . * . . . . . * . . . . . * . . . . . *

OutX      1  MECPTEPAAGIKDYVYHNGTLGGTIDHGTNHPAKPYIDHFFVNSIDTDMHNVRFHFM
PehX      1  MDPEDTEN-ITDYLIONGDLGLASONHDCRSPAKPYISAFYKS-DAAMFHRIVLGN
          * . * . . . * . . . * . * . . . * . . . * . . * . . . * . . . * . . *

OutX      1  FKVHLSPETEYRFTVRLYDDGKESVDDETVGKTTATPABLHVNYGAKGCVYNDIV
PehX      1  AKVDGLKAGDTYQFTVRYADGTTENDSNTVTTTIANPVEINISOTGAKGDTLNTS
          ** * . . . * . . . . . * . . . * . . . * . . . * . . . * . . . * . .

OutX      1  AIGKAIIDCTPAAYPKGCKVWVEGTFKGTGALFLHSDHTFEVAKGATLLGRAGDDYPLA
PehX      1  AIGKAIIDAC-----PTGCRISVPAQVFTFALMLKSDHTLLLOGATLLGSDAADYDGA
          ***** * . . . . . * . . . . . * . . . . . * . . . . . * . . . . . *

OutX      1  RGYLYPYSPKPKRPPSLINVLEADDDGSSHAGTFKIRIVDQGTIDGMDHTGICKAS
PehX      1  --YKIVSYSQV---RPAALLN---AIDKNSAVGTFKIRIVGKGIIDGCKVRSADAK
          * . * . * . * . . . * . . . * . . . * . . . * . . . * . . . * . . . *

OutX      1  DTNITEIDELKWEPLRYBASKAAMVGGDGLADDTAKAVAEQHLDEAYEMRERBLNLT
PehX      1  -----DELGHTLPGYKSDHSEVSGDGLAKNVAAMAVATGDKTAYSDRERBLVTL
          *** * . . . * . . . * . . . . . * . . . * . . . * . . . * . . . * . .

OutX      1  RGVSMYLEGTLILMPAFHGVNVLSEBHVAMMALVHTTYDAMRAGDIEFGKSDHNVRFPM
PehX      1  RGVNAYIADVTIRNPAHNGIPLSEBHVVSRIHGTFRAMHGDQVEFGNSDNIHVPNS
          *** * . . . * . . . * . . . * . . . * . . . * . . . * . . . * . . . * . .

OutX      1  FPDGDDVHFAAGYCAEVATLQKADRGAMIFNVPFRCHGAVVTCGAMIEKIVAE
PehX      1  VFDGDDSIHFAAGHGDQAK--GEPDRAHLFNFFHNGAVVLGHTGAGIYDVLAE
          ***** * . . . . . * . . . . . * . . . . . * . . . . . * . . . . . *

OutX      1  DSVNRITDVGLRMESHRYGGESDVPFRHNRMDI VNEPFPVTIEYKADV---DTGPA
PehX      1  HNVITDNDVGLBAKSAPIGGANGIVFRHSAMNLAGAVIVLRY-ADMCTIDYTPA
          . . . . . * . . . * . . . * . . . * . . . * . . . * . . . * . . . * . .

OutX      1  AEPARFDVTVSNHTVGTAKRNSINVDGHTVAEMADAYKFPGRDATHGQLFENHFK
PehX      1  KVPARFYDFTVENVT-----VDSSTCNPALIEITDSEKDIWNHSPIFRAMELLE
          ** * . . . * . . . * . . . * . . . * . . . * . . . * . . . * . .

OutX      1  RVEATDITFLKNSFKMVFENVRA---LNFCHIEHIFLEDHVKDAALITSGDETITR
PehX      1  CVSPTSISGLSDSOPHNLTFBMLRSGSPLEFQTVENV-----TVGKTVTP
          * . . . * . . . * . . . * . . . * . . . * . . . * . . . * . .

OutX      1  RAATEU
PehX      1  -----
    
```

References: OutX: this study, PehX: He and Collmer (1990).

OutX was also found to be homologous to a range of other Peh enzymes (of the endo type: EC 3.2.1.15), from various bacteria and plants. These, together with some other interesting homologies are listed in table 7.4.2a. A dendrogram, indicating the relatedness of the proteins is shown in figure 7.4.2b, while a multiple alignment is shown in figure 7.4.2c.

The homology of endo-Peh proteins of *Ecc* and tomato (*Lycopersicon esculentum*) has been discussed previously (Hinton *et al* 1990). Peh is involved in tomato ripening: causing disruption of the middle lamella, leading to the separation of the two primary cell walls, and hence softening. Since purified Peh causes green tomatoes to ripen (Crookes and Grierson 1983), this is thought to be the major cell wall degrading enzyme involved in tomato ripening. The homology between the Peh proteins of *Ecc* and tomato has been taken to indicate that the genes encoding these proteins arose from a common ancestor (Hinton *et al* 1990). This proposal can be extended, following the discovery in this study, of homologues of bacterial Peh in evening primrose, maize and avocado.

The homology with two lipoproteins was interesting, in view of the proposed lipoprotein processing site found in OutX (7.4.1). The homology of OutX to the VirB9 protein of *Agrobacterium tumefaciens* suggests that similarities between the *out* cluster and the *virB* operon (involved in the transfer of T-DNA across the bacterial membrane) extend further than previously thought (1.9). Similarly, homology with a Hrp protein of *Pseudomonas syringae* shows that the similarities between the *hrp* and *out* clusters were not limited to *hrpH* and *outD*.

A comparison was made between PulA and OutX. Although both are large and thought to be lipoproteins, only very small stretches of homology (not thought to be significant) were found. It was therefore concluded that the upstream region of the *out* cluster, encoding OutX, is very different from the corresponding region of the *pul* cluster.

An extremely interesting discovery is the homology between OutX and the PicA protein of *Agrobacterium tumefaciens*. The *pica* gene is at a chromosomal locus which

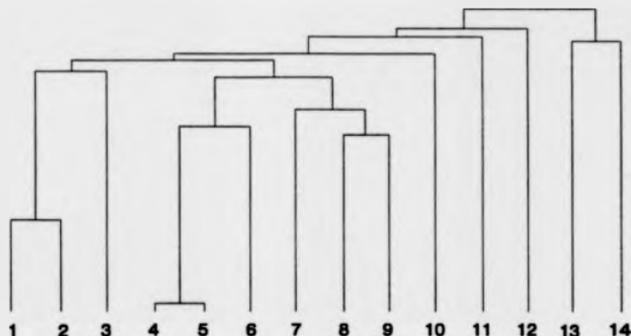
TABLE 7.4.2a: HOMOLOGUES OF OUTX.

% IDENTITY TO OUTX	OVERLAP	PROTEIN	ORGANISM	REFERENCE
46.9	401	Exo-Peh	<i>Ech</i>	He & Collmer (1990)
25.8	229	Endo-Peh	<i>Ecc</i>	Hinton <i>et al</i> (1990)
26.6	229	Endo-Peh	<i>Ecc</i>	Saarijoki <i>et al</i> (1990)
23.3	227	PicA	<i>Ag. tumefaciens</i>	Rong <i>et al</i> (1991)
18.8	202	Endo-Peh	Tomato	Grierson <i>et al</i> (1986)
21.2	156	Endo-Peh	Evening primrose	Brown <i>et al</i> (1990)
21.3	221	Endo-Peh	<i>Ps. solanacearum</i>	Huang & Schell (1990)
21.8	211	Endo-Peh	Maize	Niogrot <i>et al</i> (1991)
15.4	273	Ripening- related protein	Avocado	McGarvey <i>et al</i> (1990)
32.1	53	Major OM lipoprotein	<i>Erwinia amylovora</i>	Yamagata <i>et al</i> (1981)
29.8	47	Major OM lipoprotein	<i>Proteus mirabilis</i>	Ching <i>et al</i> (1986)
33.3	54	VirB9	<i>Ag. tumefaciens</i>	Rogowaty <i>et al</i> (1990)
18.6	102	Hrp protein	<i>Ps. syringae</i>	Mukhopadhyay <i>et al</i> (1988)

FIGURE 7.4.2b

DENDROGRAM TO SHOW THE RELATIVE SIMILARITIES
OF OUTX HOMOLOGUES.

The dendrogram was obtained using the CLUSTAL sequence analysis programme.



The proteins which were compared to form the dendrogram are listed below, together with references.

- 1 OutX, *Ecc* (this study)
- 2 PehX, *Ech* Exo-Peh (He and Collmer 1990)
- 3 Hrp, *Ps. syringae* protein involved in the hypersensitive response (Mukhopadhyay *et al* 1990)
- 4 PEcc1, *Ecc* Endo-Peh (Hinton *et al* 1990)
- 5 PEcc2, *Ecc* Endo-Peh (Saarilahti *et al* 1990)
- 6 PPs, *Ps. solanacearum* Peh (Huang and Schell 1990)
- 7 PTom, Tomato Peh involved in fruit ripening (Grierson *et al* 1986)
- 8 PPrim, Evening primrose Peh involved in pollen development, germination and tube growth (Brown *et al* 1990)
- 9 PMaize, Maize Peh (Niogret *et al* 1991)
- 10 PicA, *Ag. tumefaciens* Peh (Rong *et al* 1991)
- 11 PAvo, Avocado Peh involved in fruit ripening (McGarvey *et al* 1990)
- 12 VirB9, *Ag. tumefaciens* virulence protein (Rogowsky *et al* 1990)
- 13 LipEr, *Erwinia amylovora* major OM lipoprotein (Yamagata *et al* 1981)
- 14 LipPr, *Proteus mirabilis* major OM lipoprotein (Ching *et al* 1986)

FIGURE 7.4.2c

SEQUENCE ALIGNMENT OF OUTX HOMOLOGUES.

Gap penalty = 4 Cut-off = 10

Variable gap penalty = F Window = 10 Filtering = 2.5

+ :-> Exact match across 3 or more sequences, including OutX

:-> Exact match across 4 or more sequences, including OutX

```

OutX      1  NTDLPRTNLTLCATLVLSL IARCMHDSIPGVGORSAPPHLKVPTLAYDSSVPLA
PohX      1  -----NEVITPBBBRLASIVSTCLN--STPALAATAC-APQKLOIPTLSTDDHNRVLY
PEcc1     1  -----
PEcc2     1  -----
PicA      1  -----
PTom      1  -----
PPria     1  -----
PPa       1  -----
PNaIze    1  -----
PAvo      1  -----
VirB9     1  -----
Nrp       1  -----NLGVDSGSLCL
LipPr     1  -----

```

*

```

OutX      1  MKEPTPAAR IEDVYVHRTLLQST IQRHTSPAPKY IHPVHSICDGMHVVSPH
PohX      1  MTPEDTIN -ITGVIVKSHL IGLASNNHNSAPKPY IAPYCS-DAAPFNRIVLGN
PEcc1     1  -----
PEcc2     1  -----
PicA      1  -----
PTom      1  -----
PPria     1  -----
PPa       1  -----
PNaIze    1  -----
PAvo      1  -----
VirB9     1  -----
Nrp       1  KATP IRRTKVPSMATH ILYGQMLTKGN-PPKPHQSLPS--SLPKAMHVTSH I
LipPr     1  -----

```

* * * * *

```

OutX      1  PKVANLSPETEYRFTVALYDQGE-----SVDRETVNKTATPASLNTYS-----
PohX      1  AKVGLKAGTDYQFTVSTVADDTI-----SMDSHVTTTTTAVKVISITGV-----
PEcc1     1  -----
PEcc2     1  -----
PicA      1  -----
PTom      1  -----
PPria     1  -----
PPa       1  -----
PNaIze    1  -----
PAvo      1  -----
VirB9     1  -----
Nrp       1  RR Y I L L L M Q D T I - V A G V H K G I L P V R H M L V L S H I T Q Y F N T A L Q M P V A L S T I L
LipPr     1  -----

```

* * * * *

Figure 7.4.2c continued: Sequence alignment of OutX homologues

+ :> Exact match across 3 or more sequences, including OutX

@ :> Exact match across 4 or more sequences, including OutX

```

OutX      1  -----
PehX      1  -----
PEcc1     1  -----
PEcc2     1  -----
PicA      1  -----
PTam      1  -----
PPriA     1  -----
PPa       1  -----
PmaIze    1  -----
PAvo      1  -----
VirB9     1  -----
Hrp       1  L L F C L F C W S A G F T A L N C F L L L T S R Q K Y R I S G A S A G N E P I E E G A R T A L W P I C H E D V
LipPr     1  -----
LipPr     1  -----
    
```

```

OutX      1  -----AKDGGV-----TNDTVAIQEIQDCTPAATPKGGCVV-
PehX      1  -----AKDGT-----TLNISAIQEIQDAC----PTGCRID-
PEcc1     1  -----MEYSGCKVLSLSLGLIGLPSASAFAS
PEcc2     1  -----MEYSGCKVLSLSLGLIGLPSASAMAS
PicA      1  -----
PTam      1  -----HVIQNSILLIIPASSIYCCSNVIRDLFKNVSSILDGFANDFNAVLS
PPriA     1  -----
PPa       1  -----MHNRYTLALAAALAGANATGIVTAPMGEVAEPLPADSAVCKLSASITPI
PmaIze    1  -----NACTNANRALFLVLFPC
PAvo      1  -----
VirB9     1  -----
Hrp       1  PRVFAGLRATFESVAATGLDRDFVFLSDTNETDIAVAGDQALDYCREKSGGIIYVH
LipPr     1  -----
LipPr     1  -----
                * * * * *
    
```

```

OutX      1  -----VEGDTFKTGLFLNDDNTFVAKGAT
PehX      1  -----VPAGVFKTGLWLSDDTLNLGGAT
PEcc1     1  DRRVDSPEAPRNCYVLEGGDTATSTIRKALNCCGRKAVLSAGES--SVFLDGPLSL
PEcc2     1  DRRVDSPEPTDRCYVLEGGDTATSTIRKALNCCGRKAVLSAGES--SVFLDGPLSL
PicA      1  -----
PTam      1  YLQKIDGRRIRGVGDSIEVIVLDFRKKDGRYVRIAPRAGNACSKSTYPPQVWV
PPriA     1  -----DSTQALTALKEACAASRPSTILV
PPa       1  KGVDSVDGHP-----RRRPRRSHIRRIIRNCPADRKAVLVEGRAGEGFLDGLKIL
PmaIze    1  IYHGEKEEKGIKAKARPRRFRITLIRRRHCKTDSTKAVWAMASAGDTOKQITLI
PAvo      1  -----
VirB9     1  -----
Hrp       1  RRRRVKRSKGLDQFCRNGGDTYRNVVLDADSVHSGECLTSLVELNATPDAGIQTA
LipPr     1  -----
LipPr     1  -----
                * * * * *
    
```


Figure 7.4.2c continued: Sequence alignment of OutX homologues

* i-> Exact match across 3 or more sequences, including OutX
 # i-> Exact match across 6 or more sequences, including OutX

```

OutX      1  EFGNSMNVFNNFFDTGDSVNFAGYGAEVATLQDAGSGAM|-----
PehX      1  EFGNSMNVFNVFDTGDSVNFAGNSGQDAG--GFPRMAM|-----
PEcc1     1  DPHNSKNIJAYNSIATGDSNVIAKAYKORSETRNIDLMNFQT|-----
PEcc2     1  DPHNSKNIJAYNSIATGDSNVIAKAYKORSETRNIDLMNFQT|-----
PicA      1  NPHNSMNVISGVFVGDCCIAVKADKSPQGDGDLHARTSIDTVRSLMSP|-----
Ptom      1  NPHNSIETIDSDIATGDDCISIVGSSD---EHWGNTITCOP|-----
PPrim     1  NIGNSGVNIINTEITGDDCISLGGSK----EINHTITCOP|-----
Ppa       1  DPGDSTVNLVLAISVINTGDSNVAVKASSP---TRNLLFARNFY|-----
Pmaize    1  NHDSSGIIJTHVIVGDDCISIPGYS----KVNITGVTCOP|-----
PAvo      1  MESTFFIRHLPVHNSIPCLTDEHNNVKEFAEKLEKAEVLDLCLBLBLEEYER|-----
VirB9     1  PATTVGAEDKMHVYAGDGRLLPLEVFDGDTTVFHPGGVRIPIVYIIPRKSAMR|-----
Nrp       1  NRTLAPLPPFLVSTALLAVNTLNEPTTYFLPGLYLPQWNPKEAVALFSTIIVLL|-----
LipR      1  VILGCTLLAGCSB--NAKIDGLSTVDTLNAKVDGLSDVTAIRSDVNS--AKDGAARMS|-----
LipPr     1  VILAGCTLLAGCSBHNALDQISRDVHRLTGVGLSDVVGSA--NAGAKAYAAARMS|-----
          ++@#++ ++ + ###++ ## + * + * +

```



```

OutX      1  -----FNNFRGH-----
PehX      1  -----FNNFRGH-----
PEcc1     1  -----GH-----
PEcc2     1  -----GH-----
PicA      1  -----GH-----GGLVI
Ptom      1  -----GH-----
PPrim     1  -----GH-----
Ppa       1  -----GH-----
Pmaize    1  -----GH-----
PAvo      1  AFAGTTGLPTGTGVSHYPPCPRELKGLRHTDAGLLFDQDRVAGLLKIDGV|-----
VirB9     1  YNRSDYVRISDVRMSLSDGTVLCLMRTAYDPMRPFCTCTVNPDKVLEKVC|-----
Nrp       1  FLPELLSVLLNAKAGKGFQGFVYVHRLDGLVYLL|-----
LipR      1  RLDGARSYRE-----
LipPr     1  RLDGVITTYE-----
          ##

```



```

OutX      1  GAVYTGHTG-----AMIEKIVAEQVWNETDVLNKEKRPYYDGSRDVFNH|-----
PehX      1  GAVYLGHTG-----ADIVDLAENVI|TQNDVGLNAKSAPAIQDGAQIVFNS|-----
PEcc1     1  GNSIGSETHGVYNTVDGLINTGTT---B-----GLRISKDKSAAGVYNG--SY|-----
PEcc2     1  GNSIGSETHGVYNTVDGLKNGTT---B-----GLRISKDKSAAGVYNG--SY|-----
PicA      1  GSENSGVNVTVGDCMIGTDRGLKTKGARRGGVGHITRNVLDGQVITLBARAH|-----
Ptom      1  GIGIGLGGHSEAYVSHVYVEAKIIGAEV-----QVRIGETWGGSDA--NIEPL|-----
PPrim     1  GIGVGLGRVYKNERVYQIYVKNCTITGDSH-----QVRIKTVPKSEPGA--SENFQ|-----
Ppa       1  GLRIGSETHGVNRLVTDLITQDSSAGH-----QLRIKSDARAGQVYHII--VYD|-----
Pmaize    1  GIGIGLGRVYDREVDITQVDCITLKEKTP-----QVRIKAYEAAAVLTVNIEIP|-----
PAvo      1  VQVPPNKRIVINLQDQVEVITQKQYEVNHRVVAITDQNSHLARFHPGSDAVIPAP|-----
VirB9     1  APVNLPTDPLAFLQMATMSPQDSDPTMISMGCHHPPTLQACMILLVPLK|-----
Nrp       1  -----
LipR      1  -----
LipPr     1  -----
          @ ++ + ++ + * ++ # ++@# @+ + +@+ + + +

```

```

OutX      1 A-----MDIVHEPFFVTIETKADVY---DTPAAEPAQFQCVTVSMVTVGDTAK
PehX      1 A-----MKLAKGAVIVTLVY-ADNNGTIDTTPAKVPAFTYDFYKVMVT-----
PEcc1     1 NVVYKKNVAK-----P|V|D|T|V|Y|E|K|E|G|S|H|V|D|S|D|I|T|F|K|D|I|T|-----SQTGQVV
PEcc2     1 NVVYKKNVAK-----P|V|D|T|V|Y|E|K|E|G|S|H|V|D|S|D|I|T|F|K|D|V|T|-----SETKQVV
PicA      1 YKCDADGDDHVVGMHPAPVMDGTFVDDG|T|V|E|V|E|I|N|L|A|N|A|G|V|L|G|L|P|D|V|P|A|T|S|S
PTom      1 NVHEMDDVY-----P|I|D|D|Y|C|D|R|V|E|P|C|I|G|D|F|S|A|V|G|V|K|N|V|Y|E|N|K|G|T|S|A|T|E|A|I|
PPrim     1 D|I|T|N|N|V|G|T|-----P|I|D|D|G|Y|C|P|N|G|C|T|A|E|V|P|S|V|L|K|B|I|S|F|E|N|K|G|T|S|T|K|E|A|V
PPa       1 C|I|D|N|V|K|E|-----P|L|V|D|F|Y|S|E|V|K|G|L|Y|N|F|H|I|V|K|N|F|H|L|G|S|A|K-----S|I|K|T|N|T
PMaize    1 N|I|K|H|E|D|S|A|N|-----P|F|D|N|Y|C|P|N|K|L|T|A|N|G|A|S|K|V|T|K|D|V|F|K|N|I|G|T|S|E|T|P|E|A|V
PAvo      1 A|L|V|E|K|E|A|E|K|E|N|Y|F|F|V|E|D|Y|N|L|Y|A|G|L|K|T|O|A|K|E|P|F|E|V|N|K|E|A|V|E|T|A|L|P|I|T|I|T|----
VirB9     1 -----
Hrp       1 D|P|F|L|M|L|A|P|I|V|E|L|N|L|I|P|V|N|I|E|N|T|L|S|V|K|A|D|D|E|K|F|L|I|P|S|P|P|P|H|L|I|Y|D|S|T|Y|T|E
LipEr     1 -----
LipPr     1 -----

```

* * * * *

```

OutX      1 KNEIHYDQHTVAEMADATKFSFORDATNGGLRFENKFFENVEATDITFLKNSFENVI|F|E
PehX      1 -----VQDSTGSPAIETIGDSSKQ|W|S|Q|F|P|S|N|K|L|S|G|V|P|T|S|I|S|D|L|S|D|S|Q|F|N|L|T|F|E
PEcc1     1 VLNGEAKKPF|E|V|T|N|K|N|V|E|L|T|S|D|S|T|W|I|K|N|V|T|V|E|K|-----
PEcc2     1 VLNGEAKKPF|E|V|T|N|K|N|V|E|L|T|S|D|S|T|W|I|K|N|V|T|V|E|K|-----
PicA      1 A|T|S|P|I|V|S|H|P|S|A|V|A|T|P|P|I|N|A|D|V|P|H|N|N|L|V|F|E|G|A|V|V|D|D|P|A|L|L|D|A|P|N|I|S|D|V|P|----
PTom      1 K|F|C|D|E|N|F|P|C|E|I|N|E|I|N|L|V|-----G|G|K|P|E|A|T|C|N|N|F|H|A|N|N|V|N|P|C|T|S|I|I|S|E|D|E|
PPrim     1 K|L|V|C|K|E|F|P|C|N|G|E|L|A|D|I|L|T|Y|-----S|G|K|G|P|A|T|S|V|C|E|N|I|E|P|T|I|E|N|R|I|P|A|I|C|D|S|A|N|A|K|A|----
PPa       1 F|L|G|Y|K|A|N|K|N|K|P|L|I|T|L|D|N|V|V|F|D|L|P|A|F|E|G|N|Y|G|G|P|A|P|H|Q|V|I|T|F|N|Y|D|V|P|F|A|D|A|I|V
PMaize    1 S|L|L|C|T|A|K|V|P|C|T|V|T|H|D|V|W|V|E|Y|-----S|G|T|N|K|T|N|A|I|C|T|N|A|E|D|T|E|N|C|L|E|L|A|C|F|-----
PAvo      1 -----
VirB9     1 -----
Hrp       1 N|H|H|A|L|K|D|P|I|N|A|V|D|P|P|H|A|L|A|L|A|T|@|H|H|L|S|L|E|L|C|N|D|N|V|S|I|H|H|L|I|V|H|S|A|I|R
LipEr     1 -----
LipPr     1 -----

```

* * * * *

```

OutX      1 NVVRA---W|N|F|G|I|E|N|I|L|E|D|R|V|K|D|A|L|T|S|D|E|T|I|T|S|E|A|T|E|U
PehX      1 N|L|S|E|G|S|P|K|F|C|I|K|K|V|-----T|V|D|G|K|T|V|P|-----
PEcc1     1 -----
PEcc2     1 -----
PicA      1 -----
PTom      1 L|L|Y|N|Y|-----
PPrim     1 -----
PPa       1 T|S|T|D|V|T|V|T|G|T|P|G|T|A|A|V|D|C|K|A|F|V|L|E|V|A|P|T|S|P|I|-----
PMaize    1 -----
PAvo      1 -----
VirB9     1 -----
Hrp       1 N|A|-----
LipEr     1 -----
LipPr     1 -----

```

*

Key to sequences for figure 7.4.2c:

OutX, *Ecc*: this study; PehX, *Ech*: (He and Collmer 1990); PEcc1, *Ecc*: (Hinton *et al* 1990); PEcc2, *Ecc*: (Saarialhti *et al* 1990); PicA, *Ag. tumefaciens* (Rong *et al* 1991); PTom, Tomato (Grierson *et al* 1986); PPrim, Evening primrose (Brown *et al* 1990); PPs, *Ps. solanacearum* (Huang and Schell 1990); PMaize, Maize (Niogret *et al* 1991); PAvo, Avocado (McGarvey *et al* 1990); VirB9, *Ag. tumefaciens* (Rogowsky *et al* 1990); Hrp, *Ps. syringae* (Mukhopadhyay *et al* 1988); LipEr, *Erwinia amylovora* lipoprotein (Yamagata *et al* 1981); LipPr, *Proteus mirabilis* lipoprotein (Ching *et al* 1986).

is plant inducible: genes being expressed in the presence of carrot root extract.

Mutations in the region caused cells to aggregate into long 'ropes' in the presence of the plant extract, suggesting that the *picA* locus is involved in determining bacterial surface properties (Rong *et al* 1990).

A *picA::lacZ* fusion was used to study the induction by plant extracts. This resulted in the identification of a region of the *picA* locus which, when present in multi-copy, inhibited the induction of the *picA* promoter by carrot root extract (Rong *et al* 1991). A 3.155 Kb region of the *picA* locus was sequenced and found to include three ORFs (two complete and one partially sequenced), named *pgl*, ORF1 and ORF2, predicted to encode proteins as follows: *pgl*: 34 KDa, ORF1: 26 KDa and ORF2: > 21 KDa (all approx.). No homologues of the proteins encoded by ORF1 and ORF2 were identified, but the apparent negative regulator, Pgl, was found to be homologous to various polygalacturonases (Rong *et al* 1991).

It is interesting to note the organization of the *picA* locus, with a *peh* gene, followed by at least two other genes, all transcribed in the same direction and thought likely to be part of an operon (Rong *et al* 1991). Because of the similar organization of the upstream region of the *Ecc ou* cluster, the proteins encoded by *picA* ORF1 and ORF2 were aligned with the Out and Pul, C and D proteins, as shown in figures 7.4.2d and 7.4.2e.

Although the homologies are low, other evidence suggests a link between the *picA* and *our* gene clusters. Both have the same general organization, and a gene encoding Peh in a region thought to be involved in regulation. *PicA* ORF1 is believed to affect cell surface properties, while the ORF2 protein is believed to be membrane-associated, as is OutD.

Of particular interest, is the inducibility of the *picA* locus in the presence of plant extract, and the fact that strains with a mutation in this region showed altered virulence: being more virulent under inducing conditions than the wild-type (Rong *et al* 1991). The fact that induced mutants aggregated into 'ropes' led to the proposal that they were

FIGURE 7.4.2d

SEQUENCE ALIGNMENT OF THE PROTEIN ENCODED BY *picA*(ORF1),
WITH OUTC AND PULC.

Gap penalty = 4 Cut-off = 10

Variable gap penalty = F Window = 10 Filtering = 2.5

* -> Exact match across all seqs.; . -> Conservative matches across all.

```

OutC      1  MARIQAFKDFHSLVATFRSLPIIBFVYGLILLI|COQLAVLTMRFLLPDSRI|GVGS
PulC      1  HSEGIKHNHSMRLTIPNKKI|INYPHIVTSL|LFF|COOLAQLTKI|LPMVFTDHALS
PicA(ORF1) 1  -----NGLPFQIEYGQTTQRP|IEDDALRQFS
                                     . . . * . * . *
    
```

```

OutC      1  ---VTPADAKEKPATPCDFTLFGH-----APQDASTVNDALSGD|---PLTSL
PulC      1  SAQTSIPAAPSAETALPRFTLFDL-----AEETBAS-----APGHLGDGAPVSAI
PicA(ORF1) 1  AALALATADAGGLVYHGYDSRHWQWAMPASGKSPA|MARAVGMAAMALVDALVILPDCSA
                                     * . . . * . * . *
    
```

```

OutC      1  HSLTGVLAGDAKRS|A|IAKDSQOYSRNVGDA|PGYEA|VT|SADRIVVLQYGGYEA
PulC      1  BLVITGLASTDPERA|A|MKCGRQOVELG|QDSTPGGEAK|I|A|SPDRI|VNYRGRNEA
PicA(ORF1) 1  ----TAE|BERTRRLLACI|IARDQAGLMMVLDNQG|LGHYAE|TSA|SANFAYAL|LAAR
                                     * * * . * * * . * . *
    
```

```

OutC      1  LNLVQEEA|T|CAPSSSGAFHGVKDE|QKDPFSAQD|YLT|SPVTEEEVLKGYQLHPGKNPD
PulC      1  IRLFMPPAVCKE|EAAPPARN|LQELRAAPQH|LHYLN|SPVNVNKL|ECVRLHPGQDPA
PicA(ORF1) 1  LGLLRGEEAKAAL|SACRQALAA|LETR-----LELDGGVARI|TG
                                     * * * . * . * . *
    
```

```

OutC      1  LFFRAELGDHGLAVSLNGHQLRDADGAGGAMAGLGHSEKFL|TVERDGGQGD|IYLDAGD
PulC      1  LFRGGSLRRLA|A|LWGLD|DDEKAGQVLA|PEL|TE|LTVSRDGGKMD|IYLRDDE
PicA(ORF1) 1  |VHVAGLGGFDG-----HYRQTPQTYL|TEPVVSDAKQVGLNMA|YAESL|LAR---
                                     . * * * . * * . * . *
    
```

```

OutC      1  NU
PulC      1  U-
PicA(ORF1) 1  --
    
```

References.

- OutC: this study.
- PulC: d'Enfert *et al* (1989).
- PicA(ORF1): Rong *et al* (1991).

FIGURE 7.4.2e

SEQUENCE ALIGNMENT OF THE PROTEIN ENCODED BY
picA(ORF2), WITH OUTD AND PULD.

Gap penalty = 4 Cut-off = 10

Variable gap penalty = F Window = 10 Filtering = 2.5

* :> Exact match across all seqs.; . :> Conservative matches across all.

```

OutD (del)  :  -----NLLLSGSVLLMASLAWA---RFRASPECTDIDDFINTVSKLNKTVIIDPSV
PulD (del)  :  LIIAHVIRFSLTLLFAALLPSPAARFRFRASFKGTDIDDFINTVSKLNKTVIIDPSV
PicA(ORF2)  :  -----NSQSPFRFILLDDGIRGVAALFIVHRHAQFFGRDPAS
                *      *      *      *      *
                *      *      *      *      *

OutD (del)  :  E-----GTITVRSYDNHMEEGYGFLEVLVDVYGFVIMH
PulD (del)  :  R-----GTITVRSYDNHMEEGYGFLEVLVDVYGFVIMH
PicA(ORF2)  :  EYLAVDLFPALSGFVLAHAYGCKLYEGITTPGFLEKARFARLYPLTLALALMAAYFICL
                ****      *      *      *      *
                *      *      *      *      *

OutD (del)  :  DRNVLKIISSKRAKSTHPLATDQPGIDDEVVTVVPPVHVVAARDGQSSR-VEGRVA
PulD (del)  :  HNCVLEVRBEDAKTAAVVYASDAAPCIDDEVVTVVPLTNVAARDLAPLLRGLMDNAGV
PicA(ORF2)  :  YVGLG-----PTPIDDLNRLSDPGELAFALVTGLLFLPAPFTLT-
                *      *      *      *      *

OutD (del)  :  MDVMLBTCERRRDOOPRGVIAVHTI|VERVDQDGDNRVTTIPLSYASSTEVKCR
PulD (del)  :  GSVVHYEPSNVLMTGRAAVIKRLTI|VERVDNAGDRSVTVPLS-----
PicA(ORF2)  :  --LIGALFLVSPAMSLFHELVVNAVYARWGRATNKQTVLVAVSAYVNVAAAEF
                .      .      .      .      *
                .      .      .      .      *
    
```

Only the N-terminal regions of OutD and PulD are given, since the sequence of the C-terminal region of picA(ORF2) has not yet been published.

References.

OutD: Reeves *et al* (in press).

PulD: d'Enfert *et al* (1989).

PicA(ORF2): Rong *et al* (1991).

more adherent: binding to each other, and perhaps to plant cell walls more efficiently than wild-type cells.

The role of Peh in the regulation of the *picA* cluster is not yet fully understood. The *A. tumefaciens* strain used in the analysis of *picA* cannot use PGA as a sole carbon source and does not secrete an active Peh, although the presence of intracellular Peh activity has not been investigated. There is evidence to suggest that the inducing molecule in carrot root extract is an acidic polysaccharide related to PGA, effective at such low concentrations (1-10 μM) as to suggest that it is a true inducer molecule, and not simply a metabolite (Rong *et al* 1991). PGA itself can induce *picA*, but the degree of polymerization of the molecule is important, with chains of 6-16 subunits being the most effective (Rong *et al* 1990). It was proposed that over-expression of *pgl* on a multi-copy plasmid, might cause PGA to be rapidly degraded into polymers too small to induce *picA* (Rong *et al* 1991). This hypothesis could be extended to propose that Peh could cleave PGA to produce molecules of the optimum length, and so cause induction of *picA*. These theories obviously depend on whether the Peh is found to be active. There is also the question of how large PGA polymers could enter the cell, to be broken down by intracellular Peh. Other enzymes could be involved in this step.

The presence of a *pgl* gene at a plant inducible locus of *A. tumefaciens* might help to explain the *peh* gene found in the upstream region of the *ouX* cluster. Exo-Peh, believed to be the product of *ouX*, cleaves PGA to yield dimers (DGA) which are thought to induce Pel synthesis (Tsuyumu 1977, Collmer and Bateman 1981, Chatterjee *et al* 1985, Condemine *et al* 1986). Although the work described earlier (5.4), attempting to determine the effect of DGA on the expression of *ouX* genes was inconclusive, it seemed possible that OutX is involved in regulation. It was necessary to obtain further information on the *ouX* product.

7.5 *IN VIVO* EXPRESSION OF *OUTX*.

7.5.1 EXPRESSION OF *OUTX* USING THE T7 RNA POLYMERASE/PROMOTER SYSTEM.

The principles of the T7 RNA polymerase/ promoter system have been described previously (6.4).

The 3.5 Kb *EcoRV* fragment containing *ouX* was isolated from pSW2 by digestion, followed by agarose gel electrophoresis and trough elution. The fragment was ligated into *SmaI*-cut, phosphatased pT7-5 and transformed into DH1. Plasmid DNA was extracted from some of the transformants. Restriction analysis identified a construct carrying the 3.5 Kb *EcoRV* fragment in the correct orientation relative to the T7 RNA polymerase promoter. This plasmid, named pT7-5SW200 is shown in figure 7.5.1a.

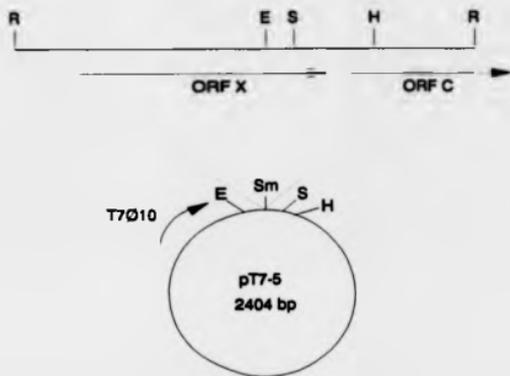
K38(pGP1-2, pT7-5SW200) was generated by electroporation, plating on to NBA Kn Ap. Single colonies were used for expression experiments, as described previously (2.22, 6.4). Control experiments were performed, looking at protein production in the absence of an insert in pT7-5, in the absence of Rif, and without induction (no heat shock). The resulting autoradiograph is shown in figure 7.5.1b.

The sample treated with Rif produced a surprising number of proteins. The experiment was repeated, using an additional culture: K38(pGP1-2, pT7-5SW100) as a control. The result (data not shown), suggested that the expression system was working efficiently and that the multiple bands were caused by pT7-5SW200. Further experiments were carried out to confirm this.

Plasmid DNA was extracted from a single colony of K38(pGP1-2, pT7-5SW200). The DNA was digested with *HindIII* and analysed by agarose gel electrophoresis, alongside *HindIII* digests of pGP1-2 and pT7-5. Comparison of the digests suggested that the correct plasmids had been present in the strain used for expression. The extracted DNA was re-introduced into K38. When the expression experiment was repeated, multiple protein products were observed as before (data not shown).

Figure 7.5.1a

pT7-5SW200

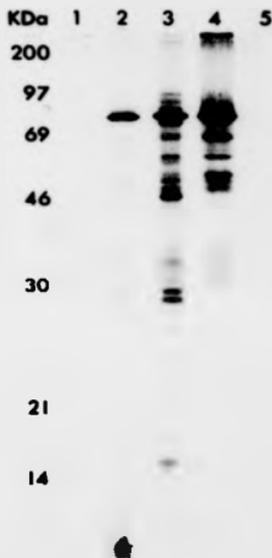


Vector and insert DNA not drawn to scale

FIGURE 7.5.1b

EXPRESSION OF *OUTX* USING THE T7 RNA
POLYMERASE/PROMOTER SYSTEM.

The photograph shows an autoradiograph (24 hr exposure), giving the result of an experiment to express *oufX*, using the construct pT7-5SW200.



KEY TO TRACKS: construct and growth conditions used

- 1 Molecular weight standards (sizes given in kDa)
- 2 pT7-5SW200, uninduced, + Rif
- 3 pT7-5SW200, induced, - Rif
- 4 pT7-5SW200, induced, + Rif
- 5 pT7-5, induced, + Rif

The cloning event which generated pT7-5SW200 was extremely rare: over 100 colonies had to be checked before the construct was isolated. This was consistent with previous findings that DNA fragments from the upstream region of the *ouX* cluster were difficult to clone (6.3, 7.2). It was possible that a mutation in the vector had allowed the creation of pT7-5SW200, and that this was causing the unusual expression results. To test this hypothesis, the 3.5 Kb fragment had to be removed from pT7-5SW200. The *EcoRV* sites had been destroyed by cloning into the pT7-5 *SmaI* site. *PstI* and *SacI* cut (at unique sites) in the pT7-5 polylinker, on opposite sides of the *SmaI* site. Once restriction analysis had proved that neither of these enzymes cut within the 3.5 Kb *EcoRV* fragment, they were used to digest pT7-5SW200. The DNA was end-repaired (2.21.1.1), self-ligated and transformed into DH1, plating on to NBA Ap. Extraction of DNA from transformant colonies, followed by restriction analysis revealed that the 3.5 Kb insert had been lost. The resulting plasmid, named pT7-5SW0, only differed from pT7-5 in that it had lost a portion (30 bp) of the polylinker. When K38(pGP1-2, pT7-5SW0) was used in an expression experiment (with the addition of Rif), no bands were seen. This proved that the multiple protein products observed previously were due to the insert in pT7-5SW200.

There were several possible reasons for the unusual results seen when *ouX* was expressed using the T7 RNA polymerase/ promoter system. These included a change in the sensitivity of the cells to Rif, and the degradation of the *ouX* product. The experiments used to test these theories are described below.

7.5.2 RIFAMPICIN, AND *OUTX* EXPRESSION.

It seemed possible that the over-expression of *ouX*, particularly if the product was exported, brought about a change in the permeability of the host cells to Rif. Since the inhibition of DNA-dependent RNA polymerase is proportional to the amount of Rif in the cell (Wehrli *et al* 1968b), this could cause partial Rif resistance. It was thought that the use of a higher concentration of Rif, might allow more of the antibiotic to enter the cells.

TABLE 7.5.2a

TREATMENTS TO DETERMINE THE EFFECT OF RIFAMPICIN
ON CELL GROWTH

CULTURE	GROWTH TEMP. (°C)	RIFAMPICIN	TIME RIF. ADDED (MIN)
1	30	-	NA
2	30	+	0
3	30	+	30
4	42	-	NA
5	42	+	0
6	42	+	30

Identical cultures were incubated under noninducing, or inducing conditions (30°C and 42°C respectively), in the presence and absence of Rifampicin (Rif). The Rif was added either at the start of incubation, or after 30 min of growth.

NA = not applicable

Expression experiments on K38(pGP1-2, pT7-5SW200) were set up, adding varying amounts of Rif, to give a final concentration of up to 800 $\mu\text{g/ml}$ (four times the usual amount). Increasing the level of extracellular Rif had no effect on the products of the expression experiment (data not shown).

If the permeability of K38 had been altered by pT7-5SW200, its sensitivity to a range of antibiotics might have been affected. To test this, K38(pGP1-2, pT7-5SW200) and K38(pGP1-2, pT7-5SW100) were used to make top lawns on iso-sensitest agar (Oxoid), Kn Ap plates, and multi-test antibiotic discs (Mastrings: Mast Labs) were applied. Plates were set up in duplicate and incubated at either 30°C or 42°C (non-inducing or inducing conditions). Neither the plasmid present, nor the growth temperature affected the antibiotic sensitivities of K38. Interestingly, both strains were sensitive to Rif.

To see whether Rif sensitivity was dependent on culture conditions, liquid cultures of K38(pGP1-2, pT7-5SW200) were studied. Six cultures were set up (by diluting an overnight culture 1:40) and treated as set out in table 7.5.2a. In each case, Rif was added to 200 $\mu\text{g/ml}$. The OD of each culture was followed, taking readings (600 nm, Corning spectrophotometer 258) over a period of 6 hr (approx.) Graphs were plotted of bacterial densities against time (data not shown), which proved that K38(pGP1-2, pT7-5SW200) was inhibited by Rif in liquid culture, under all the conditions tested.

7.5.3 PULSE-CHASE LABELLING OF OUTX.

It was proposed that OutX gave rise to the intense band on the autoradiographs (see figure 7.5.1b), corresponding to a protein of 77 KDa (approx.). If the protein was highly unstable, the other bands might be due to degradation products. Pulse-chase labelling was used in an attempt to determine whether degradation occurred.

The experimental procedure has been described previously (2.22.2). Cultures were pulsed with ^{35}S -Met for 1 min, then chased with cold Met for 0, 5, 15 or 60 min. The resulting autoradiograph showed no signs of a gradual breakdown of the 77 KDa

protein; the relative intensities of the different bands being unaffected by chase time (data not shown).

Pulse-chase labelling of the products of K38(pGP1-2, pT7-5SW200) did not explain the background bands seen after T7 RNA polymerase/ promoter expression. Either the bands were not due to the breakdown of OutX, or the degradation was so rapid, that it was completed by the end of the 1 min pulse step.

7.5.4 EXPRESSION OF DELETION DERIVATIVES OF *OUTX*.

Various deletions were made in pT7-5SW200, as shown in figure 7.5.4a. The constructs: pT7-5SW201, pT7-5SW202 and pT7-5SW203 were obtained by digestion of pT7-5SW200 with the required enzyme, followed by religation. The DNA was transformed into DH1, plating on to NBA Ap, and single colonies were used for plasmid DNA extractions. The identities of the constructs were confirmed by restriction analysis.

A series of expression experiments was carried out, using pT7-5SW200, its three deletion derivatives and pT7-5SW0: each in the presence and absence of Rif. The resulting autoradiograph is shown in figure 7.5.4b.

The *Hind*III deletion (pT7-5SW201) had no effect on the background bands in the presence of Rif, confirming that these were caused by a product of the DNA lying upstream of *owC*. The *Sal*I deletion (pT7-5SW202) resulted in the loss of the intense band corresponding to a protein of 77 KDa. Instead, a band was seen at 60 KDa (approx.). This shift was presumed to be due to the truncation of OutX, and suggested that the 77 KDa protein seen previously was the *owX* gene product. pT7-5SW202 still produced background bands, although the banding pattern differed from that seen with pT7-5SW200. The *Eco*RI deletion removed the N-terminal portion of *owX* from pT7-5SW203. Expression of this did not yield any of the products seen with the other plasmids.

Figure 7.5.4a

Deletion Derivatives of pT7-5SW200

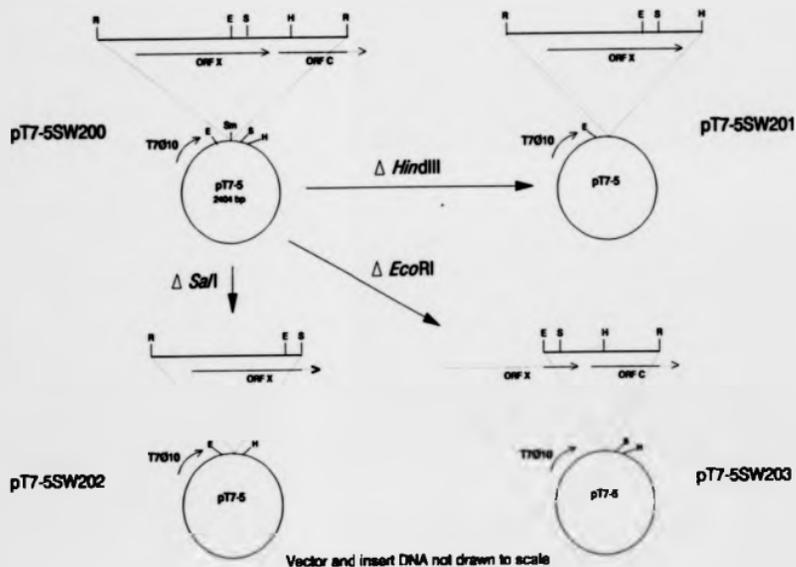
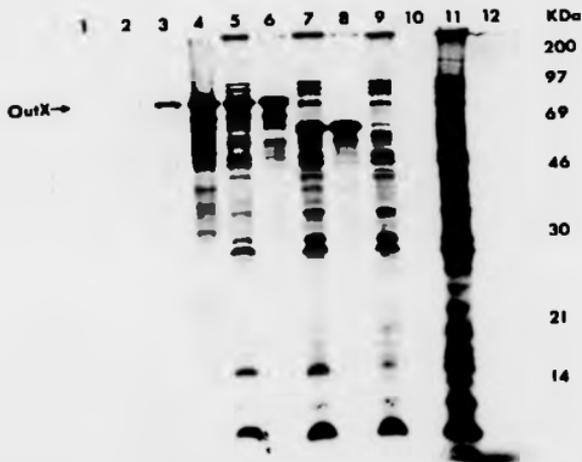


FIGURE 7.5.4b

T7 RNA POLYMERASE/PROMOTER EXPRESSION OF
DELETION DERIVATIVES OF *OUTX*.

The photograph shows an autoradiograph (24 hr exposure), giving the results of an experiment to express *outX* and partial deletions of this, using derivatives of the construct pT7-5SW200.



KEY TO TRACKS: construct and growth conditions used.

- | | | | |
|---|------------------------------|----|----------------------------|
| 1 | MW standards | 7 | pT7-5SW202, induced, - Rif |
| 2 | pT7-5SW200, uninduced, + Rif | 8 | pT7-5SW202, induced, + Rif |
| 3 | pT7-5SW200, induced, - Rif | 9 | pT7-5SW203, induced, - Rif |
| 4 | pT7-5SW200, induced, + Rif | 10 | pT7-5SW203, induced, + Rif |
| 5 | pT7-5SW201, induced, - Rif | 11 | pT7-5SW0, induced, - Rif |
| 6 | pT7-5SW201, induced, + Rif | 12 | pT7-5SW0, induced, + Rif |

7.5.5 CONCLUSIONS/ DISCUSSION.

The plasmid pT7-5SW200 was constructed and used for T7 RNA polymerase/promoter experiments for the expression of *outX*. A protein of 77 KDa (approx.) was identified, in reasonable agreement with the 72 KDa predicted from the DNA sequence analysis (7.4.1). Gene expression of the *SaII* deletion plasmid: pT7-5SW202 produced a protein of 60 KDa (approx.), compared to 53 KDa predicted for the OutX truncate from the sequence data. The predicted and observed sizes of the proteins varied, probably due to inaccuracies of sizing such large proteins by SDS-PAGE. However, the shift in size caused by the deletion confirmed the identity of the *outX* product.

In addition to OutX, a large number of other protein bands were observed, although the cause of these was not clear. One possibility was that K38(pGP1-2, pT7-5SW200) was resistant to Rif, although various experiments showed that the strain was unable to grow in its presence. Rif acts on a cell in two distinct ways. In addition to its inhibitory effect on DNA-dependent RNA polymerase, it acts as a detergent: disrupting membranes. It might therefore be possible for the antibiotic to prevent cell growth without abolishing protein synthesis. In fact, it seems that Rif not only entered the cells, but affected protein synthesis. This can be seen most clearly in the autoradiograph shown in figure 7.5.4b, in which the patterns of protein bands are different, depending on whether or not Rif was used.

Another theory was that OutX degraded. Although this could not be proved by pulse-chase labelling, the background proteins were always smaller than the major product: most obvious in the pT7-5SW202 tracks of figure 7.5.4b. The addition of Rif eliminated all the bands running above that corresponding to the *outX* product, leaving those below it. Further support for the degradation theory comes from the fact that while pT7-5SW200 and pT7-5SW201 (both containing the entire *outX* gene) gave rise to similar background bands, the pattern was different with pT7-5SW202. This was believed to be because degradation of the OutX truncate yielded different products.

7.6 IN VITRO EXPRESSION OF *OUTX*.

As mentioned earlier (6.4.1), previous attempts to express genes of the *out* cluster using the Zubay system had failed. This was thought to be due to the lack of a promoter on the cloned DNA fragments. Since an apparent promoter region had been identified, upstream of *outX* (7.4.1), it seemed possible that this gene could be expressed *in vitro*.

The protocol used for bacterial cell-free coupled transcription-translation (de Vries and Zubay 1967, Zubay 1973, Collins 1979) has been described previously (2.22.3). The system was used to analyse the protein products of pSW2 (work performed by V. Mulholland), using pBR322 as a control. The ³⁵S-Met labelled proteins were run alongside those produced by the T7 RNA polymerase/ promoter expression of genes on pT7-5SW200 and the control plasmid pT7-5 (7.5.1). The resulting autoradiograph is shown in figure 7.6.a.

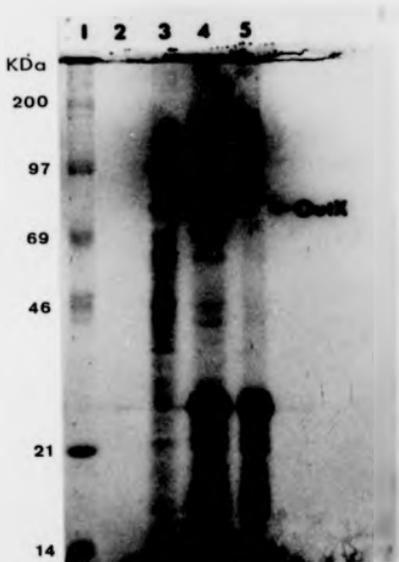
The Zubay expression experiment resulted in the production of various proteins, including those involved in resistance to Ap and Tc, encoded by pBR322. A protein of 60 KDa (approx.) was observed, which was frequently seen after Zubay expression experiments using various plasmids (data not shown) and presumed to be an artifact of the system. There were two protein bands unique to pSW2, corresponding to proteins of 77 KDa and 67 KDa (approx.). The 77 KDa protein produced by the Zubay system co-migrated with that produced by the T7 RNA polymerase/ promoter expression of pT7-5SW200, confirming that this was the *outX* product. This was the first time that an *Ecc out* gene had been successfully expressed using the Zubay system. Since the orientation of the insert in pSW2 ruled out the possibility of read-through from a vector promoter, this result confirmed the predictions from the DNA sequence analysis (7.4.1) that the DNA fragment from the upstream region of the *out* cluster, cloned in pSW2, included an *out* gene promoter.

Although the 67 KDa protein (also seen in the pT7-5SW200 track) was close to the size predicted for mature OutX (69 KDa following cleavage after residue 24), it could

FIGURE 7.6a

IN VIVO AND *IN VITRO* EXPRESSION OF *OUTX*.

The photograph shows an autoradiograph (36 hr exposure), allowing a comparison to be made between the results of T7 RNA polymerase/promoter and Zubay experiments for the expression of *OUTX*.



KEY TO TRACKS: construct and expression protocol used.

- 1 MW standards (sizes given in kDa)
- 2 pT7-5: *in vivo* expression
- 3 pT7-5SW200: *in vivo* expression
- 4 pSW2: *in vitro* expression
- 5 pBR322: *in vitro* expression

not have been produced by post-translational processing of the 77 KDa protein. This is because leader peptidases, which require membranes for their activity (1.5.2.5) cannot function in the Zubay, cell-free system. The second band was most likely to be due to proteolytic cleavage.

The multiple protein products, seen after the T7 RNA polymerase/ promoter expression of pT7-5SW200 were not seen when *outX* was expressed by the Zubay system. Since the favoured explanation for these bands was the rapid degradation of OutX (7.5.5), it seems that this protein was more stable when produced by the *in vitro*, as opposed to the *in vivo* method. It is possible that there was lower protease activity in the S-30, cell-free extract, than in the K38 host of the *in vivo* system. It is however possible, that the different expression protocols did not affect OutX stability, but that the low level of protein expression *in vitro*, relative to that driven by the strong T7 RNA polymerase promoter, meant that background bands could not be seen, even after longer exposure times.

7.7 CONCLUSIONS/ FINAL DISCUSSION.

OutX was sequenced and found to encode a protein of 72 KDa (approx.), in agreement with the results of both *in vivo* and *in vitro* protein expression experiments. The expression of *outX* by the Zubay system confirmed that there was a promoter upstream of *outX*, as predicted from the sequence data. It is not reliable to locate a promoter, simply by looking for the best match with the consensus sequence. Experiments such as S1 nuclease mapping and primer extension studies would have been useful to confirm the start sites and lengths of transcripts from the *ou* cluster, but such experiments were beyond the scope of this project.

The predicted sequence of OutX suggested that this (unlike other Peh proteins studied, or the Pel enzymes of *Erwinia*), was a lipoprotein. The amino acid residue immediately following the N-terminal glyceride Cys of a mature lipoprotein, determines whether this is targeted to the inner or outer membrane (Yamaguchi *et al*

1988). OutX, having a Ser residue at position +2, should be transported to the outer membrane, while Pula, with an Asp residue, would remain on the inner membrane, if it was not for the specific secretion factors encoded by the *pul* cluster (Yamaguchi *et al* 1988).

Homology studies and enzyme assays suggested that OutX is an exo-Peh. The protein is homologous to various Peh enzymes including one, encoded by a plant inducible locus of *Agrobacterium tumefaciens*, thought to be involved in regulation.

It is proposed that OutX is a lipoprotein: modified, then processed by signal peptidase II and transported to the outer membrane (independent of the Out apparatus, due to its lipoprotein outer membrane target sequence), where it remains due to the extreme hydrophobicity of its fatty acyl groups (unless there is a mechanism for its release, as for Pula).

Outer membrane lipoproteins are used by Gram-negative bacteria (especially pathogenic species) to interact with the cells' surroundings: recognising factors produced by eukaryotic cells. The best characterized example is the TraT lipoprotein (reviewed by Sukupolvi and O'Connor 1990). It is proposed that the exo-Peh protein OutX, breaks down PGA from plant cell walls, releasing DGA which then induces Pel synthesis. It would be advantageous for such a signalling protein to remain attached to the cell wall: ensuring that the cell and substrate were in close proximity. This would raise the effective concentration of substrate and allow the rapid entry of inducing molecules into the cell.

If this theory was correct, the expression of *outX* and the other *out* genes might be affected by DGA along with the *pel* structural genes. It seems likely that *outX* would be constitutively expressed at a low level, which might explain the apparent induction of Pel synthesis by PGA in Out⁻ mutants (reported previously: 5.4). The role of Peh in induction could have been confirmed by the use of EDTA. Previous reports suggest that in *Ech*, induction occurs in the presence of EDTA, due to exo-Peh (Collmer *et al* 1982), while in *Ecc*, induction takes longer, although it does occur (Tsuyumu 1977). However, EDTA inhibits *Ech pel* induction by plant cell walls (Collmer *et al* 1982),

suggesting that a basal level of Pel is required to facilitate Peh activity. In the presence of PGA, as cells grew faster, elongation would lead to an increased requirement for exo-Peh and Out proteins, suggesting that OutX might be induced by its own products. This induction might cause the stem loop structure down stream of *outX* to melt, allowing transcription of the rest of the *ouu* cluster. The induction of *outX* could have been tested by assaying for Peh activity (7.3.1) in PM-grown cultures.

The model highlights a possible difference between the *ouu* cluster of *Ecc* and the clusters encoding other secretory apparatus. This difference at the regulatory level, might explain why the gene clusters are not functionally interchangeable (1.7.2, 1.9.12).

Although the proposed model fits existing evidence, further confirmation is required. There are various ways of proving that OutX is a lipoprotein. Signal peptidase II is inhibited by globomycin. This antibiotic is thought to be a structural analog of the lipid-containing prolipoprotein (due to its hydrophobicity and structural similarity to the lipoprotein signal peptide). Alternatively, globomycin may bind to lipids and hence interfere with processing by the signal peptidase (Tokunaga *et al* 1982). Treatment with globomycin causes the accumulation of glyceride-modified, unprocessed prolipoproteins. This might be detected by autoradiography, following *in vivo* expression in the presence of globomycin, although the background bands (discussed above) might cause problems. Other possible experiments include site-specific mutagenesis of the cleavage site (Inouye *et al* 1983b), or the use of a strain with a temperature sensitive mutation in the structural gene for signal peptidase II (Yamagata *et al* 1982), although the latter experiment has proved most effective with the major outer membrane lipoprotein. In addition to showing whether OutX is processed by signal peptidase II, it would be possible to determine whether the protein was fatty acylated, by performing expression experiments (as described previously: 7.5, 7.6), labelling with ^3H -glycerol or palmitate (Tokunaga *et al* 1982).

The translocation of OutX to the outer membrane could be confirmed using OutX::PhoA hybrids (in a PhoA⁻ strain). If PhoA was exported, blue colonies would be obtained on XP plates. In addition, cell fractions could be immunoblotted, using

anti-PhoA antibodies. Fusion data should be interpreted with caution, since the conformation of the protein would be altered. Since the protein conformation (determined by the signal peptide) is thought to be crucial for recognition by signal peptidase II (Inouye *et al* 1983a), some fusions might not be processed in the normal way (Ghrayeb *et al* 1985).

If the transportation of OutX to the outer membrane was established, it would be necessary to determine whether the protein remained membrane-associated. This would be possible by radiolabelling (^{35}S -Met), followed by cell fractionation and SDS-PAGE. As OutX is probably normally produced in small amounts, a T7 RNA polymerase/ promoter expression experiment could be used, in an *Ecc* host to ensure normal processing and targeting. This experiment would depend on the lipoprotein processing and targeting systems being able to cope with the over-expression of *outX*. It is possible to distinguish between lipoproteins bound to the inner and outer membranes, by treating the membrane fraction with sodium sarcosinate which specifically solubilizes the cytoplasmic membrane (Inouye *et al* 1983a). Alternatively, the membranes could be separated by sucrose density gradient centrifugation.

Although circumstantial evidence suggests that *outX* really is part of the *out* cluster, this remains to be proved. Some mutants such as RJP208, not fully complemented by cHIL208, were complemented by cHIL208/5, indicating that genes upstream of *outC* were required. This evidence is supported by the apparent absence of a consensus promoter sequence between *outC* and *outX*, and the existence of one upstream of *outX*. Complementation analysis is currently being performed to see whether a plasmid carrying only *outX* complements any of the Out^- mutants (Housby). If OutX is involved in *out* gene regulation via the stem loop, complementation might not be achieved *in trans*. However, if the proposed mode of action of OutX is correct, catalytic activity in the presence of PGA would release DGA and might allow induction to occur, depending on the type of mutation in *outX*. A mutation in *outX* might also be overcome by the addition of DGA. It is unfortunate that the experiments looking at regulation of the *out* cluster in the presence of DGA, using PhoA fusions (5.4) were inconclusive. It

might also be interesting to study the effect of a mutation in the stem loop region (generated by site-specific mutagenesis) on the expression of the downstream *ou* genes.

Evidence for the role of DGA (via *ouX*) in the regulation of the *ou* cluster might be obtained by analysing the mRNA produced in the presence and absence of the putative inducer. Experiments might include primer extensions and S1 nuclease mapping (as used in the study of transcriptional regulation of the *A. tumefaciens picA* locus (Rong *et al* 1991).

Although many more experiments are needed before any firm conclusions can be reached, the discovery that *ouX* appears to encode an outer membrane exo-Peh provides an appealing model for the regulation of extracellular enzyme synthesis and secretion by *Ecc* in response to plant-derived molecules.

CHAPTER EIGHT.

FINAL DISCUSSION / CONCLUSIONS.

8.1 INTRODUCTION.

At the start of this project, it was known that the soft-rot erwinias secrete enzymes such as Pel and Cel (major pathogenicity determinants) into their surroundings, and that this secretion is required for pathogenicity. It was proposed that secretion occurs via the two-step pathway, since *Out*⁻ mutants had been isolated which accumulated Pel and Cel in the periplasm, and a periplasmic intermediate of Pel had been identified in wild-type *Ech*. It was of particular interest to discover how the second step of secretion (across the outer membrane) occurs. Cosmids had been obtained, which complemented *Out*⁻ mutants, and so were thought to carry the *out* genes required for secretion across the outer membrane of *Erwinia* spp.

The initial aim of this project was to characterize the synthesis and secretion of Pel by wild-type *Ecc* (HC131), to allow comparisons to be made with *Out*⁻ strains. In particular, it was hoped to find evidence of a periplasmic build-up of Pel early in the growth phase, which would support the two-step secretion model. The rest of the project aimed to characterize the *out* cosmids, in particular looking for a regulatory region.

The major findings of the work are summarized below.

8.2 MAJOR FINDINGS OF THE WORK.

1 Technical problems meant that it was difficult to gain reliable information on the sub-cellular localization of enzyme activity for samples taken early in the growth phase. The results showed that cell fractionation and enzyme assay data is most reliable for stationary phase cells. However, it was found that the activity of Pel in *Ecc* throughout the growth cycle differs from that reported in *Ech*. The synthesis of Pel by *Ecc* was not obviously growth-phase dependent, and no evidence was found for a transient build-up of the enzyme within the periplasm (chapter 3).

- 2 Restriction mapping and hybridization analysis revealed that various *Ecc out* cosmids all carried inserts from the same region of the chromosome: supporting the theory that there is a single cluster of *out* genes in *Ecc* (chapter 4).
- 3 cHIL208 was found to contain all the DNA carried by other *out* cosmids, and yet it failed to fully complement any *Out*⁻ mutants. This was thought to imply that the cosmid encoded a regulatory protein (chapter 4).
- 4 Transposon (*TnlacZ* and *TnphoA*) mutagenesis created inserts in the *out* gene cluster, although this did not lead to the identification of a regulatory region (chapter 5).
- 5 It was confirmed that Pel synthesis in *Ecc* is switched off at 37°C, and that induction occurs in the presence of PGA and DGA. The inducers still had some effect at 37°C, suggesting that the temperature switch is not absolute (chapter 5).
- 6 Chromosomal gene fusions (*TnphoA*) in *outD* and *outG* were used to study *out* gene expression. It was found that the *out* genes studied were not co-regulated with the *pel* structural genes: not being switched off at 37°C. There was some evidence to suggest that *out* gene expression may be affected by PGA and DGA, and that the *out* genes may be differentially regulated (chapter 5).
- 7 DNA from the upstream region of the *out* cluster was sequenced. The two genes identified: *outC* and *outX*, were expressed, and their protein products were identified by SDS-PAGE (chapters 6 and 7).
- 8 Sequence analysis showed that *OutC* (predicted to be a trans-inner membrane, but predominantly periplasmic-protein), is homologous to *PulC*: a protein involved in the secretion of pullulanase by *Klebsiella* sp. (chapter 6).

9 A palindromic sequence was identified between *oucC* and *outX*: predicted to form a stem loop structure in the mRNA transcript. This might account for the inability of CHIL208 to fully complement *Out⁻* mutants (chapter 6).

10 *OutX* is predicted to encode an exo-Peh enzyme, although it had previously been thought that only the endo-form of Peh is produced by *Ecc* (chapter 7).

11 A consensus promoter sequence was identified upstream of *outX*, suggesting that the start of the *out* operon has been found (chapter 7).

12 Sequence analysis suggested that *OutX* is a lipoprotein. The enzyme is likely to be transported out of the cell by the lipoprotein targeting mechanism (independent of the *Out* apparatus), then anchored to the outer membrane. It is proposed that the membrane-bound exo-Peh is involved in the regulation of *Pel* synthesis (and possibly *out* gene expression), by releasing DGA from pectate (chapter 7).

8.3 FINAL COMMENTS.

During the course of this project, a better understanding of the *Ecc* secretory apparatus has been reached. The *out* genes were found to encode proteins belonging to a family of 'traffic wardens' which are involved in transporting a diverse range of macromolecules across the Gram negative cell envelope. Hence, the *Ecc Out* system may represent the major route out of the Gram negative cell.

This project identified a point at which the *Ecc out* cluster differs from the other related clusters identified so far. The proposal that the first gene of the *Ecc out* putative operon encodes a membrane-bound exo-Peh provides a model for the regulation of *pel* and *out* gene expression, and may also account, at least in part, for the species-specificity of traffic warden systems.

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