Development and application of molecular genetic techniques in *Erwinia carotovora* subsp. *carotovora*

by

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Doctor of Philosophy

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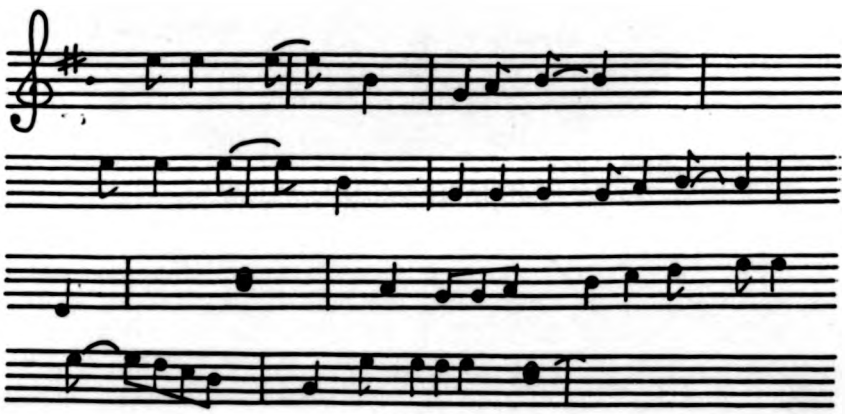
For Deborah, without whom the story
would have been very different

If you're lonely, I will call
If you're poorly, I will send poetry
I love you
I am the milkman of human kindness
I will leave an extra pint

Billy Bragg



Contents

Summary

Erwinia carotovora subsp. *carotovora* (*Ecc*) is an important bacterial phytopathogen, and a member of the group of "soft rot erwinias". Methods of molecular genetic analysis were applied to *Ecc* strain SCRI193 to enable the future study of the process of extracellular enzyme production.

Following work with a number of transformation sytems, the "Hanahan" procedure proved applicable to SCRI193, and was used to obtain up to 4×10^4 transformants per μg of pBR322 DNA. The method was optimised and used for the direct introduction of *in vitro*-manipulated plasmids to *Ecc*.

Two Tn*5* mutagenesis systems were developed for SCRI193. First, the pJB4JI system was used to obtain a variety of mutants, including HC500, which had a Cys^-, Pel^-, Cel^-, Prt^- phenotype on assay media. Second, a pBR322::*lamB*$^+$ plasmid was introduced to SCRI193 making the strain sensitive to λ::Tn*5* infection, and allowing the isolation of several Tn*5*-induced mutants.

Extracellular enzyme production by SCRI193 and HC500 was studied. It appeared that the Pel^-, Cel^-, Prt^- phenotype of HC500 was attributable to growth under sulphur-limited conditions.

The Tn*5* element and flanking DNA was cloned from HC500 and used to obtain the wild-type homologue. This wild-type *cys*$^+$ gene "complemented" the *cysB* lesion of *E. coli*, and the significance of this is discussed.

A pectate lyase structural gene was cloned from SCRI193 and studied physically and biochemically. Alternative genetic approaches in SCRI193 involved attempts to isolate a transducing phage, and the selection of nonsense suppressor mutants.

The work presented in this thesis demonstrates that the molecular genetic tools for the detailed analysis of *Ecc* are now available; SCRI193 is currently being used as a model system for the study of protein secretion.

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Acknowledgements

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Declaration

This thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself, except for reversion frequencies of mutants HC513 to HC523 (table 4:6:1) which were determined by Mark Sowden. All sources of information have been specifically acknowledged by means of reference.

Jay Hinton.

Jay Hinton

Abbreviations

Ap^S	ampicillin-sensitive
Ap^R	ampicillin-resistant
Cel	cellulase
Cfu	colony-forming units
d	day
DKII	3-deoxy-D-glycero-2,5,-hexodiulosonate
DMA	Davis minimal agar
DMF	dimethylformamide
DMSO	dimethylsulphoxide
EDTA	diaminoethanetetraacetic acid
EMS	ethanemethylsulphonate
h	hour
IEF	isoelectrofocusing
min	minute
Mr	relative molecular mass
NBA	nutrient broth agar
Ogl	oligogalacturonate lyase
PAGE	polyacrylamide gel electrophoresis
Peh	polygalacturonase
Pel	pectase lyase
Pem	pectin methylesterase
pfu	plaque-forming units
PGA	polygalacturonic acid
Pnl	pectin lyase
Prt	protease
sec	second
SDS	sodium dodecyl sulphate

TEMED	N,N,N',N'-Tetramethylethylenediamine
TPR	transfer per recipient
Tris	Tris(hydroxymethyl)aminoethane
ts	temperature-sensitive
U	unit
YE	yeast extract

Restriction endonuclease abbreviations:

AI	*AvaI*
EI	*EcoRI*
EV	*EcoRV*
BII	*BglII*
PII	*PvuII*
SaI	*SalI*
SpI	*SphI*

Antibiotic abbreviations are listed in table 2:2:2.

Glossary

Pathogenicity:

The production, or the ability to produce disease by a bacterium.

Virulence:

The relative pathogenicity of a phytopathogen.

Export:

Protein translocation to any extracytoplasmic compartment of the bacterial cell (e.g. plasma membrane, periplasm or outer membrane) (Pugsley & Schwartz, 1985).

Secretion:

A specialised type of protein export which involves the translocation of protein to the extracellular environment (Pugsley & Schwartz, 1985).

"Complementation"

Genetic complementation in a *recA*$^{+}$ strain.

Chapter 1

General Introduction

1. Preface

The soft rot Erwiniae are an important group of phytopathogens that have recently become the subject of intensive study. A great deal of research is currently directed towards analysis of the genetics, biochemistry and physiology of extracellular enzymes, and their secretion with a view to the analysis of phytopathogenicity itself. This research relies on molecular genetic technology that has recently been applied to *Erwinia carotovora* ssp. *carotovora* (*Ecc*) and *E. chrysanthemi* (*Echr*).

However, when this project was initiated, tools for the genetic analysis of *Ecc* were not available. This thesis describes the development and application of a number of genetic techniques to *Ecc* strain SCRI193.

It has become apparent that no review describing current knowledge of the pathogenicity, biochemistry or genetics of the soft rot Erwiniae is available. Consequently, this chapter has been written in the form of a review, and is intended to summarise recent *Erwinia* research. Furthermore, sections on *Erwinia* taxonomy, pathogenicity and extracellular enzyme biochemistry are included, to put recent work into context, and to justify the current interest in the soft rot Erwiniae. It is anticipated that this review will serve as a source of reference for the ongoing *Erwinia* research at the University of Warwick.

1.1 *Erwinia* taxonomy

The *Erwinia* genus belongs to the Enterobacteriaceae and contains a diverse collection of bacterial species. It includes both human and animal pathogens as well as many plant pathogenic and saprophytic epiphytic species (Chatterjee & Starr, 1972). The genus was originally proposed to segregate all phytopathogenic and plant-associated bacteria from bacteria of other genera (see Chatterjee & Starr, 1980). Therefore it is not surprising that a genus formulated on such a basis has posed a number of taxonomic problems.

The eighth edition of "Bergey's Manual" recognised three groups within the erwinias: the "*carotovora*", "*amylovora*" and "*herbicola*" groups (Lelliott, 1974). However, the segregation of the genus into such groups is of questionable taxonomic significance (Mergaert *et al.*, 1984), and has not been continued in the current edition of the manual which recognises fourteen distinct species within the diverse *Erwinia* genus (Lelliott & Dickey, 1984). This generic heterogeneity is emphasised by Brenner (1984), who used the criterion of DNA homology to assess divergence within the Enterobacteriaceae. Most *Erwinia* spp. had just 35% homology at the DNA level, whereas all *Klebsiella* spp., for example, had at least 90% homology.

Although species grouping is not taxonomically correct, the "soft-rot" or "carotovora" groups of erwinias has been referred to extensively throughout the literature (Pérombelon & Kelman, 1980). This group is generally described as containing four species, *E. carotovora*, *E. chrysanthemi*, *E. cypripedii* and *E. rhapontici*. Relatively little is known of the latter two species, and the term "soft-rot erwinias" will

be taken here to refer to *E. chrysanthemi*, and the three subspecies of *E. carotovora* (*E. c.* ssp. *carotovora* , *E. c.* ssp. *atroseptica* (*Eca*), and the recently described *E. c.* ssp. *betavasculorum* (*Ecb*)) (Skerman *et al.*, 1980; Lelliott & Dickey, 1984).

The soft rot erwinias are characterised by their ability to produce large amounts of extracellular enzymes, which may play a role in pathogenicity (see section 1.3). On a diagnostic level, these four erwinias have many similarities, but may be easily differentiated by certain biochemical tests (table 1:1).

DNA reassociation and numerical taxonomical studies have shown that *Eca* and *Ecc* are closely related, and that *Echr* should stand as a distinct species (Brenner *et al.*, 1973; Mergaert *et al.*, 1984). Little comparative work has been performed on *E. c.* ssp. *betavasculovum* since its discovery (Thomson *et al.*, 1981).

Serological studies indicate that *Ecc*, *Eca* and *Echr* are related, but there is a greater degree of similarity between *Ecc* and *Eca* than with *Echr* (see Pérombelon & Kelman, 1980). Recent work with monoclonal antibodies specific to lipopolysaccharide has allowed the differentiation of *Eca* from other pectolytic erwinias (Helk & De Boer, 1985).

The current major interest in the soft rot erwinias stems from their role in phytopathogenicity and biodeterioration (section 1:2). A very important pathogenicity trait in *Erwinia* is the production of extracellular enzymes (section 1:3). Consequently, *Erwinia* is also currently used as a model system with which to investigate the mechanisms of protein translocation. Obviously enzyme secretion and

TABLE 1:1 Biochemical differentiation of the soft rot erwinias

Test	*Echr*	*Ecc*	*Eca*	*Ecb*
Growth at 36°C	+	+	-	+
Reducing substances from sucrose	-	-	+	+
Acid production from maltose	-	-	+	+
Acid production from α-methyl glucoside	-	-	+	+
Acid production from trehalose	-	+	+	?
Acid production from palatinose	-	-	+	?
Utilisation of raffinose	+	+	+	-
Utilisation of malonate	+	-	-	-
Utilisation of tartrate	+	-	-	?
Phosphatase production	+	-	-	?
Sensitivity to erythromycin	+	-	-	-

From Lelliott & Dickey, (1984) and M. Perombelon (pers. comm.)

phytopathogenicity are closely related events and so the molecular analysis of membrane traffic in *Erwinia* is fundamental to an overall understanding of plant attack. Aspects of *Erwinia* pathogenicity and extracellular enzyme production are presented in sections 1:2 and 1:3.

1:2 Soft rot erwinias as phytopathogens

The study of the soft rot erwinias may be justified by consideration of the enormous crop losses they cause. They are one of the most common groups of prokaryotic plant pathogens reported in the U.S. (Kennedy & Alcorn, 1980). Although accurate estimates of loss are not available, they are thought to total $50-$100 million annually, on a worldwide basis (Pérombelon & Kelman, 1980).

1:2:1 Factors in procaryotic pathogenicity

A number of reviews concerning theoretical aspects of the infection of plants by bacteria have been published which recognise several elements involved in pathogenesis (Chatterjee & Starr, 1980; Keen & Holliday, 1982; Kelman, 1979). These include mutual recognition of the pathogen and host; the "invasiveness" of the pathogen (i.e. its ability to overcome host defence mechanism); and its ability to produce substances that can harm the host (e.g. toxins or plant cell-degrading enzymes).

Studies of the inheritance of plant resistance to particular pathogens have led to an understanding of the genetics of many host-pathogen interactions, and the formulation of the gene-for-gene hypothesis (Day, 1974; Flor, 1956). Several of these well-characterised host-pathogen interactions are now being used as model systems for the study of the

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molecular basis of particular aspects of pathogenicity, such as host recognition (Panopoulos & Peet, 1985). Model systems currently under investigation include *Pseudomonas syringae* pv. *glycinea* (causal agent of bacterial blight of soybean; Staskawicz *et al.*, 1984), *P. s.* pv. *syringae* (causal agent of bacterial brown spot), *P. s.* pv. *phaseolicola* (causal agent of halo blight of bean) (Niepold *et al.*, 1985) and *Xanthomonas campestris* pv.*campestris* (causal agent of crucifer back rot; Turner *et al.*, 1985).

Specialised gene-for-gene systems are though to have arisen through co-evolution of the host and pathogen, which led to the development of specific mutual recognition mechanisms (Day, 1974). However, such recognition systems have not been identified in the plant-*Erwinia* interaction, and this may suggest (as discussed in the next section) that this interaction is still relatively primitive (Keen & Holliday, 1982).

1:2:2 Aetiology and epidemiology of soft rot disease

Because the area has been extensively reviewed elsewhere (Pérombelon, 1982, 1985; Pérombelon & Kelman, 1980), this section only summarises present knowledge on the ecology of the soft rot erwinias and aetiology of the disease they cause. Although host specificity is poorly developed, the four soft rot erwinias (section 1:1) have different host ranges which probably reflect their temperature tolerances and geographical distribution.

Echr is a pathogen of a wide range of tropical and sub-tropical crops, as well as those grown in greenhouses in temperate regions. These plants include african violet, carnation and leopard lily as well as

field crops such as maize, pineapple and potato (Pérombelon & Kelman, 1980). Potato is only a host for *Echr* when it is cultivated in hot climates.

Little is known about the host range of *Ecb*, except that it is a soft rot pathogen of sugar beet in California, USA (Thomson *et al.*, 1981). However, of all the erwinias, *Ecc* and *Eca* make the greatest economic impact in the U.K. (particularly in Scotland) and these subspecies are discussed below. *Eca* appears to be almost completely restricted to potato, where it is the most important soft rot pathogen in cool climates. It causes a characteristic stem rot in the field (blackleg), and soft rot of tubers in store. *Eca* is rarely isolated from the environment, and many aspects of its pathogenicity are not understood (M. Pérombelon, pers. comm.).

In contrast, *Ecc* has a much broader host range than other members of this group, causing soft rot of a number of crops including brussels sprout, carrot, celery, cucumber, green peper, potato and turnip. *Ecc* is commonly isolated from various environmental sources, including the air in rural areas, river and drainage water, weed roots and soil. Wind-borne, soil-borne and insect-borne dispersal mechanisms have been implemented in *Erwinia* epidemiology (Harrison & Brewer, 1982; Pérombelon & Kelman, 1980). The most severe crop losses caused by *Ecc* in the U.K. occur in potato. Unlike *Eca*, it does not cause blackleg in the field, but it is responsible for a large proportion of the soft rotting of tubers in store (Pérombelon, 1982).

Potato tubers are often contaminated with both *Ecc* and *Eca*, and their relative abundance appears to be temperature-dependent. At lower

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Potato tubers are often contaminated with both *Ecc* and *Eca*, and their relative abundance appears to be temperature-dependent. At lower

temperatures (< 22°C), *Ecs* predominates, whereas at higher temperatures the reverse is true. *Echr* can also be found on tubers in some countries, and can cause symptoms similar to blackleg, as well as tuber soft rot, but only where ambient temperature is > 25°C (Pérombelon, 1982, 1985; Pérombelon & Kelman, 1980). This differential effect of temperature could reflect variations in the growth rate of the bacteria, or in the rate of extracellular enzyme production (Pérombelon, 1985), but its basis remains undetermined.

The symptoms of the blackleg disease appear as either a dark slimy rot at the base of the stem of the potato plant or as generalised wilting, depending upon weather conditions. Blackleg symptoms result from systemic infection of the plant and tubers by the pathogen. Disease incidence is related to the degree of contamination of seed by *Ecs*, but blackleg development is dependent upon certain environmental factors, particularly soil water status. No effective disease control method is available, except measures which tend to reduce seed contamination levels. Although seed certification (based on visual inspection of the crops for blackleg) is practised, seed is commonly contaminated with *Ecs* (Pérombelon, 1985).

Soft rot symptoms are caused by the maceration of parenchymatous tissue of the tuber, and can involve its complete collapse. Infection of the tuber often occurs immediately post-harvest. Following lifting, erwinias which are already present on tuber surfaces, can enter directly through open lenticels or wounds. Once inside the tuber, the bacteria can be viewed as being in a latent state (Pérombelon, 1982, 1985). Whether a rot is or is not initiated in a contaminated tuber depends solely upon environmental factors within the potato store, such as

oxygen tension, temperature and humidity (Pérombelon & Kelman, 1980). Under favourable conditions, a single *Erwinia* cell can cause a rot (Pérombelon, 1972). The rot and collapse of a tuber provides a massive source of inoculum, and can act as a focus for a progression of the disease throughout the store (Pérombelon & Kelman, 1980).

The control of soft rot in potato stores can be achieved by use of sophisticated devices to control temperature and humidity, and by segregation of tubers to contain infection. However, such methods are not practical on the large scale of industrial ware production, which commonly involves storage of tubers for up to 10 months (M. Pérombelon, pers. comm.).

To conclude, the classical plant pathological approach to the study of the soft rot erwinias, and the diseases they cause, has led to an understanding of aspects of the aetiology and epidemiology of blackleg and soft rot. Unfortunately this approach has not led to a direct control method. The classical plant pathology approach is essentially "phenomenological", in that it involves the observation of the bacteria, and the infected plant without discovering *how* the bacteria actually cause disease. An understanding of the plant-bacterial interaction at the molecular level could lead the way to a control method in the future.

Ecc and *Eca* as opportunistic pathogens

The soft rot erwinias should be considered as opportunistic pathogens of potato, because they enter the tuber by passive means, and remain latent until the host becomes "compromised" (Pérombelon, 1982). However, if soft rot simply reflects opportunistic infection by pectolytic bacteria,

it is interesting to consider why soft rot erwinias, rather than other pectolytic bacteria, are responsible for most cases of the disease (pectolytic *Pseudomonas*, *Clostridium* and *Bacillus* spp. are commonly isolated from tubers). It seems likely that soft rot erwinias are simply able to grow faster, and produce more pectolytic enzymes than other bacteria under the conditions found in the tuber (Chatterjee *et al.*, 1979; M. Pérombelon, pers. comm.; see section 1:3:7:2).

Ecc and *Ecs* do not exhibit cultivar specificity in soft rot (Perombelon, 1985), and so they do not make ideal pathogens with which to study host specificity. *Echr* may be more suitable for study of host specificity, because a degree of cultivar specificity upon ornamental plants has been reported (Dickey, 1981; Lelliott & Dickey, 1985; A. Kotoujansky, pers. comm.). However, as opportunists, *Ecc* and *Ecs* offer a chance to study a primitive pathogenic system. Pathogenesis results from changes in environmental conditions which cause plant stress and so facilitate bacterial multiplication, and the production of large amounts of extracellular enzymes. Study of the *Erwinia*-potato interaction could yield information concerning the mechanisms by which the bacteria overcome host resistance.

Despite the primary role of pectolytic enzymes in pathogenesis (section 1:3), the use of a genetic approach should allow the identification of other components in the pathogenic process. The isolation of a series of "non-pathogenic" mutants should allow dissection of the pathogenic process at a molecular genetic level.

1:3 Extracellular enzymes of the soft rot Erwiniae

1:3:1 Do extracellular enzymes have a role in pathogenicity?

The soft rot erwinias have been shown to produce a wide variety of extracellular enzymes, which have proved interesting to plant pathologists, biochemists and industrialists. The enzymatic basis of soft rot was established in 1909 and has been studied intensively since then (Bateman & Basham, 1976). A number of *Erwinia* extracellular enzymes have been described, including pectinases (Rombouts & Pilnik, 1980), cellulases (Garibaldi & Bateman, 1973; Boyer *et al.*, 1984a). DNAases (Graham & Hodgkiss, 1967), proteases and phopholipases (Mount *et al.*, 1970). The properties and potential pathogenic role of these enzymes will be discussed later. That discussion is preceded by a review of the structure of the plant's primary line of defence against infection, the plant cell wall.

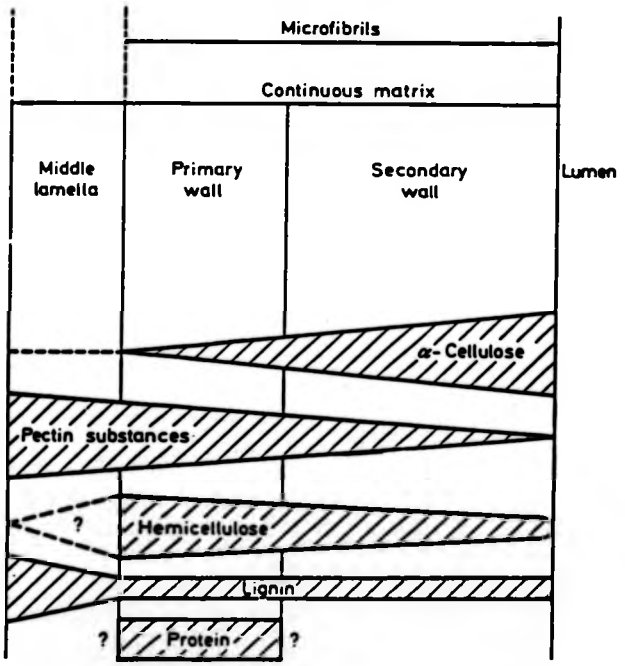
1:3:2 The plant cell wall

The cells of higher plants are surrounded by a cellulosic wall which encases the plasmalemma and cell protoplast. The dicotyledonous cell wall is traditionally divided into three structural, functional regions, the middle lamella, primary and secondary walls (Fig. 1:3:2a). However, there is no clear cut demarcation between these three regions, and they should be viewed as a continuum.

D-galacturonans (pectic polymers) predominate in the middle lamella, and are important structural components in the primary wall. These polymers are made up of long chains of α-1,4-linked galacturonan interspersed with α-1,2-linked rhamnogalacturonan. The carboxyl group of galacturonan may or may not be methylated to make a pectin or pectate

Figure 1:3:2a

A diagrammatic representation of the plant cell wall (from Bateman & Basham, 1976).



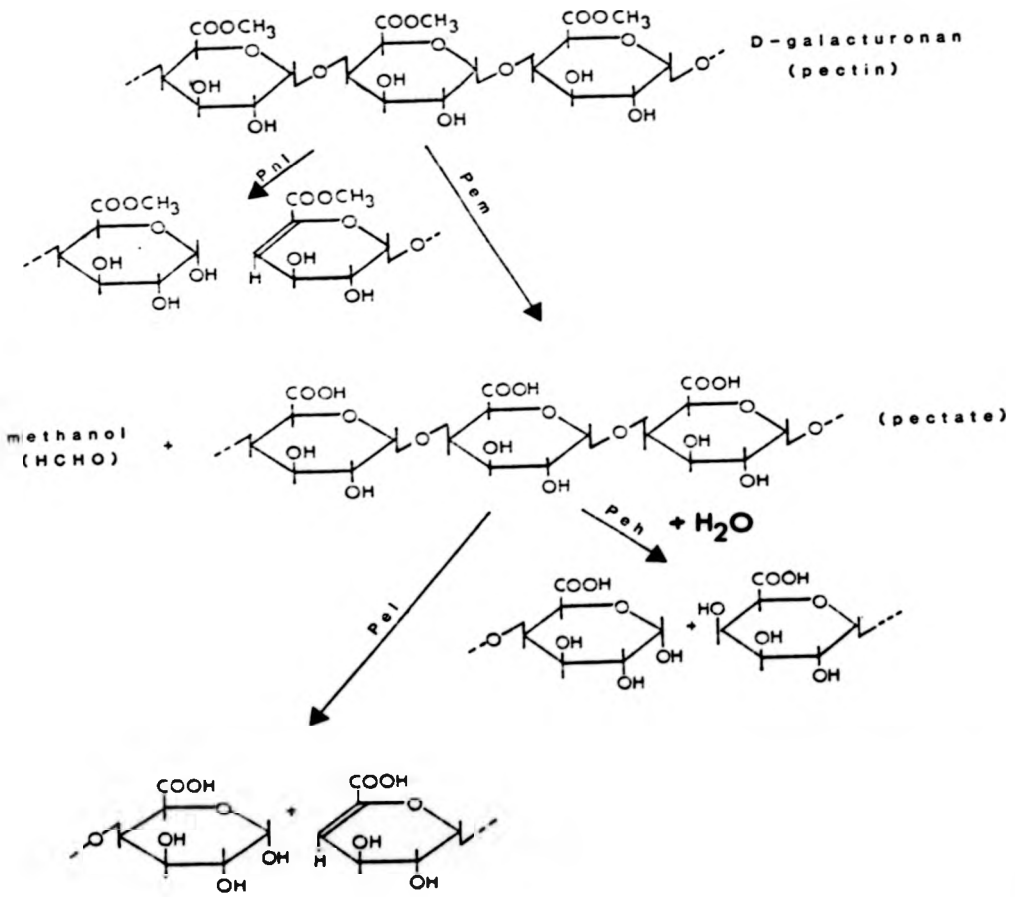
respectively (see Fig. 1:3:2b). Alternatively the galacturonan may be cross-linked by calcium, accounting for the characteristic gel-forming ability of this polymer. Arabinogalactan side chains are covalently linked to the rhamnogalacturonan backbone, accounting for the structural properties of the polymer (Cooper, 1983).

The current model of cell wall structure envisages the primary wall as a tight mesh of cellulose fibres ensheathed in hemicellulose polymers, that are interconnected by galacturonan chains (McNeil *et al.*, 1984). The cellulose microfibrils contain both amorphous and crystalline regions, and are bounded by hemicelluloses, particularly xyloglucan and arabinoxylan. However, the exact nature of the interaction between these hemicelluloses and the galacturonan polymers has not been determined. It was thought that xyloglucan cross-linked with the arabinogalactan of the galacturonan polymer, and that other arabinogalactan side chains connected with glycoprotein to form a stable wall structure (Keegstra *et al.*, 1973). Recently, the nature of the precise interaction of these polymers has been disputed (McNeil *et al.*, 1984).

Secondary wall structure is less conserved than that of the primary wall, and varies from plant to plant. In comparison with the primary wall, it generally contains more cellulose fibres arranged in parallel arrays, with less hemicellulose and galacturonan polymers. The plant cell is bounded by the middle lamella, which consists primarily of galacturonan molecules, with some hemicelluloses (Keegstra *et al.*, 1973). This is seen as a "cementing" layer which holds cells together to form coherent tissue structure.

Figure 1:3:2b

The mode of action of four pectolytic enzymes; endo-Pel, endo-Peh, Pem and Pnl.



In summary, the current understanding of the plant cell wall involves an association between galacturonan polymers, cellulose molecules and perhaps glycoproteins, to maintain the integrity of the cell. Obviously, enzymes capable of breaking down these substances could cause plant cell wall degradation. Enzymes of this type which are produced by the soft rot erwinias are described below. Experimental evidence for the role of these enzymes in pathogenesis is discussed in section 1:3:7.

1:3:3 Pectolytic enzymes

Pectin is the methylated, single chain, α-1,4-linked galacturonan polymer. Pectate (or polygalacturonic acid (PGA)) is its demethylated derivative (see Fig. 1:3:2b). The biochemistry and function of pectolytic enzymes has been extensively reviewed previously (Rombouts & Pilnik, 1980; Chesson, 1980; Collmer *et al.*, 1982), and so this section is intended only as a summary of current knowledge. These pectolytic enzymes ("pectinases") are involved in the sequential breakdown of pectin, which can lead to its assimilation into cellular carbon, as shown in Figure 1:3:3. The most important pectolytic enzymes are described in table 1:3:3. Pectolytic enzymes are produced by a number of phytopathogenic bacteria and fungi, as well as some species pathogenic to humans and animals (Rombouts & Pilnik, 1980; Bagley & Starr, 1979). The enzymes involved attack the α-1,4-glycosidic bond of the pectin or pectate molecules in either an endo (random) or exo (terminal) fashion. Both endo and exo types of pectate lyase (Pel) and polygalacturonase (Peh) have been described, and generally these are active on pectate. The endo form of these enzymes occurs more commonly than the exo form, and is more active in plant cell wall degradation (see 1:3:7). Only the endo types of pectin methylesterase (Pem) and pectin lyase (Pnl) have been reported, and these are only active on

Figure 1:3:3

Major pathways of pectate catabolism in bacteria.

Enzymes for the catabolic step are: (1) Peh, (2) Pel, (3) oligogalacturonate hydrolase, (4) Ogl, (5) uronate isomerase, (6) altronate oxidoreductase, (7) altronate hydrolase, (8) 4-deoxy-L-threo-5-hexoseulose uronate isomerase, (9) 3-deoxy-D-glycero-2,5-hexodiulosonate dehydrogenase, (10) 2-keto-3-deoxygluconate kinase, (11) 2-keto-3-deoxy-6-phosphogluconate aldolase (from Chatterjee *et al.*, 1985a).

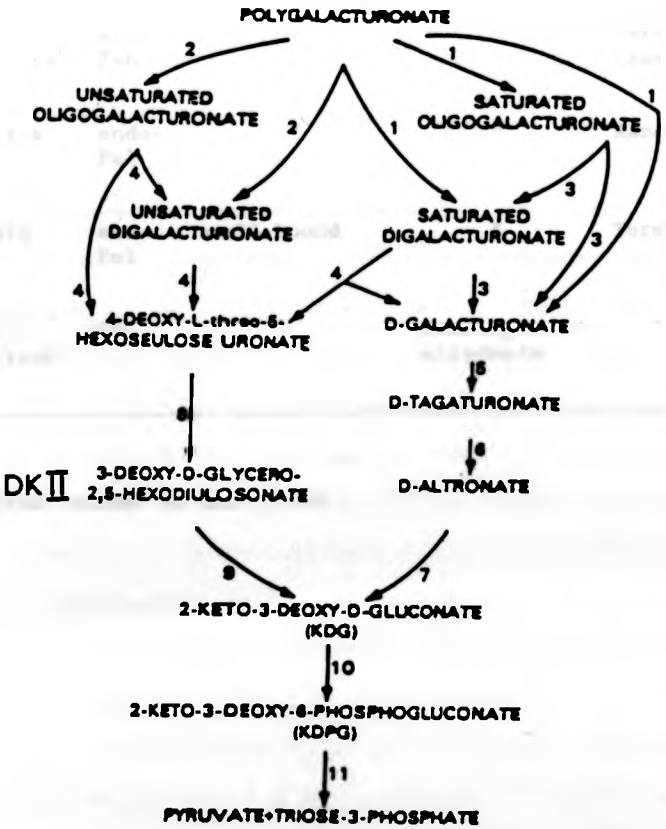


TABLE 1:3:3 Pectolytic enzymes produced by the soft rot erwinias

Enzyme	Abb.	Location	Preferred galacturonan substrate	Mode of attack	EC number
pectinmethyl-esterase	Pem	extracellular	methylated galacturonan	Random (endo)	3.1.1.11
pectin lyase	Pnl	"	"	"	4.2.2.10
endo-poly-galacturonase	endo-Peh	"	demethylated galacturonan	"	3.2.1.15
exo-poly-galacturonase	exo-Peh	"	"	Terminal (exo)	3.2.1.82
endo-pectate lyase	endo-Pel	"	"	Random	4.2.2.2
exo-pectate lyase	exo-Pel	cell-bound	"	Terminal	4.2.2.9
oligogalacturonate lyase	Ogl	"	demethylated oligomers	"	4.2.2.6

Adapted from Collmer *et al.*, 1982.

pectin (table 1:3:3). Usually, exo-type enzymes yield monomeric or dimeric products, and endo-type enzymes act randomly to generate longer oligomeric products.

Some work on the incidence of these enzymes in *Erwinia* has been published; the occurence of Pem, Pnl and the endo or exo forms of Pel and Peh in different strains of soft rot *Erwinia* spp. appears to be variable. Pem and Pnl are produced by the majority of strains (Smith, 1958; Tsuyumu & Chatterjee, 1984). All strains produce endo-Pel (the most important macerating enzyme), and some produce exo-Pel (Rombouts & Pilnik, 1980).

Isozymic forms of Pel have been reported, and their significance is discussed in section 1:3:7:1.

Regulatory studies on pectolytic enzymes have been carried out in a number or organisms, including the soft rot erwinias (Bagley & Starr, 1979; Chatterjee *et al.*, 1979; Zucker *et al.*, 1972). Peh is constitutively produced in *Ecc* (Chatterjee *et al.*, 1981). However, Pel is produced at a high basal level in *Ecc*, *Eca* and *Echr*, and is inducible by a number of substrates (Chatterjee *et al.*, 1979; Zucker *et al.*, 1972). The inducer of Pel has recently been identified as a breakdown product of pectate, 3-deoxy-D-glycero-2,5-hexodiulosonate (DKII; see Fig. 1:3:3) (Chatterjee *et al.*, 1985a; Condemine *et al.*, 1984). The basal level of Pel mediates the production of a small amount of DKII in the presence of pectate; this, in turn, induces the synthesis of a high level of Pel. The inducibility of Pel is unique to the soft rot erwinias, differentiating them from other pectolytic bacteria, and may well be important in pathogenesis (Bagley & Starr, 1979; Chatterjee *et*

al., 1979). One strain (630) of *Echr* produces exo-Peh constitutively, and the products of pectate degradation by this enzyme mediate Pel induction (Collmer & Bateman, 1982). Pel synthesis is catabolite-repressible (Chatterjee *et al.*, 1979; Hubbard *et al.*, 1978), and is also repressed by a high concentration of its inducer (DKII) (Tsuyumu, 1979). Pem is also inducible, but this inducibility has not been characterised in detail (Rexova-Benkova & Markovic, 1976). Pnl is constitutively synthesised by *Ecc* but may be "induced" by DNA-damaging agents (Itoh *et al.*, 1980; Tsuyumu & Chatterjee, 1984). This "induction" requires the presence of the *recA* gene product suggesting an involvement of the "SOS" response (Zink *et al.*, 1985a).

The four enzymes Pem, Pnl, Pel and Peh are generally found in culture supernatants. However, of these, only Pel has been proved to be secreted from *Ecc* and *Echr* by an active mechanism (Andro *et al.*, 1984; Chatterjee *et al.*, 1979). In both cases the enzyme was synthesised and secreted in mid to late log phase of growth.

1:3:4 Cellulases

Several reports of extracellular cellulase (Cel) production by soft rot erwinias can be found in the literature (Beraha & Garber, 1970; El-Helaly *et al.*, 1979; Garibaldi & Bateman, 1973). These papers refer to "Cx" activity, the ability to degrade substituted cellulosic derivatives (such as carboxymethyl cellulose), rather than crystalline cellulose (Goksoyr & Eriksen, 1980). El-Helaly *et al.*, (1979) described constitutive Cel synthesis by *Ecc* and *Echr* strains, as well as inducible synthesis by strains of *Eca*. More recently, Boyer *et al.*, (1984a,b) published detailed reports on the production, regulation, purification and characterisation of Cel from *Echr* strain 3665. In this strain Cel

synthesis was constitutive, catabolite-repressible, and the rate of synthesis increased under oxygen-limited conditions. The enzyme produced by strain 3665 was identified as endo-β-1,4-glucanase which cleaved carboxymethylcellulose to cellobiose (Boyer *et al.*, 1984a,b). The cellobiose is not catabolised further extracellularly, but transported into the cell via the phosphotransferase system, where it is degraded by β-glucosidase activity (Barras *et al.*, 1984a).

1:3:5 Proteases

A number of workers have described extracellular protease (Prt) production by *Erwinia* spp. (Mount *et al.*, 1970; Garibaldi & Bateman, 1973; Pugsley & Schwartz, 1985). Unfortunately, little detailed characterisation of the biochemistry or secretion of proteases has been undertaken. The enzyme is generally recognised by its activity on casein or skim milk, but details on substrate specificity have not been reported.

Work is currently proceeding on the characteristics of the exo-protease from *Echr* strain, 3937j (C. Wandersman, 1984; pers. comm.). This enzyme is thermostable, and able to withstand boiling in the presence of SDS or 2-mercaptoethanol without loss of activity. Its synthesis is induced by tryptone, and it is only produced during stationary phase of growth. Protease appears to be extremely efficiently secreted from *Echr*, by a different mechanism to Cel or Pel (Andro *et al.*, 1984; Pugsley & Schwartz, 1985; A. Chatterjee, pers. comm.).

1:3:6 Phospholipases

Phospholipase C is produced extracellularly by a number of *Erwinia* strains (Tseng & Bateman, 1968; Mount *et al.*, 1979). This enzyme,

traditionally referred to as phosphatidase, is known to hydrolyse diacylglycerophosphoryl compounds, and so could play a role in plant cell membrane degradation (Bateman & Basham, 1976). Although the occurrence of phospholipase C in erwinias has been reported, no information has been published on its mode of action, substrate specificity, secretion or regulation.

1:3:7 Extracellular enzymes and pathogenicity

As outlined earlier, soft rot erwinias produce various extracellular enzymes *in vitro*, which are capable of degrading plant cell wall components. A number of approaches have been used to determine if any of these enzymes are involved in *in planta* tissue maceration or cell death.

Experimental approaches. Isolated plant cells and protoplasts have been treated with purified preparations of the various enzymes, to determine any effect on plant cell viability (e.g. Tseng & Mount, 1974). Purified plant cell walls have also been isolated and treated with the various enzymes. The quantities of structural wall components, such as cellulose or galacturonans, were assayed to determine any degradation due to the action of the enzymes (e.g. Bateman & Basham, 1976). Another approach involved studying the enzymes produced *in planta*. Following infection by the pathogen, macerated plant tissue was assayed for the presence of particular enzymes (e.g. Cooper, 1983). Finally, defined mutants of the pathogen which are unable to produce particular enzymes have been isolated, and their macerating properties studied (Chatterjee *et al.*, 1985a; Chatterjee & Starr, 1977).

Such approaches have been used as a "molecular form of Koch's

postulates" (Cooper, 1983) to determine the role of each enzyme in maceration. However, much of the evidence thus produced remains circumstantial, and some disagreement exists in the literature concerning the roles of specific enzymes.

The process of plant tissue maceration appears to involve two distinct steps. Firstly, tissue disintegration occurs as a result of the degradation of the middle lamella galacturonans, and the consequent cell separation. Secondly, the separated cells die due to enzymatic action on their walls. This is characterised by electrolyte loss from the whole cells. Tribe (1955) described these two processes as being sequential, and a result of continued action of extracellular enzymes.

1:3:7:1 Pectolytic enzymes in pathogenesis

Role of Pel. High levels of endo-Pel have been isolated from erwinia-infected plant tissue (Mount *et al.*, 1970) and purified endo-Pel has been shown to have the ability to both macerate and kill parenchymatous plant tissue (Basham & Bateman, 1975a,b; Mount *et al.*, 1970). There are no reports of highly purified pectolytic enzymes which can cause cell death without causing cell wall breakdown or tissue maceration (Basham & Bateman, 1975b). It is thought that endo-Pel acts by breaking down the galacturonan polymers in the middle lamella and primary wall, and so makes the wall structurally unsound under conditions of osmotic stress, i.e. cell death results from lysis of turgid protoplasts which are no longer contained by a coherent wall (Bateman & Basham, 1976). This hypothesis is supported by the observation that plant cell protoplasts appear to be resistant to attack by endo-Pel in isotonic media (Tseng & Mount, 1974).

Purified endo-Pel can solubilise more than 50% of the total wall sugars in isolated potato cell walls (Basham & Bateman, 1975b). Exo-Pel does have some macerating ability, but it is not nearly so active as the endo form of the enzyme (Bateman & Basham, 1976).

The results of genetic experiments confirms the central role of endo-Pel in plant tissue degradation. The *Pat* mutant of *Echr* is unable to produce Pel or Cel due to a secretory lesion, but does export Prt and Peh (Chatterjee & Starr, 1977; A. Chatterjee, pers. comm.). This mutant is unable to macerate plant tissue, even though active Peh is produced. This result has been confirmed more recently (Andro *et al.*, 1984; K. Thurn, pers. comm.), suggesting that either Pel or Cel is required to degrade plant tissue.

The structural genes of various isozymes of Pel have now been cloned, from *Ecc* and *Echr*, on plasmid and phage vectors (see section 1:6). *E. coli* cells carrying these clones are able to macerate potato tissue, inducing similar symptoms to the original *Erwinia* strain, although with varying severity (section 7:5:1). Physiological experiments have been conducted with other pectolytic enzymes, and have suggested that these enzymes do not play a role in soft rot.

Role of Pem. Endogenous Pem is present in some healthy plant tissue (Chesson, 1980). Thus, detection of the enzyme in diseased tissue (Bateman & Millar, 1966) is not necessarily significant, since the Pem could be derived from the plant itself (Chesson, 1980). Pem is thought to potentiate the action of Pel *in planta*, by de-esterifying the methylated galacturonans, thereby presenting a substrate for lytic degradation (Collmer *et al.*, 1982). Complete de-esterification by Pem

is not required because endo-Pel can degrade partially methylated substrates.

Role of Pnl. Pnl has been isolated from fungi, and is capable of macerating plant tissue (Byrde & Fielding, 1968). However, Pnl from *Erwinia* spp. does not have this ability (Tsuyumu & Chatterjee, 1984). No other reports on the role of this enzyme in soft rot are available.

Role of Peh. Peh is an important macerating factor for a number of fungal pathogens (Hall & Wood, 1973; Cooper, 1983). Recent work has demonstrated that purified preparations of *Erwinia* Peh can produce plant tissue maceration (Lei *et al.*, 1985c; K. Thurn, pers. comm.). However, increased levels of Peh have not been detected in *Erwinia*-infected plant tissue (Cooper, 1983). This could be because oxidised phenolics and proteinaceous compounds found in plant tissue have been shown to serve as potent inhibitors of Peh activity (Albersheim & Anderson, 1971), and so may obscure the presence of the enzyme in diseased tissue (Cooper, 1983).

Although genetic studies by Beraha *et al.*, (1974) with mutant strains of *Ecc*, suggested that endo-Peh was a significant macerating enzyme, more detailed work with *Echr* suggested that this is not the case (Chatterjee & Starr, 1977). However, the endo-Peh structural gene from *Ecc* has been cloned recently and *E. coli* cells carrying this clone have been shown to macerate potato tissue (Lei *et al.*, 1985c; K. Thurn, pers. comm.; Zink *et al.*, 1985).

Thus, recent evidence proves that Peh has similar macerating properties to Pel. However, since the destructive effects of Pel upon plant tissue

were established some years ago, the production and regulation of this enzyme has received most attention. Clearly, the identification of Peh as a virulence determinant will stimulate work on its synthesis and regulation.

Pel as a major pathogenicity determinant

The evidence outlined above suggests that endo-Pel is an important macerating enzyme of the soft rot erwinias. It has been suggested that together with several other pectolytic enzymes, endo-Pel may act in a "pectic enzyme complex" (Stack *et al.*, 1980). The authors described a group of *Ecc* intracellular and extracellular enzymes (namely endo- Pel, exo-Pel, endo-Peh and Ogl) which could act in concert to catabolise pectate.

Similarly, the example of the indirect role of exo-Peh in the induction of Pel synthesis by strain 630 (section 1:3:3) constitutes a less intricate "pectic enzyme complex" (Collmer & Bateman, 1982).

While considering endo-Pel as a major factor in pathogenesis, it is interesting to note that it is capable of "inducing" phytoalexin formation by the plant (Davis *et al.*, 1984; McNeil *et al.*, 1984). Albersheim and coworkers have shown that Pel (or one of its products, decagalacturonide), can act as an elicitor of phytoalexins in soybean cotyledons. Thus, a fine balance must exist between avoiding the induction of host defence mechanisms and rapid plant cell killing.

The role of the pectate degradation pathways (Fig. 1:3:3) in pathogenesis has recently been questioned. Traditionally, it had been thought that these pathways allowed the bacteria to obtain nutrients via

pectate degradation *in planta* (Chatterjee & Starr, 1980). This has now been challenged, since mutants of *Echr* which are blocked in both pathways retain their pathogenic characterisation (Chatterjee *et al.*, 1985a). These mutants are unable to utilise pectate as a carbon source, but produce normal levels of Pel. Presumably, Pel-induced maceration and plant cell degradation yield sufficient nutrients for bacterial proliferation, obviating the need for pectate utilisation.

Pel isozymes

As mentioned in section 1:3:3, isozymes of Pel have been observed for *Echr*, *Ecc* and *Eca*. A sensitive technique has now been developed for the detection of isozymes by flat-bed isoelectric focusing and activity staining (Ried & Collmer, 1985a, Bertheau *et al.*, 1984; Collmer *et al.*, 1985). This has allowed the determination of the isozyme profiles of a number of strains, and has shown distinct differences between the profiles of *Echr* strains, and strains of both *Ecc* and *Eca*. Six strains of *Ecc* and *Eca* possessed similar patterns of three basic Pel isozymes. In contrast, eleven *Echr* strains produced not only basic but also neutral isozymes (Ried & Collmer, 1985a,b). A few *Echr* strains including B374 and 3937j also produce an additional acidic isozyme (Bertheau *et al.*, 1984; Ried & Collmer, 1985a,b). The selective induction of this acidic *Echr* isozyme *in planta* has been reported (Pupillo *et al.*, 1976).

It is not known why such a variety of isozymes is produced. However, Pel isozymes can differ in their physical properties, such as their ability to macerate and kill plant tissue, and these differences can often be attributed to measurable differences in the enzyme "action pattern" (Garibaldi & Bateman, 1971).

One Pel isozyme has been reported which is able to rapidly cleave galacturonan *in vitro*, in an endo fashion, but is unable to macerate plant tissue (Garibaldi & Bateman, 1971). The same workers demonstrated that some groups of isozymes varied in their "host-specificity", and this remains unexplained (Bateman & Basham, 1976). Detailed biochemical characterisation of two *Ecc* isozymes showed that they differed in temperature optima, Km, amino acid composition and molecular weight (Sugiura *et al.*, 1984; Thurn & Chatterjee, 1985a). It has been observed that isozyme profiles vary under *in vitro* and *in planta* conditions, suggesting that certain isozymic forms are more stable than others, under adverse conditions in the host (Pupillo *et al.*, 1976; Quantick *et al.*, 1983). Furthermore, it should be remembered that *in vitro* Pel assays measure the general ability to degrade purified galacturonan molecules at the optimum pH, which may not resemble the situation in the plant. In confirmation of this, it has been observed that the ability of isozymes to degrade model substrates does not necessarily reflect their ability to degrade plant cell walls (Garibaldi & Bateman, 1971). Consideration of the central role of galacturonan polymers in plant cell wall structure (section 1:3:2) raises the possibility that certain isozymes have a great affinity for the particular galacturonan linkages that are most critical for cell wall and tissue integrity (Collmer *et al.*, 1982). Such isozymes would be much more active *in planta* than *in vitro*.

1:3:7:2 Do other enzymes contribute to pathogenicity?

There have been several attempts to discover whether or not non-galacturonan-degrading enzymes are involved in plant tissue degradation. It seems doubtful that enzymes involved in the breakdown of cellulose, protein and phospholipid-based polymers are involved in plant tissue

degradation (Bateman & Basham, 1976), although Cel production is known to be an important pathogenicity factor for *Pseudomonas solanacearum* (Kelman & Cowling, 1965).

There have been several attempts to discover whether or not non-galacturonan-degrading enzymes are involved in plant tissue degradation. Purified protease and/or phospholipase C from *Ecc*, cause no damage to intact cucumber or potato tissue (Mount *et al.*, 1970; Tseng & Mount, 1974). Nevertheless, it is thought that these enzymes *are* involved in cell membrane attack: the purified enzymes do lyse cucumber protoplasts (Tseng & Mount, 1974). Both protease and phospholipase C have been isolated from infected potato tissue (Mount *et al.*, 1970), suggesting a possible role *in planta*, but this remains to be proved. Data concerning the effect of purified Cel on plant tissue is not available. However, Cel has been detected in tissue infected by *Echr* (Garibaldi & Bateman, 1970). Future experiments using the Cel and Prt clones which have now been isolated (table 1:6) may help in defining an *in planta* function for these enzymes.

Finally, Ryan and coworkers have shown that certain stimuli will induce the systemic plant synthesis of large amounts of proteins which inhibit microbial proteases (Ryan, 1978). These stimuli include mechanical damage, and the presence of certain galacturonans such as rhamnogalacturonan I (Ryan *et al.*, 1981). However, the role of protease inhibitors and endo-Peh inhibitors in plant defence systems is not clear. Thus, it seems doubtful that enzymes involved in the breakdown of cellulose, protein and phospholipid-based polymers are involved in plant tissue degradation (Bateman & Basham, 1976). In contrast, Cel production is known to be an important pathogenicity factor for

Pseudomonas solanacaerum (Kelman & Cowling, 1965).

In conclusion, from the data available, it appears that the pectolytic enzymes act as plant cell wall-modifying enzymes, rendering the non-pectic polymers of the cell wall more accessible for enzymatic decomposition. It is clear that endo-Pel (and Peh) can cause tissue maceration and cell death, but action by cellulose, protease or phospholipase may be secondary. If this is the case, the latter three enzymes are not strictly involved in "pathogenesis" but in necrosis.

In assigning functions to enzymes such as cellulase, protease or phospholipase, it is important to consider that they may be involved in another aspect of the life of the pathogen apart from plant infection, e.g. survival of the bacteria upon leaf surfaces, in soil or within infected plant material. It is unlikely that these enzymes would be produced by most soft rot erwinias (involving great energy expenditure by the cell), if they did not have some survival function. The discovery of the function(s) of these enzymes awaits further, and perhaps more imaginative, experimentation, involving both physiological and molecular genetical techniques.

1:3:8 Potential industrial uses of extracellular enzymes from *Erwinia* spp.

Mixtures of pectolytic enzymes are used commercially to clarify fruit juice, and to increase the yield of apple and grape juice (Rombouts & Pilnik, 1980). The enzymes are also used in olive oil production and in the preparation of baby food puree and fruit nectar bases (Chesson, 1980). Currently, most commercial pectolytic enzyme preparations are of fungal origin (notably from *Aspergillus niger*), and are derived from

surface cultures grown on solid media. No purification stages are involved, and the preparations are generally a crude mixture of endo-Pel, endo-Peh and Pem, with other glycan hydrolases. Approximately 10 tonnes of crude "pectinase" are manufactured annually (Priest, 1984). The soft rot erwinias could be ideal sources of these enzymes in the future, due to the development of a market for selected, defined mixtures of pectolytic enzymes for specific purposes (Chesson, 1980).

Cellulase has a number of commercial applications, particularly in the breakdown of various cellulose-rich waste-products, such as wood, plant stems and stalks. These could be used as a nutrient source for biomass production, or simply hydrolysed to yield more useful products (Goksoyr & Eriksen, 1980). *Echr* protease may prove useful in the detergent industry, due to its exceptional thermal stability (C. Wandersman, pers. comm.).

The periplasmic antileukaemic enzyme l-asparaginase is currently produced from *Echr* strain 1066 by fermentation (see Flickinger, 1985; Gilbert *et al.*, 1985). Experience with the large-scale fermentation of *Echr* suggests that the isolation of other enzymes on a production scale should be feasible. Finally, an industrial process involving the use of immobilised *E. rhapontici* cells to produce isomaltulose (palatinose) from sucrose, has been described (Cheetham *et al.*, 1985).

The potential uses of *Erwinia* enzymes explain current industrial interest in *Erwinia* research. Genetic techniques could be used to engineer enzyme-producing *Erwinia* strains, with particular properties. However, the uses of molecular genetics in the analysis of *Erwinia* phytopathogenicity and enzyme secretion, are already being realised as

outlined in sections 1:4, 1:5 and 1:6.

1:4 Molecular genetics as an approach to the study of phytopathogenicity

The genetics of phytopathogenesis is an area that has commanded a great deal of interest over a number of years. The earliest approach involved the use of plant genetic techniques to devise breeding programmes for the improvement of particular agronomic characters such as crop yield, hardiness and resistance to disease (Day, 1974). Subsequently, classical fungal and plant genetic analyses were used in conjunction, leading to the formulation of the gene-for-gene hypothesis (Flor, 1956). The hypothesis arose from work on flax and the rust pathogen *Melampsora lini*, and concerned plant cultivar and pathogen race interactions. The gene-for-gene hypothesis stated that during their evolution, host and pathogen developed complementary genic systems; that "*for each gene conditioning rust reaction in the host there is a specific gene conditioning pathogenicity in the parasite*" (Flor, 1956). Flor described specific cultivar resistance/ susceptibility alleles (*R*1 and r1) which correspond to pathogen race avirulence/ virulence alleles (*P*1 and p1); resistance was inherited as a dominant trait in the host, while virulence was recessive to avirulence in the pathogen. An interaction which involved only one parasite-host gene pair (*P/R*) specified incompatibility (i.e. no disease development), whereas other allelic pairs (e.g. *R/p*, *r/P*, *r/p*) specified compatibility (i.e. disease development).

However, the study of virulence/ avirulence genes in fungal-plant

interactions has remained rather primitive for two main reasons. First, the obligate parasitic nature of the highly specialised fungal pathogens (e.g. Basidiomycetes) limits genetic manipulation. Second, the complexity of eucaryotic genetics, the lack of sexual reproduction in many plant pathogens (the fungi Imperfecti), and the paucity of molecular biological techniques for the analysis of these fungal species has severely limited their analysis. Consequently, the use of bacterial-plant interactions as model systems for the study of pathogenesis has received increasing attention. The phytopathogenic prokaryotes offer many advantages over the fungal systems mentioned above. They may be cultured axenically, are amenable to an increasing spectrum of classical genetic techniques, and can be investigated by recombinant DNA technologies in a way that fungi cannot. Furthermore, several bacterial-plant interactions that involve gene-for-gene virulence relationships have been described, allowing complementary analysis of host and pathogen (Lacy & Patil, 1982; Panopoulos *et al.*, 1984).

A reductionist approach involving the use of non-pathogenic mutants appears to be the only practical way of studying the molecular bases of pathogenicity at the level of the bacterial-plant interaction (Staskawicz, 1983). The classical plant pathologist's approach which involves study of the events surrounding infection, or the biochemical consequences of the pathogenic process have not yielded fundamental information (Ellingboe, 1982). It has been recognised that "*too much of our current information is based on analysis of diseased tissue after the critical events in pathogenesis have occurred*" (Bateman & Basham, 1976). Only through fine dissection of the multifactorial process termed "pathogenicity" can a revolution in understanding be achieved.

It seems that genetic techniques promise to be useful tools in this dissection.

Classical approach

Genetic analyses of phytopathogenic bacteria began in the 1970s with the construction of chromosomal maps for several species (Chatterjee & Starr, 1977; Hooykaas *et al.*, 1982; Pugashetti *et al.*, 1978; see section 1:5). However, this has not contributed significantly to the analysis of pathogenesis itself.

Molecular biological approach

Recent work which involved recombinant DNA technology combined with an improved classical genetical approach has proved useful. Chemical and insertional mutagenesis techniques were used to isolate non-pathogenic mutants, and several "pathogenicity genes" have now been cloned (Daniels *et al.*, 1984; Niepold *et al.*, 1985; Staskawicz *et al.*, 1984). The cloning of such genes should be of great value in the study of the molecular basis of pathogenicity. Identification of the pathogen's *P* (or race-specificity: *rsi*) gene products may also permit the isolation of the hypothetical products of plant *R* genes (Panopoulos & Peet, 1985), or the plant cell factor that induces *A. tumefaciens vir* gene expression (Stachel *et al.*, 1986).

The isolation of non-pathogenic mutants and the cloning of "pathogenicity" genes is proceeding in many laboratories. Interest lies with several well-characterised bacterial pathogens including *A. tumefaciens* (see Panopoulos & Peet (1985) for review), *E. amylovora* (Bauer & Beer, 1985), *P. solanacearum* (Boucher *et al.*, 1985a,b), *P. syringae* pv. *glycinea* (Staskawicz *et al.*, 1984), *P. syringae* pv.

phaseolicola (Lindgren *et al.*, 1985) and *Xanthomonas campestris* pv. campestris (Daniels *et al.*, 1984; Turner *et al.*, 1985). An excellent review that summarises current knowledge of the molecular genetics of these and other phytopathogenic bacteria is now available (Panopoulos & Peet, 1985).

Plasmids and *Erwinia* pathogenicity

Plasmids are known to be associated with virulence in a number of plant pathogenic bacteria, notably *Agrobacterium tumefaciens*, *Rhizobium* spp. and *Pseudomonas syringae* pv. *savastanoi* (Panopoulos & Peet, 1985). However, analysis of exoenzyme and pathogenicity mutants of *Erwinia* spp. in a number of laboratories, has failed to implicate a role for plasmids in pathogenicity. It is known that the majority of *Ecc* and *Ecs* strains carry plasmids of various sizes, but no consistency in plasmid profiles was apparent (Forbes, 1983). Consequently, it is unlikely (but not impossible) that any *Erwinia* pathogenicity functions are plasmid-coded.

1:5 Genetic systems in *Erwinia* spp.

1:5:1 Development of genetic systems

Genetic systems are currently available in a range of phytopathogenic bacteria (Chatterjee & Starr, 1980; Leary & Fulbright, 1982; Panopoulos & Peet, 1985; Salmond, 1985; See table 1:5 and Chapter 4). However, when beginning work on a species or strain that is genetically uncharacterised, no assumptions can be made with regard to genome organisation (van Gijsegem, 1985) or the applicability of particular genetic techniques. Comparison with other closely-related species or strains in which useful genetic systems are available may well suggest a

TABLE 1:5 A chronology of genetic systems in *Erwinia* spp.

Species	Strain	System	Vector	Reference
E. amylovora	EA178	"Hfr" gene transfer	F'*lac*$^{+}$	Chatterjee & Starr, 1973
Echr	EC16	"	"	Chatterjee & Starr, 1977
Ecc	SCRI193	Chromosome mobilisation	R68::Mu	Perombelon & Boucher, 1978
E. herbicola	L244	Plasmid transformation	pBR322	Lacy & Sparks, 1979
Echr	EC16 + EC183	Chromosome mobilisation	R68.45	Chatterjee, 1980
Echr	EC183	Generalised transduction	ϕErch-12	Chatterjee & Brown, 1980
E. herbicola	Eh112Y	Tn*5* mutagenesis	pJB4JI	Gantotti *et al.* 1981
Echr	EC16	Chromosome mobilisation	F'::Tn*5*	Chatterjee *et al.*, 1982
Echr	3937j	"Hfr" gene transfer	F^{ts}*lac*::Tn*10*	Kotoujansky *et al.*, 1982
E. stewartii	SS104	Chromosome mobilisation	pDC752.1	McCammon & Coplin, 1982
E. amylovora	EA345	Transformation	pBR322	Bauer & Beer, 1983
Echr	EC16	Tn*5* mutagenesis	pJB4JI	Chatterjee *et al.*, 1983
Ecc	SCRI193	Chromosome mobilisation	R68::Mu, F'*lac*$^{+}$Tc	Forbes & Perombelon, 1985; Forbes, 1983
Echr	B374 + 3937j	"	pULB113	Schoonejans & Toussaint, 1983

Table 1:5 continued

Species	Strain	System	Vector	Ref
E. amylovora	various	Tn*5* mutagenesis	pJB4JI	Ste Bee
Ecc	Brig-P1A	Gene fusions	Mu d(Ap*lac*)	Jay 198
Echr	B374 + 3937j	Generalised transduction	ϕEC2	Res 198
Ecc & *Eca*	Ecc71 + Eca12	Tn*5* mutagenesis	pJB4JI	Zin 198
Ecc *Eca* *Echr* *E. amylovora*	Ecc193 Eca12 EC183 EA303	Chromosome mobilisation	pULB113	Cha *et*
Ecc	SCRI193	Plasmid transformation	pBR322 etc.	Hin 198
Ecc	"	Nonsense suppressor mutants	(pLM2)	Hin 198
Echr	3937j	Gene fusions	Mu d(Aplac)	Hug Pattat & Robert-Baudouy, 1985b
Echr	B374	Tn*9* mutagenesis	Mu*S*::Tn*9*	van Gijsegem *et al.*, 1985a
Echr	B374 + 3937j	Plasmid transformation	pBR322	Reverchon & Robert-Baudouy, 1985
Echr	CUCPB1237	Marker-exchange mutagenesis	pBR322::*pel C*::Kan	Roeder & Collmer, 1985b
Echr	3937j	"	pUC9	Diolez & Coleno, 1985
Ecc	SCRI193	Tn mutagenesis	λ	Salmond *et al.*, 1986
Eca	SCRI31			
Echr	B374 + 3937j			

Table 1:5 continued

Species	Strain	System	Vector	Reference
E. amylovora	various	Tn*5* mutagenesis	pJB4JI	Steinberger & Beer, 1983
Ecc	Brig-P1A	Gene fusions	Mu d(Ap*lac*)	Jayasawal *et al.*, 1984
Echr	B374 + 3937j	Generalised transduction	ϕEC2	Resibois *et al.*, 1984
Ecc & *Eca*	Ecc71 + Eca12	Tn*5* mutagenesis	pJB4JI	Zink *et al.*, 1984
Ecc *Eca* *Echr* *E. amylovora*	Ecc193 Eca12 EC183 EA303	Chromosome mobilisation	pULB113	Chatterjee *et al.*, 1985b
Ecc	SCRI193	Plasmid transformation	pBR322 etc.	Hinton *et al.*, 1985a
Ecc	"	Nonsense suppressor mutants	(pLM2)	Hinton *et al.*, 1985b
Echr	3937j	Gene fusions	Mu d(Aplac)	Hugouvieux-Cotte-Pattat & Robert-Baudouy, 1985b
Echr	B374	Tn*9* mutagenesis	Mu*S*::Tn*9*	van Gijsegem *et al.*, 1985a
Echr	B374 + 3937j	Plasmid transformation	pBR322	Reverchon & Robert-Baudouy, 1985
Echr	CUCPB1237	Marker-exchange mutagenesis	pBR322::*pel C* : Kan	Roeder & Collmer, 1985b
Echr	3937j	"	pUC9	Diolez & Coleno, 1985
Ecc	SCRI193	Tn mutagenesis	λ	Salmond *et al.*, 1986
Eca	SCRI31			
Echr	B374 + 3937j			

worthwhile approach. Two technologies (namely mutant isolation and gene transfer systems) which are commonly used in genetic analyses are discussed below.

Firstly, mutant isolation has proved useful in the study of innumerable systems throughout biology. Bacterial mutants can be generated by physical, chemical or insertional mutagenesis. The use of chemical or physical mutagens is straightforward and has been applied to a wide range of bacteria. However, the formation of multiple mutations may occur, thus complicating further analysis (e.g. the use of nitrosoguanidine; Guerola *et al.*, 1971). The choice of mutagen is dependent upon the types of mutants desired, and the uses to which they will be put. The use of insertional mutagens has a number of molecular genetical advantages, and these are outlined in Chapter 4. Unfortunately, the techniques for transposon mutagenesis are not generally applicable, and vary in their suitability for different species or strains.

Another common requirement in genetic investigations is a gene transfer system that allows linkage analysis. Enterobacterial systems include "Hfr" and "R-prime"-mediated chromosomal transfer, and both generalised and restricted transduction. These allow the mapping of particular mutations, and the introduction of specific mutant genes to new genetic backgrounds. Some examples of the development and application of such systems in *Erwinia* spp. are shown, in chronological order, in table 1:5.

The application of molecular genetic methods to *Erwinia* spp. particularly the soft rot erwinias, has proved quite straightforward. Various conjugation, transduction, transformation, plasmid isolation and

transposon mutagenesis systems are available (see tables 1:5, 3:1:1 and 4:3). However, all these systems exhibit great strain dependence, particularly transposon mutagenesis (D. Cardy, pers. comm.; Zink *et al.*, 1984). Despite this limitation, the application of genetic technology to *Erwinia* spp. has proved to be much simpler than for phytopathogenic *Pseudomonas* and *Xanthomonas* spp. or plant-associated *Agrobacterium* and *Rhizobium* spp. This reflects the taxonomic position of the *Erwinia* genus as discussed earlier (section 1:1). Because *Erwinia* is a member of the Enterobacteriaceae, it is not surprising that methods developed in other enterics have proved so useful in *Erwinia*. The genetic relatedness between *Erwinia* spp. and *E. coli* is demonstrated by the maintenance of the narrow host-range ColE1 replicon in many *Erwinia* strains (Bauer & Beer, 1983; Diolez & Coleno, 1985; Hinton *et al.*, 1985a; Lacy & Sparks, 1979; Roeder & Collmer, 1985b), and this fact alone has proved important for the development of gene cloning technology in *Erwinia*, as described in section 1:6.

A great problem in *Erwinia* genetics lies in the variety of strains of *Echr* and *Ecc* that are used in different laboratories. It is known that strains of the same species can be fairly dissimilar genetically, e.g. the genetic maps of the *Echr* strains EC16, B374 and 3937j (van Gijsegem, 1985). Therefore, information derived from one strain may not relate directly to another strain, and this may hamper progress of *Erwinia* research as a whole.

1:5:2 Application of genetic systems

The systems described in table 1:5 have been used to analyse various aspects of *Erwinia* metabolism and pathogenicity. Genetic maps have been constructed for three *Echr* strains (EC16, 3937j, B374) and one *Ecc*

strain (SCRI193) (see van Gijsegem, 1985). Details of cellulase and pectate degradation by *Echr* have been defined genetically (see Mills, 1985; Panopoulos & Peet, 1985) and biochemical and genetical aspects of lactose metabolism have been studied in *Echr* (Hugouvieux-Cotte-Pattat & Robert-Boudouy, 1985a).

However, only two examples of the use of genetic techniques to analyse *Erwinia* pathogenicity *per se* are available. First, Jayasawal *et al.*, (1984, 1985a) used Mu d(Ap*lac*) to mutagenise *Ecc*. Colonies (4700) were screened on potato tubers (under aerobic conditions), and 38 non-pathogenic mutants were obtained. Only three of these mutants proved to be non-pathogenic at low (0.2%) oxygen concentrations, of which two mutants were shown to be secretion-deficient for Pel, Peh and Prt or Peh and Prt (Jayasawal *et al.*, 1985a; Panopoulos & Peet, 1985). The third mutant was shown to be deficient in UDP glucose-pyrophosphorylase, which accounted for its non-pathogenicity (Jayasawal *et al.*, 1985a).

Pathogenicity was studied, indirectly, by Expert and Toussaint (1985). These workers selected mutants of *Echr* 3937jRH which were resistant to nine different bacteriocins. The mutants were non-pathogenic on african violet plants and fell into different classes, lacking one to three outer membrane proteins. Production of the outer membrane proteins was greatly enhanced under iron-limited growth conditions, suggesting a possible role for siderophores (Expert & Toussaint, 1985). These outer-membrane mutants may be the first host-recognition mutants of *Erwinia* to be isolated.

In summary, the mutants described above confirm the central role of Pel and Peh in soft rot and demonstrate a UDP glucose-pyrophosphorylase

requirement for *Ecc* pathogenicity. In addition, the bacteriocin-resistant mutants of *Echr* may reflect host recognition involvement in the *Erwinia*-plant interaction.

1:6 Gene cloning in *Erwinia* spp.

A number of reports concerning the molecular cloning of *Erwinia* genes have appeared recently (see table 1:6). The major breakthrough came in 1984, when it was shown that *Erwinia* genes which complemented particular lesions of *E. coli* could be cloned by direct selection. Furthermore, it was demonstrated that *E. coli* could express the structural genes for *Erwinia* extracellular enzymes, and it was possible to detect these enzyme activities by plate assay (European Molecular Biology Organisation (EMBO) Workshop on Soft-Rot Erwiniae; Marseille-Luminy, France, 23-27 July, 1984). These discoveries simplified all cloning experiments that involved extracellular enzyme structural genes, and other genes from *Erwinia* spp.

As discussed earlier, there is a great deal of interest in *Erwinia* research which involves Pel, because of its role in pathogenicity, secretion and pectate catabolism. However, repeated attempts to obtain mutations in the structural *pel* genes had proved unsuccessful, reflecting the existence of multiple *pel* genes in most strains (A. Chatterjee, pers. comm.; Andro *et al.*, 1984). Gene cloning technology has proved a means for the isolation of pel^+ and other structural genes, without the need for indirect assays or screening procedures to obtain particular recombinants. *Pel* genes have been cloned from ten strains of *Erwinia* spp. (table 1:6) and they exhibit

TABLE 1:6 Molecular cloning of *Erwinia* spp. genes in *E. coli*

Species	Strain	Gene(s)	References
Echr	EC16	*pelE*$^+$ *pelBC*$^+$	Keen *et al.*, 1984 Thurn & Chatterjee, 1985a
"	3937j	*pelADE*$^+$ *pelBC*$^+$ *cel*$^+$ *pem*$^+$	Kotoujansky *et al.*, 1985
"	B374	*pelADE*$^+$ *cel*$^+$ *prt*$^+$ *clb*$^+$	Reverchon *et al.*, 1985
"	"	*recA*$^+$	Faelen *et al.*, 1985
"	"	*cya*$^+$	Hedegaard & Danchin, 1985
"	CUCPB1237	*pelBC*$^+$ *pelDE*$^+$	Collmer *et al.*, 1985
"	3665	*pel*$^+$ *cel*$^+$ *clb*$^+$	Barras *et al.*, 1984b
"	3937j	*prt*$^+$	C. Wandersman, pers. comm.
"	1066	asparaginase	Gilbert *et al.*, 1986
Ecc	Ecc71	*pel*$^+$ (exo & endo forms) *peh*$^+$ *recA*$^+$	Zink & Chatterjee, 1985 Zink *et al.*, 1985
"	"	*prt*$^+$	Willis & Chatterjee, 1985
"	EC	*pelABC*$^+$ *peh*$^+$	Lei *et al.*, 1985a,c
"	"	*araB*$^+$ *araC*$^+$	Lei *et al.*, 1985b
"	EC100	*recA*$^+$	Keener *et al.*, 1984
"	EC153	*oriC polA*$^+$	Takeda *et al.*, 1982
"	SCRI193	*cysB*$^+$	Hinton *et al.*, 1985c
"	"	*pel*$^+$ *peh*$^+$ *pem*$^+$	G. Plastow, pers. comm.
"	EC14	*pel*$^+$ *peh*$^+$	Allen *et al.*, 1985

Table 1:6 continued

Species	Strain	Gene(s)	Reference
Eca	SR8	*pel*$^+$	Roberts *et al.*, 1985
E. amylovora	EA322	*recA*$^+$	Norelli *et al.*, 1985
"	?	*lpp*	Yamagata *et al.*, 1981
E. herbicola	ATCC39368	pigment operon	Perry *et al.*, 1985
"	265R6	*ice*	Orser *et al.*, 1985
E. stewartii	?	*cps*	Dolph & Coplin, 1985

Genotypes are as Bachmann (1983) wherever possible. The nomenclature used for *pel* is designated in accordance with the "proposals for uniform genetic nomenclature", EMBO Workshop on Soft Rot Erwiniae, Marseille-Luminy, July 1984. Genes A to E refer to Pel isozymes with acidic to basic pI.

differences in gene organisation as well as showing variations in the cellular localisation of the isozyme products, and plant tissue maceration properties. Up to five structural *pel* genes have been isolated from individual strains, confirming the presence of separate structural genes for different Pel isozymes. Clusters of the genes *pel*ABC$^+$, *pel*ADE$^+$, *pel*BC and *pel*DE$^+$ have been identified in different strains. However, the *pel*BC$^+$ and *pel*ADE$^+$ clusters have been shown to contain genes which are transcribed in opposite directions suggesting that they are not polycistronic (Collmer *et al.*, 1985; A. Kotoujansky, pers. comm.). Within strain CUCPB1237, different *pel*$^+$ structural genes share some homology, as determined by DNA hybridisation (Schoedel *et al.*, 1985), but no homology was detected between *pel*C$^+$ and *pel*E$^+$ of strain EC16 (Keen *et al.*, 1984). Recently, the nucleotide sequence of *pel*D$^+$ and *pel*E$^+$ from *Echr* strain have been determined (B. Staskawicz, pers. comm.). Comparison of the two sequences reveals four highly-conserved domains within the PelD and PelE proteins (G. Salmond, pers. comm.).

The Pel$^+$ clones encode periplasmically-located isozymes in *E. coli*, and usually cause maceration of potato tubers (Collmer *et al.*, 1985; Keen *et al.*, 1984; Kotoujansky *et al.*, 1985; Reverchon *et al.*, 1985). Variable amounts (up to 75%) of Pel activity have been detected extracellularly in some *E. coli* clones, but it is not clear whether this represents autolysis or active secretion (Collmer *et al.*, 1985; Keen *et al.*, 1984; M. Mount, pers. comm.). Several Pel$^+$ *E. coli* clones have been unable to macerate potato tubers, although the enzyme appears to be periplasmic (G. Lacy, pers. comm.; Lei *et al.*, 1985a). Collmer *et al.*, (1985) suggested that certain isozymes (e.g. PelC) have reduced macerating ability.

The Pel$^+$ clones are catabolite-repressible in *E. coli*, but are not induced by PGA (Collmer *et al., 1985; Keen et al.*, 1984; Kotoujansky *et al.*, 1985). However, the enzyme is not over-produced when clones are present in multicopy, perhaps suggesting some type of negative regulation.

Insertion mutagenesis has been used to inactivate cloned *pelC*$^+$ genes from two strains of *Echr* (Diolez & Coleno, 1985; Roeder & Collmer, 1985a,b). The *pelC*$^+$ gene cloned from strain CUCPB1237 was inactivated *in vitro*, and returned to the *Echr* chromsome by homogenotisation. The resulting strain did not produce PelC, but its virulence on potato tubers was unaffected (Roeder & Collmer, 1985a,b). As an alternative approach, the cloned *pelC*$^+$ gene from strain 3937j was inactivated by Mini-Mu-*lac* mutagenesis, and incorporated into the *Echr* chromosome, to make a mutant which lacked the PelC isozyme. PelC regulation was studied by assaying β-galactosidase levels under various conditions (Diolez & Coleno, 1985).

In the course of all of the cloning work described above, the utility of λ, ColE1 and RSF1010-derived vectors for the cloning of *Erwinia* genes was demonstrated. Subsequently, recombinant derivatives of the vectors pMMB33, pBR322 and pBR329 have been transferred to *Ecc* and *Echr* and successfully used in complementation studies (Faelen *et al.*, 1985; Roeder & Collmer, 1985a,b; Zink *et al.*, 1985). These experiments demonstrated that *Erwinia* genes could be cloned and manipulated in ColE1-derived vectors developed for use in *E. coli*, and did not require the use of specific broad host-range vectors (e.g. Franklin, 1985).

Tables 1:5 and 1:6 show that there are now no technical problems in manipulating *Erwinia* genes at the genetic or molecular level. Consequently, the use of *Erwinia* spp. as model systems for the study of pathogenesis and secretion should prove extremely productive.

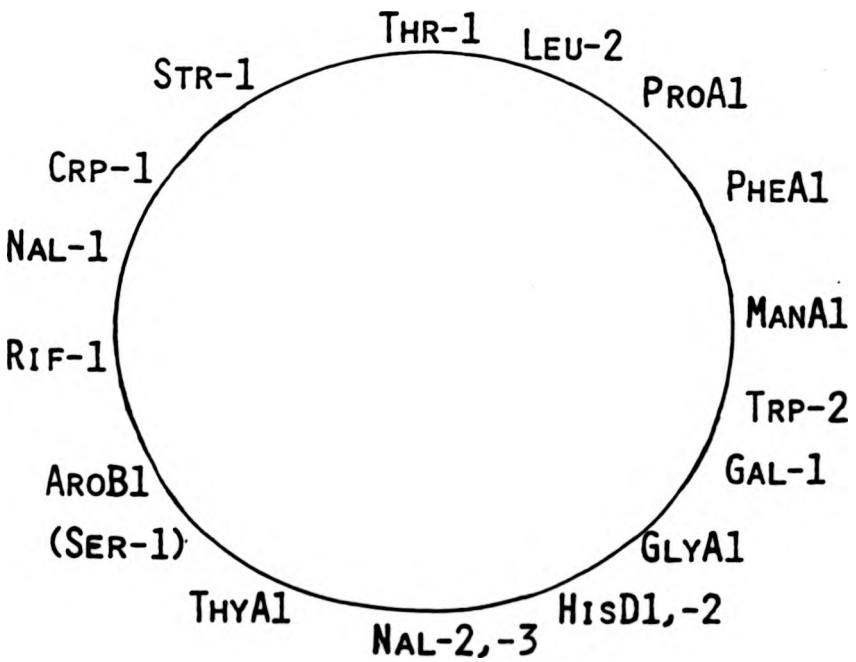
1.7 Aims of this work

This project was proposed and initiated in 1982, at a time when genetic analysis of *Ecc* was rudimentary (table 1:5). A classical genetical characterisation of *Ecc* strain SCRI193 had already been carried out (Forbes, 1983), and involved the conjugal mapping of chemically-induced mutations to give a seventeen marker genetic map (Fig. 1:7, table 1:5; Forbes & Perombelon, 1985; Perombelon & Boucher, 1978). Furthermore, standard plasmid screening techniques revealed no indigenous plasmids in SCRI193 (Forbes, 1983).

The long-term aim of the project was to study the genetics of phytopathogenicity of *Ecc*. However, in the short-term, work had to be directed to the development of genetic systems in SCRI193 to facilitate this genetic analysis. The priorities were the application of transposon mutagenesis and recombinant DNA technologies to SCRI193. It was hoped that, in the longer term, such genetic techniques would be applied to the analysis of *Ecc* phytopathogenicity and protein secretion.

Figure 1:7

Genetic map of SCRI193 (from Forbes, 1983, pers. comm.).



Chapter 2

Materials and Methods

2:1 Bacterial strains, bacteriophage strains and plasmids

The bacterial and bacteriophage strains, and the plasmids used in this study are listed in tables 2:1:1, 2:1:2 and 2:1:3. Derivatives of strains CSH26ΔF6, CB64, HC500 and SCRI193 which carried various pBR322-based plasmids were constructed by transformation, and are defined in the text.

2:2 Media

Solutions, growth media and assay media are listed in table 2:2:1, and were prepared in double-distilled water. To prevent precipitate formation, and to ensure reproducibility, all components of assay media were added in the order indicated. Media and solutions were sterilised by autoclaving at 121°C for 20 min. PGA and skim milk are degraded if autoclaved for longer periods. NB, LB, LM and DMM were solidified with 1.5% (w/v) Bacto agar, when necessary. Double Difco medium was used for phage work; DDA and soft DDA contained 1% and 0.6% (w/v) Bacto agar respectively. Minimal medium was supplemented with amino acids (20 μg/ml, final concentration) and sugars (0.2% (w/v), final concentration) when required. M9 minimal medium was only used in MIM, for spectrophotometric pectinase assays. Davis minimal medium was used routinely in minimal agar (DMA). MIM was supplemented with bacto yeast extract (YE), as defined in the text. Antibiotics were prepared as 100x final concentration stocks, and used at the concentrations shown in table 2:2:2. Rifampicin, tetracyline and chloramphenicol were dissolved in 50% (w/v) ethanol, and stored at -20°C. Nalidixic acid was dissolved in 30 mM NaOH and trimethoprim in 1% (w/v) lactic acid. All other antibiotics were

Table 2:1:1 Bacterial Strains

Strain	Characteristics	Plasmid phenotype	Source	Reference
E. coli K12				
LE392	F⁻,*hsd*R514(r_k^-,m_k^+), *sup*E44,*sup*F58,*lac*Y1 or Δ*lac*(IZY)6,*gal*K2, *gal*T22,*met*B1,*trp*R55, λ⁻	-	G.Mackinnon	de Bruijn & Lupski, 1984
QD*sup*F	F⁻,*pro*,*sup*F,λ⁻	-	G.Salmond	Wilson *et al.*, 1979
ED8812*rec*A	F⁻,*thr*,*leu*,*thi*,*lac*Y, *lacZ*(M15Δ),*ton*A,*sup*E, *hsd*S($r_k^-m_k^-$),*rec*A,λ⁻	-	E.Hitchin	N.Murray, pers. comm.
W3110	F⁻,sup°	-	D.Gill	Bachmann, 1972
DH1	F⁻,*rec*A1,*end*A1,*gyr*A96, *thi*-1,*hsd*R17($r_k^-m_k^+$), *sup*E44,*rel*A1?,λ⁻	-	C.Oakley	Hanahan, 1983
HB101	F⁻,*hsd*S20(r_B^-,m_B^-), *rec*A13,*ara*-14,*pro*A2, *lac*Y1,*gal*K2,*rps*L20, *xyl*-5,*mtl*-1,*sup*E44,λ⁻	-	N.Maitland	Boyer *et al.*, 1968
CSH26ΔF6	F⁻,*ara*,Δ(*lac*-*pro*),*thi*, *rps*L,Δ(*rec*A-*srl*)F6, *sup*°,λ⁻	-	D.Gill	Jones & Holland, 1984

Table 2:1:1 continued

Strain	Characteristics	Plasmid phenotype	Source	Reference
RB308	F^+,*deo*C,*lac*Y,*thy*A, Str^S,λ^-	-	D.Gill	Jones & Holland, 1984
DG37	HFr PO45,*rel*A1, Δ(*pts*I-*cys*A)127, *spo*T1,*thi*-1,λ^-	-	B.Bachmann	-
CB64	F^-,*trp*-75,*cys*B93,*tfr*-8	-	B.Bachmann	-
JM81A	F^-,*cys*C92,*tfr*-8?	-	B.Bachmann	-
JM221	F^-,*pro*-50,*his*-97,*trp*-74,*cys*D91,*arg*A,*ilv*A, *lac*,*gal*T47,*Xyl*,*mtl mal str*A *tsx*	-	B.Bachmann	-
JM15	F^-,*cys*E50,*tfr*-8	-	B.Bachmann	-
AT2455	Hfr PO1,*thi*-1,*cys*G44, *mal*-18,*rel*A1,*spo*T1,λ^-	-	B.Bachmann	-
JM246	F^-,*cys*I53,IN(*rrn*D-*rrn*E)1,λ^-	-	B.Bachmann	-
JM96	F^-,*thr*-1,*leu*B6,*trp*-1, *his*G1,*cys*H56,*arg*H1, *thi*-1,*ara*-13,*lac*Y1, *gal*-6,*mal*A1,*xyl*-7, *mtl*-2,*rps*L9,*ton*A2,λ^r, λ^-	-	B.Bachmann	-

Table 2:1:1 continued

Strain	Characteristics	Plasmid phenotype	Source	Reference
AT2427	*Hfr PO1*, *cysJ43*, *relA1* *thi-1*, *spoT1*, λ^-	-	B.Bachmann	-
UQ818	F^-, *lacZ4*, *cysS181*, *gyrA222*, *aroE24*, *metB*, *rpoB*, λ^-	-	B.Bachmann	-
J53*nal* (pJB4JI)	F^-, *pro*, *met*, *nal*	$Gm^r Sp^r Km^r$	M.Daniels	Beringer *et al.*, 1978
C600*nal* (pSP60)	F^-, *thi-1*, *thr-1*, *leuB6*, *lacY1*, *tonA21*, *supE44*, *nal*, λ^-	$Tp^r Km^r Ap^r$ Tc^r	G.Salmond	R.Simon, pers. comm.
C600*nal* (pSP601)	C600*nal*	$Tp^r Km^r Ap^r$ Tc^r	G.Salmond	R.Simon, pers. comm.
S17-1 (pSUP2021)	F^-, *thi*, *pro*, ($r_k^- m_k^+$), ::RP4-2(Tc::Mu) (Km::Tn*7*)	$Km^r Ap^r Cm^r$	R.Simon	Simon *et al.*, 1983a
OV2(pLM2)	F^-, ara_{am}, lac_{am}, $galU_{am}$, trp_{am}, *galE*, *leu*, *thyA*, *deo*, tsx_{am}, *thi*, *his*, *ilv*, $supF^{ts}$, λ^-	$Km^r Ap^{ts}$ Tc^{ts}	G.Salmond	pers. comm.
W3110(pLM2)	W3110 (ex HC107 x W3110 mating)	$Km^r Ap^s Tc^s$	This work	-

Table 2:1:1 continued

Strain	Characteristics	Plasmid phenotype	Source	Reference
QD*supF*(pLM2)	QDsupF (ex HC105 x QD*supF* mating)	$Km^rAp^rTc^r$	This work	-
J53(RP4-Km^s)	F^-,*pro*,*met*	Ap^rTc^r	A.Vivian	pers. comm.
W3110(RP4-Km^s::Tn*5*)	W3110	$Km^rAp^rTc^r$	This work	-
C600(R751)	C600	Tp^r	A.Vivian	Meyer & Shapiro, 1980
JC6310 (pUB307, pKT210)	*his*,*trp*,*lys*,*rpsL*,*recA*	Km^r,Tc^r, Sm^rCm^r	K. Derbyshire	-
Salmonella typhimurium				
PSA(pLM2)	*sup*$^+$,LT2	$Km^rAp^rTc^r$	L.Mindich	Mindich *et al.*, 1982
Erwinia carotovora subsp. *atroseptica*				
SCRI31	wild-type	-	M. Pérombelon	Hinton *et al.*, 1985a
BS209	wild-type	-		-
SCRI1043	wild-type	-	"	-
E. chrysanthemi				
B374	wild-type	-	A.Toussaint	Hamon & Peron, 1961
3937j	wild-type	-	A.Toussaint	Kotoujansky *et al.*, 1982

Table 2:1:1 continued

Strain	Characteristics	Plasmid phenotype	Source	Reference
1066	industrial asparaginase -producing strain	-	T.Atkinson	Gilbert *et al.*, 1985
E. carotovora subsp. *carotovora*				
C466	wild-type	-	M. Pérombleon	-
SCRI193	wild-type (= Kelman's SR44) isolated from potato	-	"	Pérombelon & Boucher, 1978
SCRI193 derivatives				
SCRI193 (pKT210)	from JC6310(pUB307, pKT210) x SCRI193 mating	Sm^rCm^r	This work	-
KF1005	*str*-1	-	K.Forbes	Forbes, 1983
KF1029	*rif*-1 *nal*-1	-	"	"
KF1033	*nal*-1 KF1005	-	"	"
KF1036	*str*-1,*nal*-2,*his*-2, *arg*-3,*cys*-1	-	"	"
HC105	KF1033(pLM2) from OV2(pLM2) x KF1033 mating	$Km^rAp^sTc^s$	This work	Hinton *et al.*, 1985b
HC106	*sup*-1 HC105 (EMS mutagenesis)	Km^rAp^{ts} Tc^r	This work	"
HC107	*sup*-2 HC105 (EMS mutagenesis)	Km^rAp^{ts} Tc^r	This work	"
HC122	KF1033(pJB4JI) "small" transconjugant from J53*nal*(pJB4JI) x KF1033 mating	$Gm^rSp^rKm^r$	This work	-

Table 2:1:1 continued

Strain	Characteristics	Plasmid phenotype	Source	Reference
HC123	KF1033(pJB4JI) "small" transconjugant from J53*nal*(pJB4JI) x KF1033 mating	$Gm^rSp^rKm^r$	This work	-
HC124	KF1033 *zzz*::Tn5 spontaneous "large" colony from HC122	$Gm^sSp^sKm^r$	This work	-
HC131	SCRI193(pHCP2) by transformation	Ap^r	This work	Salmond *et al.*, 1986
HC500	KF1033 *cysB*::Tn5 (Table 4:6:1; = mutant 15.29)	-	This work	Hinton *et al.*, 1985c

Other Tn*5* mutants of SCRI193 are described in table 4:6:1.

TABLE 2:1:2 Bacteriophages

Phage	Characteristics	Source	Reference
λ^+	wild-type	G. Salmond	
λ_{540}	*att*$^+$ *imm*^{21} *nin*	C. Henry	
λ_{467}	b_{221} *rex*::Tn5 cI_{857} O_{am29} P_{am80}	G. Mackinnon	de Bruijn & Lupski, 1984
$P1_{vir}$	virulent derivative of P1	K. Hussain	
T4GT7	generalised-transducing derivative of T4	G. Salmond	Wilson *et al.*, 1979
PRD1	*inc*P plasmid-specific	L. Mindich	Mindich *et al.*, 1982
PRD1*sus*-2	replication-amber derivative of PRD1	L. Mindich	Mindich *et al.*, 1982

TABLE 2:1:3 Plasmids

Plasmid	Characteristics	Phenotype	Source	Reference
pJB4JI	pPH1JI::Mu::Tn*5*	$Gm^rSm^rSp^rKm^r$	M.Daniels	Beringer *et al.*, 1978
pSP60	R751::Tn*1*,::Tn*5*, ::Tn*1771*	$Tp^rAp^rTc^rKm^r$	G.Salmond	R.Simon (pers comm.)
pSP601	R751::Mu,::Tn*1*, ::Tn*5*,::Tn*1771*	"	"	"
pLM2	RP1,Km^r,Ap^{am},Tc^{am}	variable	L.Mindich	Mindich *et al.* 1982
RP4-Km^s	Km^s derivative of RP4	Ap^rTc^r	A.Vivian	-
RP4-Km^s, ::Tn*5*	Tn*5*-carrying derivative of RP4-Km^s	$Ap^rTc^rKm^r$	-	This work
pUB307	Ap^s derivative of RP4	Km^r,Tc^r	K. Derbyshire	Bennett *et al.*, 1977
R751	-	Tp^r	A.Vivian	Meyer & Shapiro 1980
F	sex factor	-	D.Gill	Bukhari *et al.*, 1977
pBR322	multicopy cloning vector	Ap^rTc^r	Amersham	Bolivar *et al.*, 1977
pBR325	"	$Ap^rTc^rCm^r$	"	Bolivar, 1978
pKT210	broad host-range cloning vector	Sm^rCm^r	K. Derbyshire	Bagdasarian *et al.*, 1981
pSUP2021	pBR325::*mob*$^+$,::Tn*5*	$Ap^rCm^rKm^r$	R.Simon	Simon *et al.*,1983a

Table 2:1:3 continued

Plasmid	Characteristics	Phenotype	Source	Reference
pAT153	copy-number mutant of pBR322	Ap^rTc^r	M. Richardson	Twigg & Sherratt 1980
pJH1	pBR322::*cysB*::Tn*5*	$Ap^rTc^rKm^r$	-	This work
pJH3	pBR322::*cysB*$^+$	Ap^rTc^r	-	This work
pJH5	pBR322::*cysB*$^{+a}$	Ap^r	-	"
pJH6	b	"	-	"
pJH7	*Eco*RV deletion of pJH5	"	-	"
pJH10	pBR322::Tn*5* (Tn*5* insert at position 2950)	$Ap^rTc^rKm^r$	-	"
pPEL1	pBR322::*pel*-1$^+$	Ap^r	-	"
pPEL2	pBR322::*pel*-1$^+$ (opposite orientation to pPEL1)	"	-	"
pHCP2	pBR322::*lamB*$^+$	"	G.Mackinnon	Clement *et al.* 1982

a: pJH5 contained the 5 kb *Sal*I-*Eco*RI fragment from pJH3.

b: pJH6 contained the 7 kb *Sal*I-*Eco*RI fragment from pJH3.

TABLE 2:2:1 Media

Medium	Constituents per Litre[a]
Nutrient Broth (NB)	13 g Oxoid nutrient broth
Luria Broth (LB)	10 g Bacto tryptone 5 g Bacto yeast extract 5 g NaCl [pH 7.2]
LM	10 g Bacto tryptone 5 g Bacto yeast extract 10 ml 1 M NaCl 10 ml 1 M $MgSO_4$
SOB	20 g Bacto tryptone 5 g Bacto yeast extract 10 ml 1 M NaCl 2.5 ml 1 M KCl (10 ml 1 M $MgSO_4$, 1 M $MgCl_2$ filter sterile) [pH 6.8]
SOC	as SOB (+ 20 ml 1 M glucose)
Stab medium	as NB + 7 g Bacto agar
Double Difco medium (DD)	20 g Bacto tryptone 8 g NaCl (+ 10 ml 20% (w/v) maltose + 10 ml 1 M $MgSO_4$ for λ work)
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]

Table 2:2:1 continued

Media	Constituents per litre[a]
M9 Minimal Medium (M9 MM)	(100 ml 10x M9 salts) (10 ml 1 M $CaCl_2$, 0.1 M $MgSO_4$)
Davis Minimal Medium (10x) (DMM)	50 g NH_4Cl 10 g NH_4NO_3 20 g Na_2SO_4 anhydrous 30 g K_2HPO_4 anhydrous 10 g KH_2PO_4 anhydrous 1 g $MgSO_4.7H_2O$
Hershey salts	1.1 g NH_4Cl 15 mg $CaCl_2.2H_2O$ 0.2 g $MgCl_2.6H_2O$ 0.2 mg $FeCl_3.6H_2O$ 87 mg KH_2PO_4 anhydrous 12.1 g Tris-HCl [pH 7.4]
H medium	Hershey salts (20 ml 20% (w/v) glucose) (10 ml 2% (w/v) proline) (1 ml 0.1% (w/v) thiamine)
K medium	M9 MM (50 ml 20% (w/v) Bacto casamino acids) (0.1 ml 0.1% (w/v) thiamine)
Phage buffer	7 g Na_2HPO_4 anhydrous 3 g KH_2PO_4 anhydrous 5 g NaCl 2.5 g $MgSO_4.7H_2O$ 1 ml 1% (w/v) gelatin

Table 2:2:1 continued

Media	Constituents per litre[a]
Minimal Induction medium (MIM)	M9 MM (50 ml 2% (w/v) polygalacturonic acid) (9 ml 50% (v/v) glycerol) (Bacto yeast extract: see text)
Pectinase assay medium	15 g Bacto agar (5 ml 20% (w/v) Bacto yeast extract) (5 ml 20% (w/v) $(NH_4)_2SO_4$) (9 ml 50% (v/v) glycerol) (125 ml 20% (w/v) polygalacturonic acid) (100 ml phosphate buffer)
Phosphate buffer	14.96 g Na_2HPO_4 anhydrous 0.74 g $NaH_2PO_4.1H_2O$ [pH 8.0]
Cellulase assay medium	10 g carboxymethyl cellulose 15 g Bacto agar (100 ml DMM, 10x) (5 ml 20% (w/v) Bacto yeast extract) (4 ml 50% (v/v) glycerol)
Protease assay medium	15 g Bacto agar (100 ml DMM, 10x) (5 ml 20% (w/v) Bacto yeast extract) (4 ml 50% (v/v glycerol)
Freezing medium (2x)	126 g K_2HPO_4 anhydrous 0.9 g sodium citrate 0.18 g $MgSO_4.7H_2O$ 1.8 g $(NH_4)_2SO_4$ 3.6 g KH_2PO_4 anhydrous 88 g glycerol

[a]Items in brackets were added after autoclaving, from sterile stocks.

TABLE 2:2:2 Antibotics

Antibiotic	Abbreviation	Final concentration in media
sodium ampicillin	Ap	50 μg/ml (35 μg/ml to select transformants)
chloramphenicol	Cm	50 μg/ml
gentamicin sulphate	Gm	5 μg/ml
kanamycin sulphate	Km	50 μg/ml
neomycin	Nm	50 μg/ml
nalidixic acid	Nx	50 μg/ml
rifampicin	Rm	50 μg/ml
spectinomycin	Sp	50 μg/ml
streptomycin sulphate	Sm	100 μg/ml
tetracycline	Tc	10 μg/ml
trimethoprim	Tp	50 μg/ml

dissolved in double-distilled water and stored at 4°C.

TE was used in all DNA manipulations, and contained 10 mM Tris-HCl, 1 mM diaminoethanetetraacetic acid (EDTA), pH 8.0.

2:3 Chemicals

Media chemicals (table 2:2:1) were generally obtained from Fisons or BDH Chemicals and were of "Analar" grade. Polygalacturonic acid, carboxymethylcellulose, amino acids and vitamins were obtained from the Sigma Chemical Company. All other chemicals, with the exception of those listed below, were obtained from Sigma. Ethidium bromide, dimethyldichlorosilane and phenol came from BDH. Propan-2-ol, butan-1-ol, chloroform, iso-amylalcohol and acetic acid were purchased from May & Baker. Ethanol and methanol were reagent grade. Acrylamide, developer D19 and Unifix fixer were obtained from Eastman Kodak. N:N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylene-ethylenediamine (TEMED), ammonium persulphate, sodium dodecylsulphate (SDS), glycine and the protein assay concentrate were purchased from Bio-Rad. Caesium chloride and glycerol came from Fisons. Restriction enzymes and ^{35}S-methionine were obtained from Amersham International. pBR322 DNA came from Amersham International and Bethesda Research Laboratories. The chemicals for the Hanahan transformation method were all grade I from Sigma, except for glass-distilled dimethyl sulphoxide (DMSO) and hexamine cobalt (III) chloride which were purchased from Fluka.

2:4 Growth and maintenance of bacterial cultures

Culture maintenance

E. coli and *Ecc* strains were maintained on NB agar (NBA) containing appropriate antibiotics for up to 1 month at 4°C. For long term storage, strains were kept in stabs, at room temperature. Tn5 mutants were stored frozen to minimise secondary transposition events: an overnight NB culture was mixed with an equal volume of 2x freezing medium, and stored at -20°C.

Growth conditions

E. coli and *Ecc* cultures were inoculated at 37°C and 30°C respectively, unless stated otherwise in the text. Liquid cultures were generally grown in 25 ml universal tubes in a Gallenkamp Orbital Shaker (150 rpm). *E. coli* and *Ecc* cells used for transformation were grown in 250 ml flasks in a New Brunswick Gyrotory waterbath (275 rpm). Culture density was monitored at 550 nm or 600 nm with an LKB ultrospec 4050.

Cells were harvested by centrifugation in an MSE Multex or MSE Chilspin at 5000 rpm for 10 min. During manipulations involving Eppendorf tubes, centrifugation was performed in an MSE Microcentaur; cells were generally harvested by a 2 min spin at high speed.

2:5 Bacterial conjugation

Patch matings were performed by mixing a loopful of donor and recipient cells on NBA plates, incubating at 30°C overnight, and streaking onto selective media. When plasmid transfer frequencies were quantified,

donor and recipient cells were grown in static liquid culture overnight (without antibiotic selection), mixed in an Eppendorf tube (0.1 ml donor, 1 ml recipient) and concentrated by centrifugation in an MSE microcentaur (low speed, 1 min). The mating mixture was gently resuspended in 100 μl of NB, and spotted onto membranes (Gelman GA8, 0.2 μm) and incubated overnight on NBA at 30°C. Cells from the membrane were resuspended in 2 ml NB, and various dilutions spread on selective media. At the same time, appropriate media were used to determine the viable count of the recipient strain. Mating frequency was expressed as the number of transconjugants per recipient cell (TPR).
Counterselection was achieved by antibiotics (Nx, Rm or Sm) or by temperature (42°C) since SCRI193 is unable to grow at temperatures > 39°C.

2:6 Preparation of high-titre phage lysates

λ

λ_{467} lysates were prepared on the suppressing host LE392. An overnight culture of LE392 was grown in LB + 0.2% maltose, 10 mM $MgSO_4$. The culture (300 μl) was infected with 10^5-10^6 pfu of λ_{467} (i.e. 2 fresh plaques, resuspended in phage buffer) at room temperature for 10 min. Following the addition of 3 ml soft DDA, the mixture was overlayed upon a fresh, wet DDA plate (40 ml DDA per 9 cm Petri dish). Plates were incubated for *ca.* 8 hours (h), at 37°C, until confluent lysis was observed (by comparison with a phage-free control lawn). The top agar was removed with a glass spreader, and the plate washed with 3 ml of phage buffer. 0.5 ml of $CHCl_3$ (stored in foil-covered glass at room temperature) was added, and the mixture vortexed for at least 3 minutes.

Agar was removed by centrifugation in an MSE Chilspin (5000 rpm, 10 min, 4°C), and the supernatant decanted. Lysates were stored over a few drops of $CHCl_3$ at 4°C. This method yielded 1-5 x 10^{10} plaque forming units (pfu) per ml for λ_{467}. Lysates of other λ strains were made in the same way.

PRD1

Lysates of PRD1 and its derivative PRD1 *sus*-2 were prepared as described above. However, PSA (a suppressing derivative of *S. typhimurium* carrying RP4) was used as a host for this phage, and DDA medium was not supplemented with maltose or $MgSO_4$.

2:7 Use of PRD1 and PRD1 *sus*-2 to screen for Sup^+ phenotype

PRD1 (10^8 pfu) and PRD1 *sus*-2 were spotted onto seeded lawns of various strains, on unsupplemented DDA medium, and the plates were incubated at 30°C overnight. Turbid lysis indicated a positive reaction (i.e. lytic phage replication). The reversion frequency of PRD1 *sus*-2 when plated on a Sup^o strain was 3 x 10^{-8} per pfu.

2:8 Tn5 Mutagenesis

2:8:1 Transduction with λ_{467}

E. coli

The method of de Bruijn & Lupski (1984) was used to construct pBR322::Tn5 (pJH10). CSH26ΔF6 was shown to be sup^o using the pLM2 assay (appendix 1:4), and proved to be an efficient host for Tn5 mutagenesis.

Infection of 10^8 colony forming units (cfu) of CSH26ΔF6 with 10^7 plaque forming units (pfu) of λ_{467} gave rise to *ca.* 300 Km^r colonies. Using the restriction enzymes *Hin*dIII, *Pvu*II and *Sal*I, the Tn*5* in pJH10 was mapped to position 2960 bp in pBR322 (Peden *et al.*, 1983).

SCRI193

To infect HC131 with λ_{467}, a fresh overnight culture of HC131 was diluted 1:20 into 10 ml of NB Ap + 10 mM $MgSO_4$. This was incubated at 30°C (275 rpm) until a culture density of A_{550} = 0.8 was attained (*ca.* 2½ h). Cells were centrifuged in an MSE Multex (5,000 rpm, 5 min) and resuspended in 1 ml of the same fresh media, and a portion removed as a control for spontaneous Km resistance. A portion (4 x 10^8 pfu) of λ_{467} was added, and the cells were incubated at 30°C for 2 h. Subsequently, aliquots were spread on NBA Km, and incubated at 30°C for 36 h. This procedure yielded *ca.* 250 Km^r colonies per 10^7 pfu of λ_{467}.

2:8:2 Use of pJB4JI

Conjugal transfer of pJB4JI to *Ecc* recipients was done by the membrane filter method (section 2:5). Mating mixes were frozen at -20°C and used as required. Following plating in NBA Sm Km, "large" transconjugants (see section 4:4) were screened for mutant phenotypes by patching to various media containing Sm and Km. Tn*5*-induced auxotrophs were characterised by the pool plate method of Clowes & Hayes (1968).

2:8:3 Reversion of Tn*5*-induced auxotrophs

Mutants were grown in 10 ml NB cultures without antibiotic selection (2 (2 days (d), 30°C, 150 rpm), and cells were pelleted by centrifugation in an MSE Multex (5000 rpm, 10 min). Cells were resuspended in 1 ml DMM and transferred to 1.5 ml Eppendorf tubes following centrifugation in an

MSE Microcentaur (1 min, high speed). Cells were resuspended in a small volume of DMM, and plated on DMA + raffinose to select for prototrophic revertants. Revertant colonies arose after 3 d incubation at 30°C, and were tested for Km^r on DMA Km + raffinose.

2:9 Chemical mutagenesis

Chemical mutagenesis was used to isolate Sup^+ derivatives of SCRI193. A 10 ml overnight culture of HC105 in NB was treated with 200 µl of ethyl methane sulphonate (EMS), shaken vigorously to dissolve the mutagen, and maintained at 30°C for 2 h. Cells were centrifuged in an MSE Multex (5000 rpm, 10 min), and the pellet resuspended in a small volume of NB, and aliquots were spread on selective media. Viable counts were determined before and after mutagenesis, to quantify survival.

2:10 Spectrophotometric assays

Protein

Protein concentration was determined by the Bio-Rad assay method (BioRad bulletin 1069) and calibrated with bovine serum albumin standards (10-140 µg/ml).

Pectate lyase

To allow the simultaneous assay of a large number of samples, a thiobarbituric acid (TBA)-based assay was employed. The method was modified from Sherwood (1966). Supernatant or cell lysate (16 µl) was added to 80 µl substrate (1% (w/v) polygalacturonic acid, 0.25 mM $CaCl_2$,

100 mM Tris-HCl pH 8.5) and incubated in Eppendorf tubes at 37°C for 1 h. The reaction was stopped by the addition of 400 µl of 0.5 M HCl and 800 µl of 10 mM TBA reagent. Tube caps were pierced with a hot syringe needle and the tubes were placed in a boiling water bath for 1 h. A white precipitate was removed by centrifugation in an MSE Microcentaur (3 min, high speed), and the A_{548} of the supernatant was determined. A pink colour indicated Pel activity.

As an absorbance reference, active enzyme and substrate were added to HCl and TBA without incubation at 37°C. One unit (U) was defined as an increase in absorbance of 0.1 under these conditions (Bertheau *et al.*, 1984). In this assay, 16 µl of supernatant from an overnight culture of SCRI193 in minimal induction media, gave an A_{548} of 0.3-0.4.

2:11 Determination of Pel activity in culture supernatants and cell lysates

2:11:1 Determination of Pel activity throughout the growth phase of SCRI193

An overnight culture of SCRI193 in MIM + 0.1% YE was subcultured with 40 ml fresh medium in a 250 ml flask, to give a culture density of A_{600} = 0.01. This culture was incubated at 30° (275 rpm) in a New Brunswick Gyrotory waterbath. At hourly intervals, samples were removed for determination of culture density and for enzyme assay.

The procedure for the determination of Pel activity in cell lysates is essentially as Andro *et al.*, (1984). A 1 ml culture sample was transferred to an Eppendorf tube and centrifuged in an MSE Microcentaur

(2 min, low speed). The supernatant was decanted and frozen at -20°C until assay. Cells were washed once in 1 ml MIM to remove residual Pel, and then resuspended in 300 µl ice-cold 10 mM tris-HCl (pH 8.0). Lysozyme was added (10 µl of 3 mg/ml) and the tube vortexed and incubated on ice for 10 min. EDTA (25 µl of 250 mM, pH 8.0) was then added and the cells vortexed and maintained on ice until lysis. Debris and unlysed cells were removed by centrifugation in an MSE Microcentaur (5 min, high speed), and the supernatant decanted to a fresh tube. $CaCl_2$ was added to a final concentration of 20 mM to ensure an excess of Ca^{2+} ions which is required for Pel activity. Samples (16 µl) of lysate and supernatant were assayed for Pel as described previously (section 2:10:2). Lysates from samples of culture density A_{600}>1.0 contained 200-400 µg soluble protein per ml of original culture. Pel activity was expressed as units per ml of culture.

2:11:2 Rapid assay of intracellular and extracellular Pel

Aliquots (5 ml) of MIM + 0.3% YE containing appropriate antibiotics, were inoculated with 10 µl of a fresh overnight culture of the strains to be assayed. The cultures were grown at 30°C (150 rpm) for *ca.* 18 h, until a culture density of A_{600} = 1.5 was attained. Supernatant and cell lysate preparations from 1 ml culture samples were prepared as in section 2:11:1. The cell lysates contained 250-350 µg soluble protein per ml of original culture. Pel activity was expressed as units per mg soluble protein (Thurn & Chatterjee, 1985b).

2:12 Use of extracellular enzyme plate assays

Inoculation and incubation

Fresh colonies were picked and either streaked or patched onto Pel, Cel or Prt assay media containing the appropriate antibiotics. Plates were incubated for 24 h at 30°C for patches, or 48 h to allow the development of single colonies.

Prt assay

The medium described in table 2:2:1 was modified from a basic recipe of C. Wandersman (pers. comm.). A positive reaction was observed as a zone of clearing against the turbid background. Because this assay reaction did not require development with chemical reagents it was non-destructive.

Cel assay

The cellulase assay media was modified from that of Gilkes *et al.*, (1984) (table 2:2:1). Cellulase activity was revealed by flooding plates with 0.2% (w/v) Congo red for 15-20 min, followed by bleaching with 1 M NaCl for 10-15 min. Increased contrast was then obtained by flooding with 1 M HCl for 5 min. Cel^+ colonies gave a red translucent halo upon a dark blue background. The use of Congo red to stain carboxymethylcellulose was originally described by Teather & Wood (1982).

Pel assay

This assay is modified from Andro *et al.*, (1984), as described by A. Toussaint (pers. comm.) (table 2:2:1). Following incubation, plates were flooded with 1% (w/v) cetrimethyl ammonium bromide (CTAB), and agitated

gently (60 rpm) for 2-4 h. Pel^+ colonies gave a clear halo upon an opaque white background. The CTAB precipitated the non-degraded PGA to give this reaction (Jayasankar & Graham, 1970).

2:13 "Pathogenicity" testing

Tests were performed by the method of Collmer *et al.*, (1985), with modifications. Strains were grown in MIM + 0.3% YE for 20 h (30°C, 150 rpm). Cells were pelleted by centrifugation (5000 rpm, 5 min) and resuspended in the same medium at a density equivalent to A_{600} = 1.5. Store-bought potato tubers (Cultivar Maris Piper) were washed, surface sterilised for 10 min in NaOCl (5% available chlorine), and air-dried without rinsing. A 50 µl syringe (Hamilton Co.) was used to inject 10 µl of bacteria into a hole made by inserting a 200 µl micropipette tip 15 mm into an intact tuber. The injection points were then sealed with paraffin wax (heated to 60°C) and the tubers were wrapped in a triple layer of wet paper towels and cling-film. Tubers were sealed separately in polythene bags and incubated at 22°C for 3 days (this temperature was chosen to minimise rot by secondary pathogens; M. Pérombelon, pers. comm.). Following incubation, the tubers were sliced along the axis of injection, and the macerated tissue was scraped out and weighed. Non-inoculated tubers, and tubers injected with *E. coli* HB101 were used as controls. This tuber "pathogenicity" test should be considered as a bioassay, since it does not attempt to reproduce the events of the plant-pathogen interaction in the field.

2:14 Chromosomal DNA extraction from *Ecc*

The DNA extraction of Marmur *et al.*, (1961) was used with modifications. Cells were grown to stationary phase in 500 ml NB (30°C, 150 rpm, 16 h) The culture was harvested in an MSE Hi-spin 21 centrifuge using a 6 x 250 ml rotor (8,000 rpm, 10 min, 4°C). The pellet was resuspended in 36 ml TES (100 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, pH 8.0), transferred to two 50 ml polycarbonate "Oakridge" tubes, mixed with 4 ml lysozyme solution (10 mg/ml, in TES), and kept at 37°C for 30 min. SDS (20% (w/v); (2 ml) was added slowly with mixing, the tubes inverted several times, and stored at 60°C for *ca.* 15 min, until lysis. Sodium perchlorate (10 ml of 5 M) was then added to aid DNA-membrane separation. The solution was then transferred to universals and extracted with an equal volume of phenol saturated with TES; following gentle mixing of the solution to form an emulsion, the tubes were centrifuged in an MSE Chilspin (5000 rpm, 5 min, 4°C), and the upper aqueous layer decanted to a fresh universal. This extraction was repeated twice, before extraction with chloroform/iso-amyl alcohol (24:1). Sodium acetate (3 M, pH 4.8) was added to a final concentration of 0.3 M, and then an equal volume of 100% ethanol was added. Solutions were mixed gently and incubated on ice for 15 min, and the DNA collected by centrifugation in an MSE Chilspin (8000 rpm, 10 min, 4°C). The DNA pellet was dried under vacuum for 30 min, and resuspended in 15 ml TE (overnight, 4°C with gentle agitation). RNAaseA (2 mg/ml in TE, heat treated; Maniatis *et al.*, 1982) was added to a final concentration of 100 μg/ml, and the solution incubated at 37°C for 30 min. Following an extraction with phenol/chloroform (section 2:19) and an extraction with chloroform/iso-amyl alcohol, sodium acetate (3 M, pH 4.8) was added to the DNA solution to a final concentration of 0.3 M, and the DNA was

precipitated with an equal volume of 100% ethanol (ice, 15 min). Following centrifugation in an MSE Chilspin (8000 rpm, 10 min, 4°C) the DNA pellet was washed with 70% (v/v) ethanol, recentrifuged, and dried under vacuum (30 min). DNA was resuspended in 10-15 ml TE (overnight, 4°C, with gentle agitation).

2:15 Isolation of plasmid DNA

2:15:1 Large-scale preparation

The basic procedure was that of Clewell and Helinski (1970). *E. coli* cells were grown, in 500 ml NB containing appropriate antibiotics for plasmid selection, to 2-4 x 10^8 cells/ml (A_{600} = 0.5). To amplify the plasmid, spectinomycin was then added to a final concentration of 300 µg/ml, and incubated for a further 18 h. Cells were harvested in an MSE Hi-Spin 21 Centrifuge using 6 x 300 ml rotor (10,000 rpm, 10 min, 4°C), resuspended in 16.5 ml Tris-sucrose solution (0.05 M Tris-HCl, pH 8.0, 25% (w/v) sucrose) and transferred to siliconised 250 ml bottles (section 2:28). After adding 5 ml lysozyme (0.25 M Tris-HCl, pH 8.0, 5 mg/ml lysozyme), followed by storage on ice for 5 min, 4.5 ml EDTA (0.25 M, pH 8.0) was added and the solution was kept on ice for a further 5 min. Cells were lysed by the addition of 18 ml of lysis mix (0.05 M Tris-HCl, 0.0625 M EDTA, 2% Brij 58, 0.4% sodium deoxycholate, pH 8.0) and the mixture was inverted until it cleared. Incubation at 42°C for a few minutes aided this process. The lysed solution was transferred to 50 ml polycarbonate "Oakridge" tubes and the unlysed cells, cell debris and intact chromosomal DNA were pelleted in an MSE Hi-Spin 21 centrifuge using a 8 x 50 ml rotor (18,000 rpm, 20 min, 4°C).

The supernatant ("cleared lysate") was carefully decanted into a fresh tube, and was used to make three 10 ml CsCl gradients, or was frozen at 20°C until required. For a 10 ml gradient, 7.76 ml cleared lysate, 7.38 g CsCl and 0.27 ml ethidium bromide (2.5%, w/v) were gently mixed, and kept at room temperature until all the CsCl had dissolved. The R_I of the resulting solution was *ca.* 1.393, and this was poured into 10 ml Beckman polyallomer tubes and the remaining space was filled with liquid paraffin. Gradients were centrifuged in a Beckman L8 ultracentrifuge using a 70 Ti rotor (36,000 rpm, 60 h, 15°C). The plasmid DNA, visualised by long-wave UV light, was removed through the side of the tube with a size 21 needle and a 2 ml syringe. The DNA was extracted three times by vigorous shaking with an equal volume of propan-2-ol equilibrated with CsCl and TE (80 ml propan-2-ol, 20 g CsCl, 20 ml TE) followed by centrifugation in an MSE microcentaur (1 min, high speed). The upper layer, containing ethidium bromide, was discarded. The DNA solution was dialysed against 5 L of TE for 4 h, and then overnight against 5 L of fresh TE.

The procedure for the isolation of plasmid DNA from *Ecc* (SCRI193) was essentially as described above, except that cells were grown at 30°C without spectinomycin amplification, for 16 h (attempts to amplify with 300 μg/ml spectinomycin or 150 μg/ml chloramphenicol resulted in spontaneous cell lysis).

2:15:2 Rapid small-scale preparation

An identical procedure was used for the "mini-prep" of plasmid DNA from *E. coli* and *Ecc*, and is based on the method described by Maniatis *et*

al., (1982). Cultures were grown overnight in 5 ml NB with appropriate antibiotic selection Cells were harvested in an MSE Multex centrifuge (5000 rpm, 10 min), resuspended in phage buffer, and transferred to 1.5 ml Eppendorf tubes. All subsequent centrifugation steps were carried out in an MSE Microcentaur at high speed, at room temperature. After centrifugation (1 min), the supernatant was discarded and the cells were resuspended in the drop of liquid remaining in the tube. Ice-cold solution I (150 μl of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0) was added, mixed by gentle vortexing, and stored at room temperature for 5 min. Subsequently, 200 μl of solution II (0.2 M NaOH, 1% SDS; stable for 2 weeks at 4^{o}C) was added, and the tubes inverted rapidly three times and stored on ice for 5 min. Tubes were inspected, and the contents mixed by inversion as necessary to obtain an almost clear solution. Ice-cold potassium acetate (150 μl, pH 5.0; made by mixing 60 ml 5M potassium acetate with 11.5 ml glacial acetic acid and 28.5 ml of water) was added, and the tubes agitated immediately by three cycles of rapid inversion and momentary vortexing. Following at least 5 min storage on ice, the tubes were centrifuged (5 min) to remove unlysed cells, membrane-bound chromosomal DNA and cell debris, and the supernatant transferred to a fresh tube using a Gilson micropipetter. An equal volume (*ca.* 400 μl) of phenol/$CHCl_3$ (section 2:19) was added, the tubes vortexed for 5 sec, and the layers separated by centrifugation (1 min). The upper layer was transferred to a fresh tube containing 400 μl $CHCl_3$/iso-amyl alcohol (24:1), vortexed for 5 sec and centrifuged again (1 min). The upper layer was removed to a fresh tube, taking care to avoid disturbing proteinaceous material at the interface, and to this two volumes of 100% ethanol (room temperature) were added. Following 2 sec vortexing and 2 min incubation at room temperature, the DNA precipitate was harvested by centrifugation (5 min). The supernatant

was discarded, 1 ml of 70% ethanol (room temperature) was added to the pellet (but not mixed), and tubes were centrifuged again (5 min). The supernatant was carefully poured off, remaining drops were removed with a tissue, and the DNA pellet was dried for 10 min under vacuum. The DNA was resuspended in 50 μl TE by vortexing, and was then held at 65°C for 5 min. For each restriction digest 3 μl of the DNA solution was used.

2:16 Transformation

2:16:1 E. coli

Routine transformation with plasmid DNA was carried out as described previously using Grade I $CaCl_2$ (Sigma no. C-3881) (Maniatis *et al.*, 1982) throughout. This method gave a transformation efficiency of *ca.* 4 x 10^5 transformants per μg pBR322 DNA.

During the construction of gene libraries and the cloning of Tn*5* elements, certain modifications were employed. An overnight culture of HB101 was subcultured (1:50) and grown up in 50 ml SOB, in a 250 ml flask (275 rpm, 37°C) to A_{550} = 0.35. Cells were then treated as Maniatis *et al.*, (1982). Prechilled pipettes and Eppendorf tubes were used throughout. Following heat shock, cells were incubated in 10 ml SOB for 90 min at 37°C to allow antibiotic gene expression. These modifications yielded *ca.* 6 x 10^6 transformants per μg of pBR322 DNA.

2:16:2 *Erwinia* spp.

The transformation optimisation data presented in section 3:2 involved the use of the Hanahan (1983) method, except the *Ecc* cultures were grown at 30°C. The optimal method for SCRI193 is described below. This

yields between 10^3 and 10^4 SCRI193 transformants per μg of pBR322. The solutions for this procedure are described in table 2:16:2.

A single colony of SCRI193 was used to inoculate SOB and incubated without shaking at 37°C for 16 h. Next day, the culture was diluted 1:20 into 25 ml fresh SOB, and incubated in a 250 ml flask (275 rpm, 37°C) for 2-3 h. When the culture reached a density of A_{550} = 0.3 (*ca.* 5 x 10^7 cfu per ml), the flask was put on ice for 10 min. The culture was poured into a chilled universal, centrifuged in an MSE Chilspin (3000 rpm, 10 min, 4°C), and the pellet resuspended gently in 10 ml TFB. After 15 min on ice, cells were pelleted again (3000 rpm, 10 min, 4°C) and resuspended carefully in 2 ml fresh TFB. The resuspended cells were kept on ice, and 70 μl of freshly-thawed DMSO was added and dispersed by gentle swirling of the mixture. After 5 min, 70 μl of DTT stock was added, and the mixture was incubated for a further 10 min. Finally, 70 μl of DMSO was added, maintained on ice for 5 min, then dispersed in 210 μl aliquots to prechilled 1.5 ml eppendorf tubes. DNA was added in < 10 μl of TE (10-100 ng; *ca.* 5 μl of a "mini-prep") dispersed by low-speed vortexing (to prevent clumping of DNA) and stored on ice for 30 min. The DNA-cell mixture was heat-shocked by transferring the tubes to a 30°C waterbath for 6 min and then 800 μl SOC was added to each tube. Cells were incubated at 30°C for 70 min (200 rpm) to allow antibiotic gene expression, and the cells were pelleted by centrifugation in an MSE microcentaur (2 min, low speed). The supernatant was discarded, and the cells were carefully resuspended by tapping the tube several times. The transformants were carefully spread on fresh LM agar (containing 35 μg/ml Ap), with an unused bent short-form Pasteur-pipette. Colonies appeared after 24 h incubation at 30°C.

TABLE 2:16:2 Constituents of Hanahan transformation buffer (TFB)

Solution	Constituents	Source of chemical
DTT stock	2.25 m dithiothreitol (DTT)	Sigma
	40 mM potassium acetate (pH 6.0)	Sigma
TFB	10 mM K-MES (pH 6.2)	Sigma
	100 mM KCl	BDH
	45 mM $MnCl_2.4H_2O$	BDH
	10 mM $CaCl_2.2H_2O$	Sigma
	3 mM hexamine cobalt (III) chloride ($HACoCl_3$)	Fluka

The solutions listed in table 2:16:2 were prepared exactly as Hanahan (1983, 1985) in double-distilled water. KCl was substituted for RbCl; DMSO was purchased freshly-distilled (Fluka No. 41645), and stored immediately in 0.5 ml aliquots at -20°C. DMSO and DTT aliquots were discarded after a single use. TFB was made as a 5x stock, filter-sterilised and diluted in ultrapure water. TFB was stable at 4°C for at least 2 years.

2:17 Restriction endonuclease digestion

The low, medium and high salt buffers were prepared at 10x concentration and used as described by Maniatis *et al.*, (1982). BRL core buffer was used for the enzymes *Bgl*II, *Eco*RV, *Hin*dIII, and *Pst*I. After adding 1 µl of 10x restriction buffer for every 9 µl DNA, restriction enzyme was added and the digestion was carried out at 37°C for at least 90 min. RNase A (100 µg/ml) was added to restriction digests of plasmid DNA prepared by the small-scale method.

2:18 Ligation

Restricted DNAs were mixed in appropriate volumes of TE buffer. To subclone fragments, a 4:1 fragment:vector ratio was used with a DNA concentration > 50 µg/ml. To promote recircularisation, the DNA concentration was reduced to 10 µg/ml. The mixture was heated at 65°C for 5 min, and allowed to reanneal slowly on ice for 1 h. After adding appropriate amounts of 10x ligation buffer (4 mM ATP, 66 mM $MgCl_2$, 0.1 M DTT, 0.66 M Tris-HCl, pH 7.6) and T4 DNA ligase, the mixture was

incubated at 15°C for at least 18 h.

2:19 Extraction of DNA with phenol/chloroform

Phenol/chloroform mix was prepared by dissolving 100 g phenol and 100 mg 8-hydroxyquinoline in 100 ml chloroform and 4 ml iso-amyl alcohol. This was equilibrated by shaking the mixture with two changes of 0.2 volume 1 M Tris (pH 8.0) and two changes of 0.2 volume TE, before storage at 4°C. Using gentle shaking, DNA samples were mixed with an equal volume of phenol/chloroform, until an emulsion formed. The two phases were separated by centrifugation in an MSE Microcentaur (1 min, high speed). The upper aqueous phase was recovered, taking care not to disturb the interface, and the extraction process was repeated. A further extraction with chloroform/iso-amylalcohol (24:1) was carried out as above, to remove any remaining phenol. To maximise recovery, the organic phenolic phase was mixed with an equal volume of TE, mixed and centrifuged (1 min, high speed). The aqueous phase was then extracted with chloroform/iso-amylalcohol and pooled. DNA was recovered by ethanol precipitation.

2:20 Ethanol precipitation

To a DNA solution, half a volume of ammonium acetate (7.5 M, pH 7.5; Sigma # C-7262) and three volumes of ethanol (-20°C) were added, mixed by vortexing and chilled at -20°C overnight. The DNA was recovered by centrifugation in an MSE Microcentaur (10 min, high speed, room temperature). The supernatant was discarded and residual ethanol was

removed from the tube walls. The DNA pellet was dried under vacuum, and resuspended in TE buffer.

2:21 Agarose gel electrophoresis

Horizontal agarose slab gels were prepared by boiling agarose (Sigma, type I, #A-6013) in TAE electrophoresis buffer (0.04 M Tris-acetate, 0.001 M EDTA) and 0.5% (w/v) gels were used routinely. The agarose was cooled to *ca.* 50°C before pouring. DNA samples were prepared by adding 0.1 volume of loading buffer (0.25% bromophenol blue, 15% Ficoll type 400) and loaded into the gel slots. Electrophoresis was carried out with the gel completely submerged in electrophoresis buffer at 80-100 volts, or 25 volts when run overnight. DNA was visualised by transillumination with short-wave UV light (260 nm) and photographed using Polaroid Type 665 film.

Restriction fragment sizes were determined with the DNASIZE programme (G. Russell, pers. comm.), adapted from Schaffer & Sederoff (1981), and run on a BBC Model B microcomputer. Restriction mapping was performed essentially as described by Maniatis *et al.*, (1982).

2:22 Visualisation of high molecular weight plasmid DNA

This procedure was adapted from the method of Eckhardt (1978) by R. Simon (1984; pers. comm.) and employed flat-bed agarose gels. Plasmid-containing strains of *Ecc* or *E. coli* were grown in static culture overnight, in NB supplemented with appropriate antibiotics. A

0.5% flat-bed agarose gel (0.5% (w/v)) was prepared in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) without ethidium bromide. The gel was made with two sets of sample wells formed by a 3 mm pyrex strip taped behind a 2 mm sample well comb. Once the gel had set, the pyrex strip was removed, and the area behind the sample well comb was filled with molten solution I (0.8% (w/v) agarose, 2.0% (w/v) SDS in TBE). After 10 min, the front comb was removed, and the gel submerged in TBE in the electrophoresis tank. Samples of each bacterial culture (350 μl; *ca.* 10^8 cells) was centrifuged in an MSE Microcentaur (2 min, high speed), and resuspended in 40 μl of solution II (20% (w/v) sucrose, 100 μg/ml RNase A (heat-treated), 0.01% (w/v) bromophenol blue in TBE. Lysozyme was added to 0.2 mg/ml immediately before use). The resuspended cells were immediately loaded into the sample wells of the gel. Pre-electrophoresis at 5 volts for 20 min delivered the SDS to the sample wells, causing cell lysis, then the electrophoresis was performed at 100 volts for 10 h. The gel was stained for 30 min in 1 μg/ml ethidium bromide.

Plasmid molecular weight markers were R751 (51 Kb; Meyer & Shapiro, 1980), F (94.5 Kb; Bukhari *et al.*, 1977), RP4-Km^s (56 Kb; *in* Bukhari *et al.*, 1977), RP4-Km^s::Tn*5* (61 Kb; this work) and pJB4JI (98.5 Kb; Hirsch & Beringer, 1984).

2:23 Preparation of DNA fragments from agarose gels

The procedure published by Dretzen *et al.*, (1981) was used. DNA fragments were separated by electrophoresis in 0.5% to 1.5% (w/v) agarose gels (section 2:21). DNA bands were visualised with long wave

UV light only. The gel was rotated through 90°; slits made at one end of the DNA bands required, a piece of DEAE-cellulose (Whatman, DE81 paper; prewashed in 2.5 M NaCl overnight, washed several times in water, and stored in 1 mM EDTA at 4°C) placed into each split, and the gel squeezed firmly against the papers to close the incision. TAE buffer was added to the tank until it barely covered the surface of the gel. Electrophoresis was resumed until the DNA had entered the DEAE-cellulose paper strips. The DEAE-cellulose paper strips were blotted dry, placed in 1.5 ml Eppendorf tubes, and 400 µl of 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1.5 M NaCl was added. The papers were shredded by vortexing then incubated in foil-wrapped tubes at 37°C for 2 h, to elute the DNA. The mixture was transferred to eppendorf tubes containing a plug of siliconised glass wool (to trap the paper fibres). A small hole was made in the bottom of each tube, which was placed on the lip of another Eppendorf tube which in turn was placed in a universal bottle. After centrifugation for 1 min at 4000 rpm, the eluate was extracted with 3 volumes of water-saturated butan-1-ol, and the DNA was precipitated by adding 2 volumes of ethanol (section 2:20).

2:24 Construction, maintenance and screening of the SCRI193 gene library

Construction and maintenance

Chromosomal DNA from HC500 (section 2:14) and vector pBR322 DNA (section 2:15) was digested to completion with *Hin*dIII. The digested DNA was phenol-extracted and precipitated with ethanol (sections 2:19 & 2:20), and resuspended in 30 µl of TE. *Hin*dIII-cut chromosomal DNA (4 µg) was mixed with 1 µg of *Hin*dIII-cut pBR322, made up to 100 µl and ligated at

15°C for 48 h (section 2:18). Portions (12.5 µl) of the ligated mix were added to 100 µl aliquots of competent HB101 cells. Following transformation (section 2:16:1), cells were pooled and incubated in 10 ml SOB (90 min, 37°C) to allow gene expression. Then a portion was removed to determine transformation and cloning efficiency. Ap was added to the remainder (35 µg/ml), and the culture was incubated at 37°C overnight. Next day, the "amplified" library was mixed with an equal volume of 100% (v/v) glycerol, and stored at -20°C. This gene library was then screened for genes coding for Pel activity.

Screening

To isolate Pel^+ recombinants from the *Hin*dIII library of HC500, cells were diluted to give *ca.* 500 colonies per NBA Ap plate. Using velvets, colonies were replicated to NBA Ap and Pel Ap assay plates. Extracellular enzyme-producing colonies were detected as described in section 2:12.

2:25 Cloning of Tn*5* from HC500

The cloning of the Tn*5* element from HC500 was performed essentially as section 2:24:1. HC500 chromosomal DNA and pBR322 DNA were digested to completion with *Eco*RI. Following ligation, transformation and incubation in 10 ml SOB, to allow gene expression, cells were concentrated by centrifugation in an MSE Multex (3000 rpm, 10 min), and resuspended in 1 ml SOB. Aliquots (100 µl) were plated on ten NBA Km plates, to select colonies carrying the Tn*5* element.

2:26 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analysed on 10-30% exponential gradient SDS-polyacrylamide gels (Kelly & Dow, 1985; Laemmli, 1970). The constitution of buffer and solutions is given in table 2:26. Ammonium persulphate solution was always freshly prepared. Electrophoresis was carried out using a Bio-Rad Protean gel system, at a constant current of 16 mA for 12 h. ^{14}C-methylated proteins (Amersham) were used as molecular weight markers, and contained lysozyme (14,300), carbonic anhydrase (30,000), ovalbumin (46,000), bovine serum albumin (69,000), phosphorylase b (92,500) and myosin (200,000).

Following electrophoresis, gels were fixed in 10% (v/v) glacial acetic acid and 30% iso-propanol for 1 h, then transferred to a sheet of Whatman 3 MM paper, and dried in a Bio-Rad model 1125B gel drier (80^{o}C). Autoradiography was done at room temperature using Harmer film cassettes (without intensifying screens) and Kodak X-omat S X-ray film.

2:27 Gene product identification

Plasmid-encoded gene products were identified using the "Maxicell" system (Sancar *et al.*, 1979; Stoker *et al.*, 1984), by a method devised by D. Gill (pers. comm.). CSH26ΔF6 derivatives were grown in 15 ml K medium (Rupp *et al.*, 1971) to a culture density of A_{600} = 0.5 (2 x 10^8 cells/ml), and placed on ice. Cells (10 ml) were UV-irradiated using gentle agitation and a dose of *ca.* 50 J/m^2 in a Petri dish. These cells were transferred to a sterile universal, freshly prepared cycloserine was added to 200 μg/ml (final concentration), and the culture was

TABLE 2:26 SDS-PAGE buffers and solutions

A:	separating gel buffer	3 M Tris-HCl, pH 8.8
B:	stacking gel buffer	0.05 M Tris-HCl, pH 6.8
C:	low-bis acrylamide	60% (w/v) acrylamide 0.3% (w/v) N,N'-methylene-bis-acrylamide
D:	high-bis acrylamide	60% (w/v) acrylamide 1.6% (w/v) N,N'-methylene-bis-acrylamide
E:	stacking gel acrylamide	10% (w/v) acrylamide 0.5% (w/v) N,N'-methylene-bis-acrylamide
F:	electrophoresis buffer	0.025 M Tris-HCl 0.192 M glycine 0.1% (w/v) SDS
G:	sample buffer (2x)	0.0625 M Tris-HCl, pH 6.8 10% (v/v) glycerol 2% (w/v) SDS 5% (v/v) β-mercaptoethanol 0.01% (w/v) bromophenol blue

H: gel composition:

	Resolving Gel		Stacking Gel
	10 ml 30% gel	25 ml 10% gel	
Buffer A	1.25 ml	3.1 ml	-
Buffer B	-	-	2.4 ml
Solution C	5 ml	-	-
Solution D	-	4.2 ml	-
Solution E	-	-	3.0 ml
SDS, 10% (w/v)	0.1 ml	0.25 ml	0.1 ml
Glycerol, 75% (v/v)	3.65 ml	-	-
H_2O	-	17.45 ml	4.4 ml
Ammonium persulphate (10 mg/ml)	20 μl	50 μl	100 μl
TEMED	2 μl	5 μl	5μl

incubated for 14-16 h, at 37°C (150 rpm).

Cells were collected by centrifugation in an MSE Multex (5000 rpm, 5 min), washed twice with Hershey salts (Worcel & Burgi, 1974), and resuspended in 5 ml of Hershey medium (table 2:2:1). Following incubation at 37°C (150 rpm) for 1 h, 30 μCi of ^{35}S-methionine was added, and incubation continued for a further 1 h. Cells were harvested in an MSE multex (5000 rpm, 5 min), and washed twice in 10 mM Tris-HCl (pH 8.5). Finally cells were resuspended in 50 μl 10 mM Tris-HCl (pH 8.5) and added to 50 μl of 2x sample buffer (table 2:26). Samples were boiled for 5 min, to complete cell lysis and to solubilise protein, and then vortexed. Cell lysates were stored at -20°C and always reboiled before electrophoresis. The cell lysate was then loaded on to the gel (15 μl per slot).

2:28 Siliconisation of glassware

Clean, grease-free glassware was rinsed thoroughly with 2% (v/v) dimethyl dichlorosilane in CCl_4, and left to dry. Subsequently, glassware was rinsed with double-distilled water. No baking was required.

CHAPTER 3

TRANSFORMATION

3:1 Introduction

3:1:1 Historical perspective

Transformation may be defined as the introduction of DNA to competent bacterial cells, and its subsequent maintenance in the population. A transformation system is an essential tool for the introduction of non-conjugative plasmids into recipient strains. Furthermore, an efficient method facilitates the introduction of recombinant DNA molecules, thus permitting the use of a strain of interest as a host for subcloning and other *in vitro* DNA manipulations, without recourse to *E. coli* as an intermediate.

Transformation of *E. coli* with plasmid DNA was first described by Cohen *et al.* (1972), and involved the use of divalent cations at 0°C, followed by a heat shock. Subsequently, several treatments have proved effective in inducing competence for Gram-negative bacteria (table 3:1:1).

Three stages can be defined in the transformation of *E. coli*. Firstly, the DNA molecules adsorb to the bacterial cell surface (Weston *et al.*, 1981). This is followed by the heat-shock step, during which the DNA becomes insensitive to DNAase, and is presumably transported to a site within the periplasm, or inside the inner membrane (Bukau *et al.*, 1985). Finally, internalised plasmid DNA must overcome barriers within the cell (such as restriction sytems), and become established as a stable genetic element within the cell (Bergmans *et al.*, 1981).

Since its first description in 1972, a number of methods for enhancing the transformation frequency of *E. coli* have been described. Dagert & Ehrlich (1979) recommended prolonged incubation in $CaCl_2$; Reijnders *et*

TABLE 3:1:1 Plasmid transformation systems for Gram-negative bacteria

Species	Plasmid	Basis of Method	Maximum No. of transformants per μg of plasmid DNA	References
Agrobacterium tumefaciens	RP4	Freeze thaw + $MgCl_2$	10^3	Holsters *et al.*, 1978
Bordetella pertussis	RSF1010	Cohen-Wheeler broth	10^2	Weiss & Falkow, 1982
Citrobacter intermedius	native	$CaCl_2$ + $MgCl_2$	10	Prieto *et al.*, 1979
Enterobacter aerogenes	pBR322::*metA*$^+$	$CaCl_2$	10^3	Michaeli & Ron, 1983
Erwinia amylovora	pBR322	$CaCl_2$ + $RbCl_2$	10^5	Bauer & Beer, 1983
Ecc	pBR322	$CaCl_2$	1	Berman *et al.*, 1983
Ecc	pBR322	"Hanahan"	10^4	Hinton *et al.* 1985a
Echr	pBR322	$CaCl_2$ + $MgCl_2$	10^2	Reverchon & Robert-Baudouy, 1985
E. herbicola	pBR322	$CaCl_2$	10^2	Lacy & Sparks, 1979
Escherichia coli K12	pBR322	"Hanahan"	10^9	Hanahan, 1983, 1985
Klebsiella pneumoniae	pR0164	$CaCl_2$	10^3	Olsen *et al.*, 1982
Paracoccus denitrificans	RPI	$CaCl_2$ + $MgCl_2$	?	Spence & Barr, 1981
Pseudomonas aeruginosa	pR0164	$MgCl_2$	10^4	Olsen *et al.*, 1982
P. phaseolicola	pBR322	$CaCl_2$	10^3	Gantotti *et al.*, 1979
P. putida	pR0164	$MgCl_2$	10^3	Olsen *et al.*, 1982

Table 3.1.1 continued

Species	Plasmid	Basis of Method	Maximum No. of transformants per μg of plasmid DNA	References
P. solanacearum	pWI297	PEG	10^5	Morales & Sequeira, 1985
P. syringae	RSF1010	?	10^3	Lindow & Staskawicz, 1981
Rhizobium meliloti	pGV1106	$CaCl_2$ + $MgCl_2$	50	Kiss & Kalman, 1982
R. meliloti	pRK248	Freeze-thaw + $MgCl_2$	10^3	Selvaraj & Iyer, 1981
Salmonella typhimurium	pSC101	$CaCl_2$ + $MgCl_2$	10^6	Lederberg & Cohen, 1974
Serratia marcescens	pBR322	$CaCl_2$	10^3	Reid *et al.*, 1982

al. (1979) suggested the use of a $MgCl_2$ + $CaCl_2$ buffer, and Kushner (1978) proposed the inclusion of RbCl and DMSO to improve the competence of *E. coli*. For optimal transformation efficiency, a crucial factor appears to be the use of healthy cells in the exponential phase of growth (Brown *et al.*, 1979; Norgard *et al.*, 1978).

Recently, details of a novel transformation technique for *E. coli* and the effects of many of its components upon transformation efficiency, have been published (Hanahan, 1983, 1985). This technique involves the use of a defined mixture of monovalent and divalent cations and hexamine cobalt (III) chloride, with sequential addition of DMSO and DTT. When combined with the use of Mg^{2+} ions in all growth media, the Hanahan method allows transformation frequencies of 10^8 per μg of pBR322 DNA to be attained reproducibly in certain strains of *E. coli* K12. Moreover, the technique reduces the growth phase-dependence of efficient transformation, allowing greater reproducibility between experiments.

3:1:2 Mechanism of transformation

Despite the concerted efforts of a number of workers, the mechanism of transformation remains obscure. It is clear that within a population of competent *E. coli* cells only 0.1% to 1% of the cells are able to take up plasmid DNA (Sabelnikov *et al.*, 1984). Several hypotheses have been proposed to explain this; Bergmans *et al.* (1981) suggested that these cells had received minimal damage to their outer membrane. However, Hanahan (1983) proposed that the limiting factors in competence were genetic or physiological events which occurred after DNA uptake, and so did not involve the outer membrane.

It is thought that the standard $CaCl_2$ transformation procedure causes

altered membrane conformation which facilitates DNA uptake, but it is not clear whether specific outer membrane proteins, transmembrane pores or other membrane components are involved (Humphreys *et al.*, 1979). Van Die *et al.* (1983a,b) demonstrated that the heat shock step induced phase transitions of membrane lipids which could facilitate DNA uptake. The role of phase transitions was also discussed by Hanahan (1983) who postulated that they caused membrane crystallisation, thereby increasing the accessibility of certain cell envelope channels to the DNA. However, the mechanism of the Hanahan method itself has yet to be elucidated. Hanahan (1983) proposed that the ability of divalent cations to bind membranal phosphate moieties, and the ability of DMSO to stabilise ionic interactions at membrane surfaces facilitated association between the phosphate-rich DNA and the cell surface phospholipid and lipopolysaccharide. Finally, he proposed that hexamine cobalt (III) chloride acted as an analogue of vitamin B12 (cobalamin), and that DNA uptake was linked to the active transport of this vitamin (Holroyd & Bradbeer, 1984).

3:1:3 Transformation of bacterial species other than *E. coli*

Methods have been developed for the transformation of various phytopathogenic and plant-associated bacteria, including strains of *Agrobacterium*, *Erwinia*, *Pseudomonas* and *Rhizobium* (table 3:1:1). All these procedures involve the induction of artificial competence, but this has proved unnecessary for one strain of *Pseudomonas solanacearum* which is naturally competent (C. Boucher, pers. comm., Là *et al.*, 1978). At the commencement of this work, only the method of Berman *et al.*, (1983) was available for the transformation of soft rot erwinias. Extrapolation from the authors data gives a frequency of less than one transformant per μg of pBR322 DNA (in a transformation mix containing

10^8 cells). Recently, more efficient methods have been described for *Echr* (table 3:1:1, Reverchon & Robert-Baudouy, 1985).

As outlined in section 3:1:2, the basis of the induction of competence in Gram-negative bacteria has been the subject of much speculation, but remains poorly understood (Benzinger, 1978). Even the transformation systems which have been extensively characterised, exhibit a high degree of strain specificity (Hanahan, 1985; Reverchon & Robert-Baudouy, 1985). Consequently, the development of a transformation system in a new strain or species requires an empirical approach. In an attempt to find a transformation system for SCRI193, a number of published techniques were tested. It was hoped that an efficient transformation system would simplify subsequent molecular genetic manipulations of SCRI193.

RESULTS & DISCUSSION

3:2 Development of a transformation system for SCRI193

3:2:1 Preliminary experiments

Six published methods were used in an attempt to induce competence of SCRI193 (Bauer & Beer, 1983; Dityatkin *et al.*, 1972; Enea *et al.*, 1975; Fornari & Kaplan, 1982; Maniatis *et al.*, 1982; Olsen *et al.*, 1982). At the time these experiments were performed, it was not known whether the narrow host-range ColE1 replicon would be maintained in SCRI193. Consequently, transformation experiments were carried out with CsCl-purified pBR322 and pKT210 DNA (pKT210 is a broad host-range multicopy cloning vector; Bagdasarian *et al.*, 1981). After mobilisation of the plasmid from JC6310(pUB307,pKT210) to SCRI193, it was detected in the

transconjugants using a "mini" plasmid screen, and by the Sm^r Cm^r phenotype. This confirmed that the RSF1010 replicon was able to replicate in SCRI193.

Despite the use of 1 μg of each plasmid DNA per experiment, none of these six methods yielded transformant colonies. Control experiments with *E. coli* ED8812*recA*, yielded between 10^3 and 10^4 transformants per μg pBR322 DNA, for five of the six methods. However, due to the success in the use of $CaCl_2$ and/or $MgCl_2$ treatments to induce competence in a range of bacteria (table 3:1:1), two methods which involved these compounds were tried repeatedly. Both the procedures of Maniatis *et al.*, (1982; modified by substitution of 150 mM $CaCl_2$ for 100 mM $CaCl_2$) and Morrison (1979) yielded 4 transformants per μg of pKT210 DNA (Hinton *et al.*, 1985a). All transformants were Sm^r Cm^r (8/8), and carried an 11.8 Kb plasmid which comigrated with pKT210 (data not shown).

Unfortunately these systems were not efficient enough to be used routinely either for the introduction of plasmids isolated by "mini-preps", or for *in vitro*-manipulated recombinant plasmids. Therefore, experiments were continued, with the novel method of Hanahan (1983).

3:2:2 Application of the Hanahan method to SCRI193 transformation

Certain modifications of the Hanahan procedure allowed efficient and reproducible transformation frequencies to be obtained for SCRI193, ranging from 2×10^3 to 4×10^4 transformants per μg of plasmid DNA (table 3:2:2). In an attempt to optimise conditions, experiments were done to determine the effect of specific components of the system upon transformation efficiency.

TABLE 3:2:2 Transformation of various *Erwinia* spp. with the plasmids pAT153, pBR322 and pBR325 by the modified Hanahan method

Strain	Plasmid	Transformation frequency/μg DNA Range	Mean	No. of Experiments
SCRI193	pAT153	1×10^3 - 7×10^3	4.1×10^3	2
SCRI193	pBR322	1×10^3 - 8×10^3	4.2×10^3	4
SCRI193	pBR325	-	4.0×10^4	1
HC500	pBR322	1×10^3 - 4×10^4	5.0×10^3	5
KF1005	pBR322	-	1.2×10^3	1
KF1029	pBR325	-	40	1
KF1033	pBR322	-	3.4×10^3	1
KF1033	pBR325	-	4.0×10^3	1
KF1036	pBR322	-	40	1
B374	pBR322	-	10	1
SCRI31	pBR322	1×10^2 - 1×10^3	4.4×10^2	3
SCRI1043	pBR322	-	32	1

Plasmid pBR322 was used in these optimisation studies, because the ColE1-derived *mob*$^+$ vector pSUP2021 had already been shown to replicate in SCRI193 (section 4:4). Much of these results have been published previously (Hinton *et al.*, 1985a).

3:2:3 Effect of treatment on cell viability

Following treatment of 1.5 x 10^8 cfu of SCRI193 by the Hanahan method, 1.1 x 10^8 cfu remained viable. This represents a survival of 73% compared with 78% reported for *E. coli* (Hanahan, 1983), and 50% for *Echr* (Reverchon & Robert-Baudouy, 1985).

3:2:4 Effect of DNA concentration

Hanahan (1983) reported that optimum transformation efficiencies of *E. coli* were achieved with 1-10 ng of plasmid DNA and 210 μl of competent cells,but that the addition of 200 ng of DNA reduced the transformation efficiency ten-fold. To determine if SCRI193 transformation was similarly affected by DNA concentration, various amounts of pBR322 DNA (10 ng to 300 ng) were used to transform 210 μl of competent cells. However, transformation efficiency was not affected by these DNA concentrations (2-3 x 10^3 transformants per μg of pBR322 DNA).

3:2:5 Effect of growth phase

Seven cultures of SCRI193 were grown to various densities ranging from 2.5 x 10^7 to 6.8 x 10^7 cfu/ml, corresponding to early to mid-log phase of growth. Transformation efficiencies for these cultures were similar, ranging from 1-8 x 10^3 per μg of pBR322 DNA. In contrast, the transformation frequency obtained for cells taken from a late log phase culture (2.5 x 10^8 cfu/ml), was reduced to 1 x 10^2 per μg of pBR322 DNA.

These results agree with those of Hanahan (1983), who found similar efficiencies of transformation for log phase *E. coli* cultures of densities ranging from 2-9 x 10^7 cfu/ml, and a reduction thereafter (i.e. optimum efficiencies were obtained with mid-log phase cultures).

3:2:6 Effect of heat-shock

Various heat-shock regimes were studied in order to optimise the transformation system. HC500 cells were grown at 37°C before treatment, and subjected to the standard transformation protocol. The effect of different heat-shock conditions was assessed by treating aliquots of competent cells, plus 100 ng of pBR322 DNA, in parallel.

The influence of heat-shock temperature on transformation efficiency is shown in Figure 3:2:6a. The 30°C heat-shock gave the highest transformation efficiency.

To determine the optimum duration of heat-shock, aliquots were treated at 30°C for various times. The results of two independent experiments involving from 0 to 18 min heat shock are presented in Figure 3:2:6b. Best results were obtained with a heat shock of 6 min at 30°C, and these conditions were used routinely for subsequent experiments. This treatment gives three-fold more transformants of SCRI193 than the 2 min/42°C heat-shock recommended by Hanahan (1983).

The 6 min/30°C heat-shock is similar to that described by van Die *et al* (1983a) for *E. coli*, and by Reverchon & Robert-Baudouy (1985) for *Echr.*

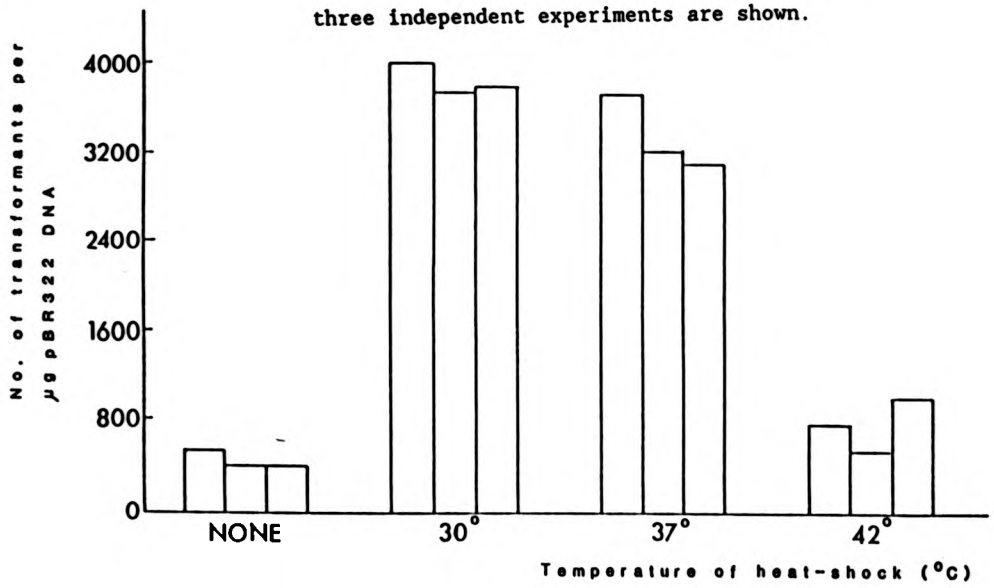
3:2:7 Effect of other components on transformation efficiency

The effect of several elements of the Hanahan procedure upon

Figure 3:2:6a

The effect of heat-shock temperature upon the efficiency of transformation of SCRI193 with pBR322.

Following transformation, cells were heat-shocked for 6 min at 30°C, 37°C or 42°C. A non-heat-shocked control was included. The results of three independent experiments are shown.



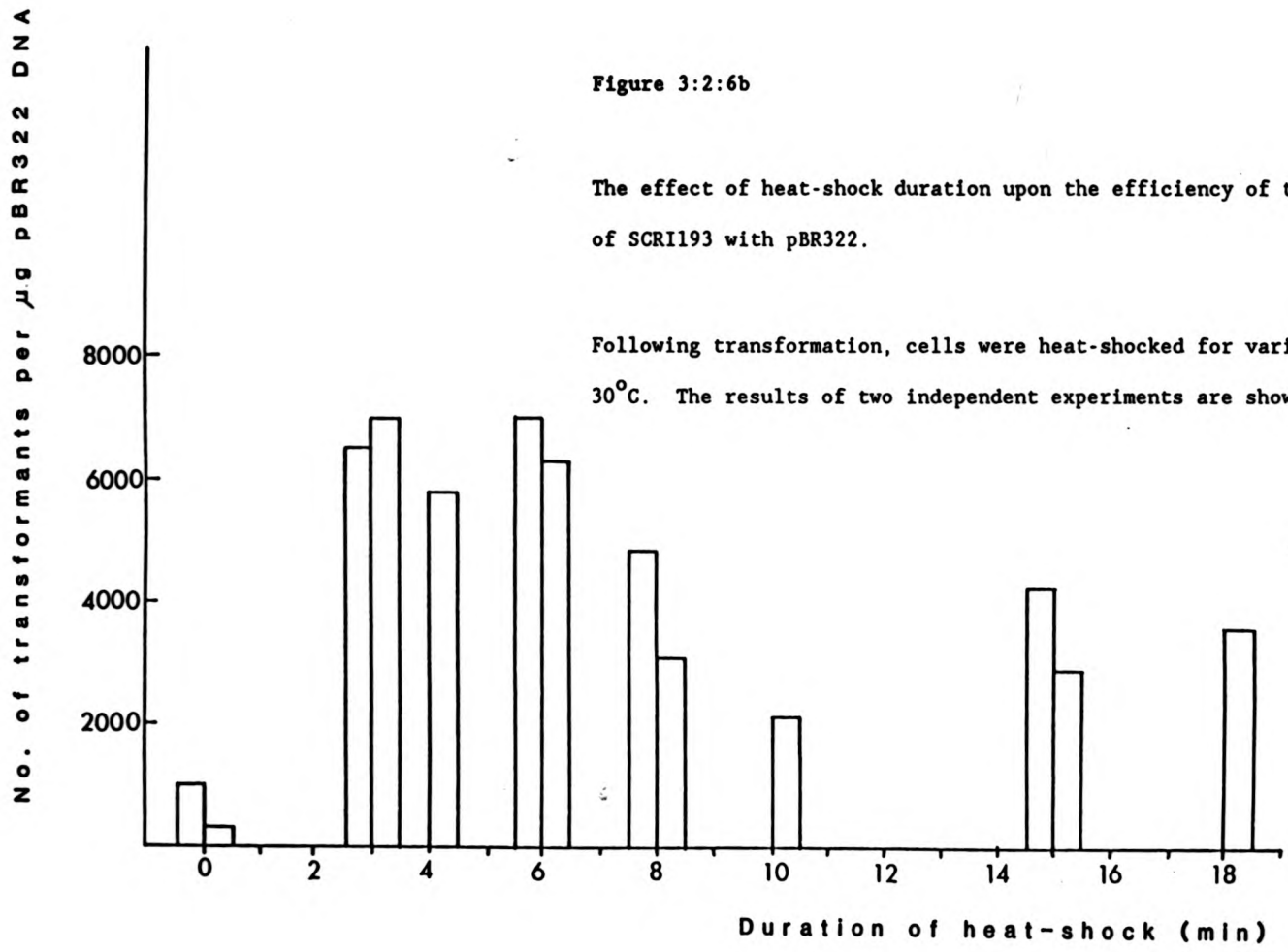


Figure 3:2:6b

The effect of heat-shock duration upon the efficiency of transformation of SCRI193 with pBR322.

Following transformation, cells were heat-shocked for various times at 30°C. The results of two independent experiments are shown.

transformation efficiency was determined by subjecting aliquots of SCRI193 cells, grown at 30°C and concentrated in TFB, to various treatments (Fig. 3:2:7). The omission of DMSO and/or DTT reduced transformation efficiency by up to ten-fold. The substitution of DMF for DMSO reduced the efficiency two-fold. Three modifications which were reported to increase transformation efficiency for *E. amylovora* (Bauer & Beer, 1983) were shown to have the opposite effect for SCRI193. Firstly, overnight incubation of cells in TFB, prior to the addition of DMSO, DTT, and DNA, resulted in a reduction of transformation efficiency by thirty-fold. Secondly, the inclusion of a 30 min incubation on ice, subsequent to heat-shock, reduced the transformation frequency by one quarter. Finally, the combination of this step with a freeze shock prior to heat shock reduced the efficiency three-fold. The importance of DMSO and DTT in inducing competence in SCRI193 was also observed for *E. coli* (Hanahan, 1983, 1985), but in contrast to the *E. coli* results, DMF could not be substituted for DMSO without reducing the transformation efficiency of SCRI193.

In summary, no alteration in the basic transformation procedure led to increased tranformation efficiency. However, it was observed that the growth of SCRI193 at 37°C prior to treatment, doubled the transformation efficiency. These findings were used to devise the optimised transformation procedure described in section 2:16:2. The effect of elevated temperature upon transformation efficiency may reflect altered phase transitions in the membrane, and similar observations have been made for *E. coli* (van Die *et al.*, 1983a,b).

Figure 3:2:7

The effect of components upon the efficiency of transformation of SCRI193 with pBR322.

The transformation treatments were:

(a) + DMSO, + DTT, cells grown at 37°C prior to transformation

(b) + DMSO, + DTT

(c) + DMF, + DTT

(d) - DMSO, + DTT

(e) + DMSO, - DTT

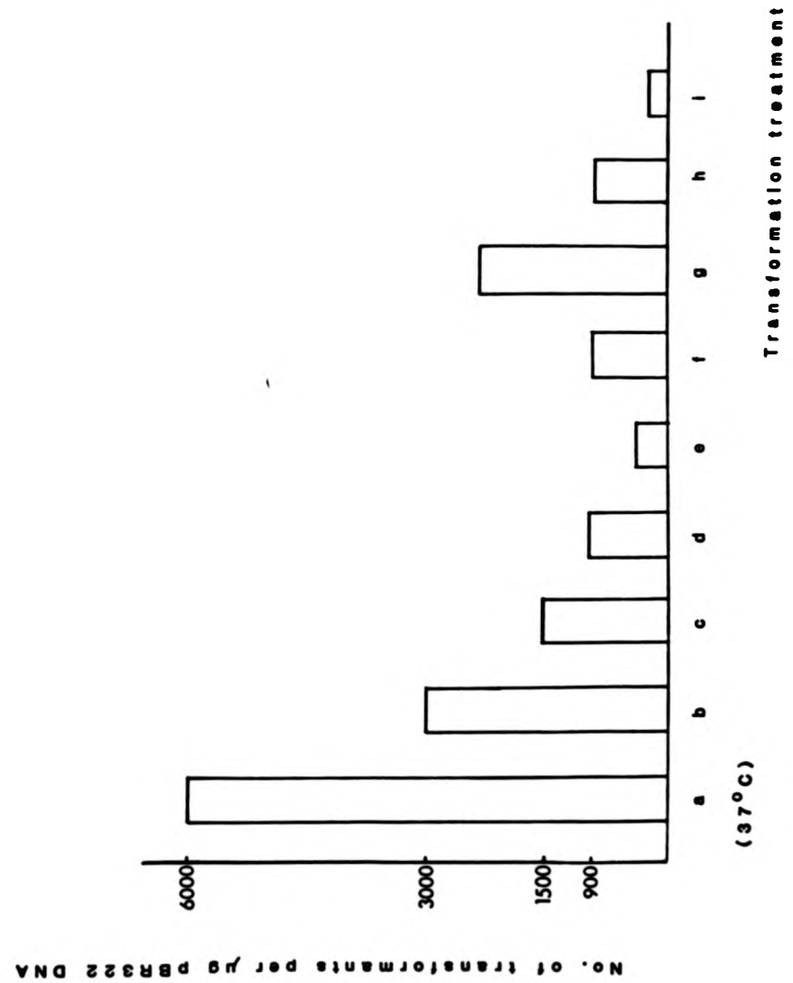
(f) - DMSO, - DTT

(g) Cells maintained on ice for 30 min post-heat-shock

(h) Cells subjected to freeze-thaw prior to heat-shock

(i) Cells maintained in TFB for 20 h (4°C) *before* transformation

For treatments (b)-(i), cells were grown at 30°C prior to transformation.



3:3 Genetical and physical studies of transformants

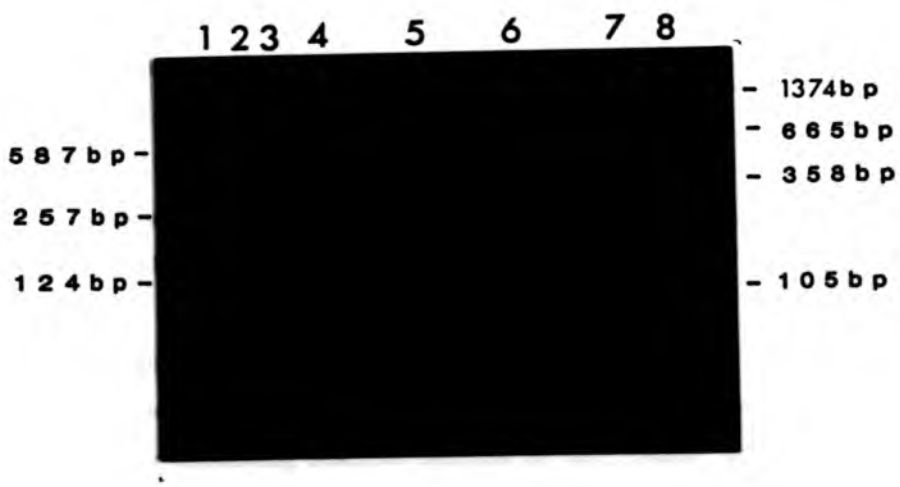
Confirmation that the antibiotic-resistant colonies obtained by the Hanahan method (section 3:2) were true transformants of SCRI193 was given by the results outlined below. Firstly, although transformants were selected on the basis of a single antibiotic resistance (generally Ap^r), they inherited the other unselected marker, in all cases, i.e. all Ap^r colonies which arose from transformation of SCRI193 with pAT153, pBR322 and pBR325 were simultaneously Tc^r (20/20, 85/85, 55/55 respectively). Secondly, no antibiotic-resistant colonies appeared when plasmid DNA was omitted from the transformation assay (spontaneous Ap^r of SCRI193 is $< 5 \times 10^9$ per cell). Thirdly, SCRI193(pBR322) and SCRI193(pBR325) transformants were Pel^+, Cel^+, Prt^+, prototrophic, able to utilise raffinose and unable to ferment α-methylglucoside (see table 1:1). The transformants also retained the ability to rot potato tuber slices (M. Perombelon, pers. comm.). Finally, the presence of the plasmids in the transformants was confirmed by physical methods. Plasmid DNA was prepared from the SCRI193(pAT153), SCRI193(pBR322) and SCRI193(pBR325) transformants by the boiling method (Holmes & Quigley, 1981), and detected after agarose gel electrophoresis. In all cases, plasmid bands were observed which comigrated with the appropriate monomeric forms of the three plasmid controls (data not shown).

A more detailed characterisation of the plasmid carried by SCRI193(pBR322) was carried out. Plasmid DNA was simultaneously isolated from this strain and DH1(pBR322), and purified on CsCl gradients (section 2:15). the plasmids were restricted with a variety of enzymes, including *Sau*3A and *Hae*III (Fig. 3:3a). (Both these enzymes cut pBR322 to give 22 fragments; Maniatis *et al.*, 1982). As Figure 3:3a

Figure 3:3a

Agarose gel electrophoresis of pBR322 DNA isolated from DH1 And SCRI193, digested with *Hae*III and *Sau*3A.

Samples were run on a 3% agarose gel. Tracks 1 & 8 = λ digested with *Hind*III; tracks 2 + 3 = undigested pBR322 DNA isolated from DH1 and SCRI193 respectively; tracks 4 + 5 = *Hae*III-digested pBR322 DNA isolated from DH1 and SCRI193 respectively; tracks 6 + 7 = *Sau*3A-digested pBR322 DNA isolated from DH1 and SCRI193 respectively.



shows, no differences were observed between the pBR322 isolated from *Ecc* and *E. coli*, suggesting that the plasmid is unaltered in *Ecc*. Figure 3:3b demonstrated the ability of SCRI193 to resolve dimeric plasmids. Monomeric and dimeric pBR322 standards are shown in tracks 10 and 2 respectively. When DH1 was transformed with dimeric pBR322, the plasmid continued to replicate as a dimer (track 4). However, after transformation of SCRI193 with the same plasmid, it assumed a predominantly monomeric conformation (track 6). When DH1 was transformed with plasmid DNA isolated from this SCRI193(pBR322) strain, the pBR322 continued to replicate as a monomer (track 8). DH1 is unable to resolve dimeric pBR322 because it carries a *rec*A mutation (Bedbrook & Ausubel, 1976). Presumably, SCRI193 resolves dimeric plasmid via an analogue of the *E. coli* *rec*A system. Proof that a *rec*A gene exists in some strains of *Erwinia* has been provided recently by the cloning of *rec*A$^+$ from *Ecc* and *Echr* by direct complementation of *rec*A mutants of *E. coli* (section 1:6).

3:4 The uses of transformation in SCRI193 and other *Erwinia* strains

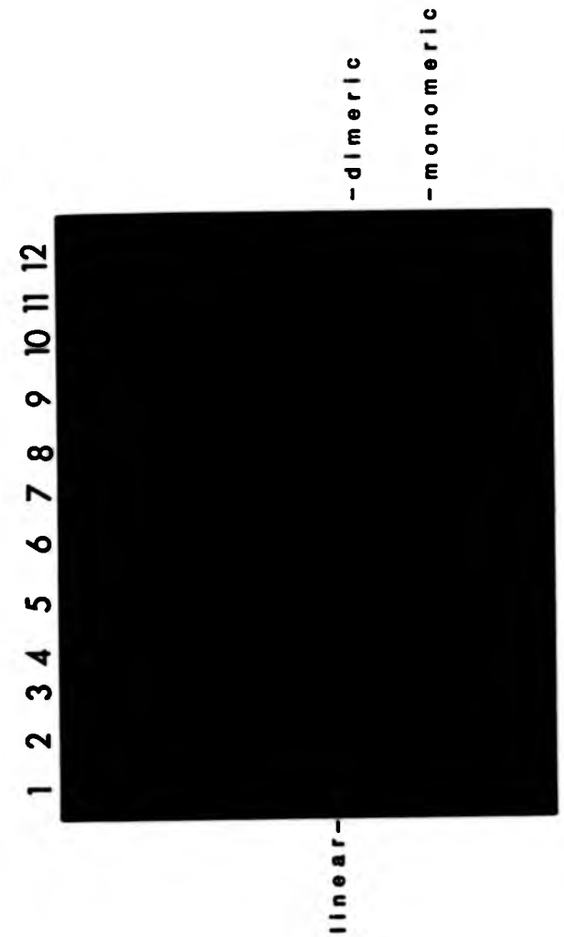
3:4:1 Introduction of various plasmids

The transformation system described here is a valuable tool for plasmid manipulations involving SCRI193 (Hinton *et al.*, 1985a; Chapters 6 & 7), and has been used to introduce various ColE1-based plasmids to SCRI193 derivatives, and strains of *Eca* and *Echr* (table 3:2:2). As with SCRI193, the nature of the SCRI31(pBR322) transformants was confirmed both genetically and physically. However, this transformation technique was not applicable to four of the strains tested: 1066 and 3937JLD

Figure 3:3b

Agarose gel electrophoresis of *E. coli* and SCRI193 transformants.

Size markers were lamda DNA digested with *Hin*dIII (lanes 1 + 12). Lanes 2, 4, 6, 8 and 10 show uncut plasmid DNA; lanes 3, 5, 7, 9 and 11 represent plasmid DNA digested with *Hin*dIII. Lanes 2 + 3: dimeric pBR322 DNA (obtained from BRL). Lanes 4 + 5: DH1 transformed with this dimeric pBR322. Lanes 6 + 7: SCRI193 transformed with dimeric pBR322. Lanes 8 + 9: DH1 transformed with monomeric plasmid from SCRI193(pBR322) (i.e. lane 6). Lanes 10 + 11: monomeric pBR322 DNA (obtained from Amersham). Electrophoresis was performed on a 0.7% gel.



(*Echr*), BS209 (*Eca*), and C466 (*Ecc*) (i.e. yielding < 10 transformants per μg of pBR322 in two separate experiments). Such strain dependence of genetic techniques, within *Erwinia* spp., has been reported previously, e.g. similar results were obtained for B374 and 3937j (Reverchon & Robert-Baudouy, 1985).

Table 3:2:2 shows a range of transformation frequencies, for different derivatives of SCRI193, ranging from 40 to 40,000 transformants per μg of pBR322 DNA. Similar variability in the "transformability" of *E. coli* K12 derivatives has been observed, but remains unexplained (Hanahan, 1983, 1985; Klebe *et al.*, 1983; Kushner *et al.*, 1978). Frequencies of *ca.* 4 x 10^4 transformants per μg of plasmid DNA were obtained in *ca.* 20% of transformation experiments involving SCRI193 or HC500. It is not clear what factor gave rise to such high efficiencies, but it does not seem to be related to the growth phase of the cells used for the experiment.

3:4:2 Application of transformation to gene cloning in SCRI193

It is well known that *in vitro* manipulation and religation of DNA substantially decreases the efficiency of transformation (Forde, 1985). this has prevented the direct introduction of *in vitro*-constructed plasmids to gram-negative species other than *E. coli* (Franklin, 1985). In order to determine the utility of SCRI193 transformation in routine gene cloning experiments, the transformation efficiency of recombinant plasmids was determined.

An example of this (the construction of recombinant plasmid pJH6) is described fully in section 6:4:1. In brief, 1 μg of *Erwinia* fragment DNA was ligated with 300 ng of vector (pBR322) DNA and used for

transformation of *E. coli* strain DH1 and *Ecc* strain HC500. Half of the ligated DNA gave rise to 7,700 Ap^r DH1 transformants but only 11 Ap^r HC500 transformants. These numbers are related to overall cellular competence, and represent about 1% of the transformation efficiency of native pBR322, for both strains. These experiments prove that *in vitro* manipulated DNA can be introduced to SCRI193 directly, and this has facilitated the construction of three subclones without the use of *E. coli* (sections 6:4:1 and 6:4:2).

To maximise the transformation efficiency of recombinant DNA molecules it would be advisable to use vector and insert DNA that had been isolated from SCRI193. This would prevent the restriction of incoming DNA by the SCRI193 recipient. Such restriction reduces the transformation efficiency, of *E. coli*-derived plasmid DNA by 100-fold, for this strain (Gilbert *et al.*, 1986).

The transformation frequencies which have been presented in this Chapter were obtained with CsCl-purified plasmid DNA. However, crudely-purified "mini-prep" DNA is used for most routine gene cloning experiments. When "mini-prep" DNA, isolated from SCRI193, was used to transform the same strain, frequencies of only 60 to 600 transformants per μg of pBR322 DNA were obtained. The reduced transformation efficiency of "mini-prep" DNA probably reflects the presence of inhibitory compounds. Thus, although the efficiencies of transformation are dependent upon the plasmid purity, these experiments show that this transformation method *can* be routinely applied to gene cloning in SCRI193.

3:5 Conclusions

The development and optimisation of an efficient transformation system for SCRI193 has been described. The system is also applicable to some strains of *Eca* and *Echr*, but other strains have proved recalcitrant.

Transformation efficiencies in SCRI31 and B374 may well be improved by optimisation studies in each strain. It is likely that a restrictionless mutant of SCRI193 would improve transformation frequencies of DNA isolated from *E. coli* (e.g. Franklin, 1985).

This system allows routine plasmid construction, subcloning and other manipulations to be carried out without recourse to *E coli* as an intermediate host.

CHAPTER 4

TRANSPOSONS AS A TOOL IN THE GENETIC ANALYSIS OF SCRI193

4:1 Introduction

Transposons are mobile genetic elements which can integrate into new target sites independently of homologous recombination. They carry genes necessary for maintenance and transposition, and often encode resistance to antibiotics. A number of transposons isolated from resistance plasmids of *E. coli* and *Pseudomonas* spp. have been described (Berg, D. & Berg, C, 1983; Mills, 1985; Kleckner *et al.*, 1977). Soon after their discovery, it was apparent that bacterial transposons offered short-cuts to a whole range of traditional genetic techniques, and allowed a number of novel manipulations (Kleckner *et al.*, 1977). Subsequently, the utility of transposons has been elegantly demonstrated in a variety of systems (Berg, D. & Berg, C., 1983; de Bruijn & Lupski, 1984), and they have been described as "one of the cornerstones of bacterial genetics" (Ely, 1985).

Transposons can generate stable mutations by insertional inactivation of a gene, thereby causing complete loss of gene function and simultaneously providing a selectable antibiotic resistance. Other examples of their use include the marking of non-selectable genes (Anderson & Mills, 1985; Salmond & Plakidou, 1984); the generation of deletions (Berg, D. & Berg, C., 1983); the cloning of non-selectable genes (Niepold *et al.*, 1985; Purucker *et al.*, 1982; Staskawicz, 1983); the study of operon organisation (Berg., C. *et al.*, 1979; de Bruijn & Lupski, 1984) and use as a portable region of homology for directed chromosomal transfer (Banfalvi *et al.*, 1983; Pischl & Farrand, 1983).

The advantages offered by transposons in the study of pathogenic bacteria have been described previously (Daniels, 1984; Salmond, 1985;

Staskawicz, 1983). The "tagging" of pathogenicity genes with the antibiotic resistance of the transposon facilitates genetic mapping. In addition, it is possible to clone DNA sequences which flank any Tn*5* insertion by making use of the fact that Tn*5* has no *Eco*RI sites (de Bruijn & Lupski, 1984). Transposons have been exploited in the analysis of human, animal and plant pathogens (Niepold *et al.*, 1985; Stapleton *et al.*, 1984; Zarenkov *et al.*, 1984; see table 4:3).

4:2 The utility of Tn*5*

A wide range of transposons have been isolated and characterised (Berg, D., 1985), but the properties of Tn*5* have been studied most exhaustively (Berg, D. & Berg, C., 1983; de Bruijn & Lupski, 1984). The advantages of Tn*5* include a low specificity of insertion (Berg, D. *et al.*, 1983, 1985; Shaw & Berg, 1979), a detailed restriction map (Auerswald *et al.*, 1981; Beck *et al.*, 1982; Mazodier *et al.*, 1985), well-characterised gene products (Rossetti *et al.*, 1984; Rothstein *et al.*, 1981), relatively stable insertions (Berg, D. & Berg, C., 1983; Berg, C. *et al.*, 1983) and its ability to cause completely polar mutations (Berg., C. *et al.*, 1979; Berg, D. *et al.*, 1980; de Bruijn & Lupski, 1984). It transposes at a high frequency (10^{-2} to 10^{-3} per cell in *E. coli*) simplifying mutant isolation. Tn*10* derivatives of similar transposition efficiency have been described recently (Way *et al.*, 1984), but Tn*10* is known to exhibit marked insertional specificity (Kleckner *et al.*, 1977). The kinetics and copy number control of transposition of Tn*5* have been studied (Johnson & Reznikoff, 1984; Rossetti *et al.*, 1984), and mechanisms of transposition have been proposed (Berg, D., 1985; Berg, D. *et al.*, 1985).

A disadvantage sometimes encountered in the use of Tn*5* lies in the ability of its two IS50 elements to transpose independently of the resistance marker, causing mutations that are not linked to kanamycin resistance (Anderson & Mills, 1985; Staskawicz *et al.*, 1983, 1984). Furthermore, Tn*5* does not transpose efficiently in all Gram-negative species, (e.g. *Rhizobium meliloti* (Forrai *et al.*, 1983; Meade *et al.*, 1982) and *Xanthomonas campestris* pv. *campestris* (Turner *et al.*, 1984), although this can be used to advantage in the construction of R-prime plasmids (Banfalvi *et al.*, 1983). The transposition efficiency of Tn*5* is strain-dependent in some species (Forrai *et al.*, 1983). Insertional specificity of Tn*5* has been reported in *Acinetobacter calcoaceticus*, where it was shown to have a single site of insertion in the chromosomes (Singer & Finnerty, 1984). Nevertheless, Tn*5* appears to have a high transposition frequency and low insertional-specificity in all enteric bacteria so far studied. In addition to kanamycin/neomycin resistance, Tn*5* also expresses bleomycin resistance (Genilloud *et al.*, 1984; Collis & Hall, 1985) as well as streptomycin resistance in many non-enteric Gram-negative species (O'Neill *et al.*, 1983).

4:3 Transposon mutagenesis

In order to achieve transposon mutagenesis of a bacterium other than *E. coli*, an unstable transposon delivery vehicle ("suicide vector"; van Vliet *et al.*, 1978) is required. Upon introduction to the recipient, the vector fails to replicate, and selection of the transposable antibiotic resistance marker ensures that all survivors carry transposon insertions. Examples of suicide vectors include phage P1 (Kuner & Kaiser, 1981; Quinto *et al.*, 1984), narrow host-range plasmids (Ely, 1985; Simon *et al.*, 1983a,b, Whitta *et al.*, 1985), plasmids containing the Mu prophage (Beringer *et al.*, 1978; section 4:3:2), and λ phage

carrying nonsense mutations in the DNA replication genes (de Vries *et al.*, 1984; Palva & Liljestrom, 1981). Other methods include the displacement of a resident plasmid by incompatibility (Elmerich, 1983), and the use of plasmids which are temperature-sensitive for replication (Kotoujansky *et al.*, 1982; O'Hoy & Krishnapillai, 1985; Rella *et al.*, 1985) (see table 4:3 for examples of the use of these vectors).

Three types of suicide vectors were studied in SCRI193, namely the narrow host-range pSUP2021 (Simon *et al.*, 1983a), pJB4JI (Beringer, 1978) and a phage λ vector (Kleckner *et al.*, 1977). A discussion of the advantages and limitations of each type of vector follows.

4:3:1 Mu-containing suicide plasmids

Suicide vectors based on broad host-range plasmids carrying the Mu prophage have been used to introduce transposons to the genome of a variety of bacterial species (table 4:3). Despite their widespread use, the basis of the instability of such plasmids in species other than *E. coli* is not understood.

Mu-containing plasmids are transferred to many bacterial species at a greatly reduced frequency compared to parental plasmids (Boucher *et al.*, 1977; Denarié *et al.*, 1977; Forbes & Pérombelon, 1985; van Vliet *et al.*, 1978). Study of RP4::Mu plasmids which were maintained in populations of *Agrobacterium* and *Rhizobium* spp. determined that all contained deletions of the *A-C* region of Mu, and flanking sequences (Casey *et al.*, 1983; van Vliet *et al.*, 1978). It is thought that this region encodes a Mu function that is lethal in many species (van Vliet *et al.*, 1978). Apart from spontaneous deletion formation, restriction of Mu DNA and zygotic induction are the limiting factors in the establishment of these

TABLE 4:3 Some bacterial species which have been mutagenised by Tn*5*

Organism	Vector	Reference
Acinetobacter calcoaceticus	pRK2013::Tn*5* pJB4JI	Ely, 1985 Singer & Finnerty, 1984
Alcaligenes eutrophus	pJB4JI; pSUP2021	Srivastava et al., 1982; Simon *et al.*, 1983a,b
Agrobacterium rhizogenes	pJB4JI	White & Nester, 1980
Agrobacterium tumefaciens	pJB4JI	Pischl & Farrand, 1983 Garfinkel & Nester, 1980
Azospirillum brosilense	pJB4JI & pJB3JI	Elmerich, 1983
Bordetella pertussis	RP4-ColE1::Tn*5*	Weiss & Falkow, 1983
Caulobacter crescentus	pJB4JI	Ely & Croft, 1982
Caulobacter crescentus	pJB4JI	Bellofatto *et al.*, 1984
Caulobacter crescentus	pRK2013::Tn*5-132*	Ely, 1985
Erwinia amylovora	pJB4JI	Steinberger *et al.*, 1983
Erwinia amylovora	pJB4JI	Steinberger & Beer, 1985
Eca	pJB4JI	Zink *et al.*, 1984
Ecc	pJB4JI	Zink *et al.*, 1984
Ecc	P1::Tn*5*	Berg, D. & Berg, C., 1983
Ecc	pJB4JI; λ::Tn*5*	Hinton *et al.*, 1985c Salmond *et al.*, 1986
Echr	pJB4JI	Chatterjee *et al.*, 1983

Table 4:3 continued

Organism	Vector	Reference
E. herbicola	pJB4JI	Gantotti *et al.*, 1981
E. uredovora	pJB4JI	Doten & Mortlock, 1985
Klebsiella aerogenes	P1::Tn*5*	Quinto *et al.*, 1984
K. pneumoniae	λ::Tn*5*	de Vries *et al.*, 1984
Methylobacterium AM1	pMO75	Whitta *et al.*, 1985
Methylophilus sp.	pLG221	Boulnois *et al.*, 1985
Myxococcus xanthus	P1::Tn*5*	Kuner & Kaiser, 1981
Pseudomonas aeruginosa	RP4^{ts}::Tn*5-751*	Rella *et al.*, 1985
Pseudomonas aeruginosa	pJB4JI	Stapleton *et al.*, 1984
Pseudomonas aeruginosa	R68^{ts}::Tn*5*	O'Hoy & Krishnappillai, 1985
P. putida	pLG221	Boulnois *et al.*, 1985
P. syringae pv. *syringae*	pSUP1011	Anderson & Mills, 1985 Kuykendall *et al.*, 1985
P. syringae pv. *phaseolicola*	pSUP1011	Anderson & Mills, 1985
P. syringae pv. *phaseolicola*	pSUP1011	Deasey *et al.*, 1985
P. syringae pv. *tomato*	pGS9	Cuppels, 1985
P. syringae pv. *tagetes*	pGS9	Kuykendall *et al.*, 1985
P. solanaceanum	pJB4JI	Boucher *et al.*, 1981
P. solanaceanum	pJB4JI	Staskawicz *et al.*, 1983
Rhizobium japonicum	pSUP1011	Hom *et al.*, 1984
Rhizobium japonicum	pGS9	Rostas *et al.*, 1984
R. leguminosarum	pSUP2021	Simon *et al.*, 1983a
R. leguminosarum	pJB4JI	Beringer *et al.*, 1978

Table 4:3 continued

Organism	Vector	Reference
R. meliloti	pJB4JI	Meade *et al.*, 1982
R. meliloti	pJB4JI	Duncan, 1981
R. meliloti	pSUP2021	Simon *et al.*, 1983a
R. meliloti	pSP601	Ali *et al.*, 1984
R. meliloti	pGS9	Selvaraj & Iyer, 1983
R. phaseoli	pJB4JI	Beringer *et al.*, 1978
R. phaseoli	pSUP2021	Simon *et al.*, 1983a
R. trifolii	pJB4JI	Beringer *et al.*, 1978
R. trifolii	pJB4JI	Walton & Moseley, 1981
R. trifolii	pSUP2021	Simon *et al.*, 1983a
Rhizobium slow-growing	pSP601; pJB4JI	Cen *et al.*, 1982
Rhodopseudomonas capsulata	pSUP1011	Kaufmann *et al.*, 1984
Rhodopseudomonas sphaeroides	pJB4JI; RK2^{ts}::Tn*5*	Weaver & Tabita, 1983
Salmonella enterides	P1::Tn*5*	Berg D. & Berg C., 1983
S. typhimurium	λ::Tn*5*	de Vries *et al.*, 1984
S. typhimurium	λ::Tn*5*	Palva & Liljestrom, 1981
Serratia marcescens	P1::Tn*5*	Berg D. & Berg C., 1983
Vibrio harveyi	P1::Tn*5-132*	Belas *et al.*, 1982
Xanthomonas c. pv. *campestris*	pSUP1011	Turner *et al.*, 1984
Yersinia pestis	P1::Tn*5*	Zarenkov *et al.*, 1984

"suicide" plasmids (Casey *et al.*, 1983; Forbes & Pérombelon, 1985; van Vliet *et al.*, 1978).

The Mu-containing suicide plasmid that has been used most commonly for transposon mutagenesis is pJB4JI (Beringer *et al.*, 1978; table 4:3). It is based on R751, with a small insert from R1033, and carries the Mu c^+ prophage. A Tn*5* element is inserted in the region encoding tail proteins of Mu (Hirsch & Beringer, 1984). *E. coli* strains containing pJB4JI are Mu-immune, but are unable to produce viable Mu phage (Beringer *et al.*, 1978). Upon introduction to many Gram-negative species, pJB4JI is unstable, and transposition of Tn*5* to the host chromosome may be selected. However, in some cases, this transposition event may be accompanied by insertion of Mu DNA, thus complicating genetic analysis and subsequent directed cloning experiments (Meade *et al.*, 1982).

pJB4JI is maintained in *Azospirillum* sp. (Elmerich *et al.*, 1983), *P. aeruginosa* (Rella *et al.*, 1985), *Rhodopseudomonas capsulata* (Kaufmann *et al.*, 1984), *X. c.* pv. *campestris* (Turner *et al.*, 1984) and some strains of *Ecc* and *Eca* (Zink *et al.*, 1984). In *Rhodopseudomonas sphaeroides*, pJB4JI appeared to be stable but was lost during serial passage on non-selective media (Weaver & Tabita, 1983). However, it has proved useful in a wide range of species (table 4:3).

Another Mu-containing suicide plasmid (pSP601) has been used in *Rhizobium meliloti* and other slow-growing *Rhizobium* species (Ali *et al.*, 1984; Cen *et al.*, 1982), and is based on the R751-derived pSP60 which carries the transposons Tn*1*, Tn*5* and Tn*1771*. pSP601 carries the Mu c^+ prophage inserted into pSP60 at an undetermined point, and encodes the

production of viable Mu phage in *E. coli* (R. Simon, pers. comm.).

The behaviour of Mu-containing plasmids has been studied in SCRI193 (Forbes, 1983; Forbes & Pérombelon, 1985; Pérombelon & Boucher, 1978). Zygotic induction has been demonstrated following intergeneric conjugation of R68::Mu c^+ to lysogenic and non-lysogenic SCRI193-derivatives. Deletion formation was observed in R68::Mu c^+ plasmids that were maintained in a *crp* derivative of SCRI193 (Forbes, 1983). The presence of the Mu prophage on RP4 was shown to reduce its transfer frequency by 10^{-4} (Forbes, 1983).

4:3:2 Narrow host-range plasmids

Alternative technologies for transposon mutagenesis involve the use of plasmid replicons that do not function in some species (Ely, 1985; Simon *et al.*, 1983a; Whitta *et al.*, 1985). It has been suggested that such vectors have advantages over Mu-based systems in which genetical complications may arise (Casey *et al.*, 1983; O'Neill *et al.*, 1983), such as stably-replicating mutants of the plasmid vector (Simon *et al.*, 1983a). Simon *et al.* (1983a,b) constructed derivatives of the cloning vector pBR325 and pACYC184 which carried the 2 Kb *oriT* region ("*mob*") from RP4. These plasmids comprise the pSUP series, and may be mobilised by RP4 functions *in trans* (Clarke & Warren, 1979). pSUP2021 is a mob^+ derivative of pBR325 that carries an insert of Tn*5* in its tetracycline resistance gene. The utility of pSUP2021 as a suicide vector is based on the host-range of the ColE1 replicon, which is confined to *E. coli* and closely-related species (Ely, 1985; O'Neill *et al.*, 1983). Similar vectors have been used successfully in *Pseudomonas* spp. (Anderson & Mills, 1985; Lam *et al.*, 1985), *Rhizobium* spp. (Bullerjahn & Benzinger, 1984; Hom *et al.*, 1984; Simon *et al.*, 1983a) and *Rhodopseudomonas* sp.

(Kaufmann *et al.*, 1984). Other examples of narrow host-range vectors have been developed, and are ColE1-based (Ely, 1985), p15A-based (Selvaraj & Iyer, 1983) or R91-5-based (Whitta *et al.*, 1985).

Apart from Mu-containing and narrow host range suicide vectors, alternative approaches have been used to displace transposon-carrying plasmids from various species. These include the use of temperature-sensitive replication plasmid mutants (Kotoujansky *et al.*, 1982; O'Hoy & Krishnapillai, 1985; Rella *et al.*, 1985) and incompatibility (Elmerich *et al.*, 1983).

4:3:3 Bacteriophages as suicide vectors

Phages have been used as tools for transposon mutagenesis in various bacterial species, ranging from *E. coli* to *Yersinia pestis* (Belas *et al.*, 1982; Berg, D. & Berg, C., 1983; Harkki & Palva, 1984; Kuner & Kaiser, 1981; Palva & Liljestrom, 1981; Quinto *et al.*, 1984; de Vries *et al.*, 1984; Zarenkov *et al.*, 1984). In particular, P1 and λ have proved extremely valuable as delivery vectors, and their uses are discussed below.

P1

The host range of P1 is normally limited to *E. coli* and closely-related enterics. Nonetheless, it is possible to select P1-sensitive mutants of different bacterial species using P1-derivatives carrying antibiotic resistance genes (Goldberg *et al.*, 1974; Jayasawal *et al.*, 1984; Murooka & Harada, 1979; Quinto *et al.*, 1984; Streicher *et al.*, 1975). Such mutants permit the efficient adsorbtion of P1 phage and the injection of its DNA, but rarely provide the host functions required for replication. P1-sensitive mutants have recently been isolated from a wide range of

Eca, *Ecc* and *Echr* strains, in this laboratory (H. Kemp, pers. comm.). P1 can be propagated on *S. typhimurium* and *Klebsiella pneumoniae*, and has been used for intergeneric transduction between these species and *E. coli* (Quinto *et al.*, 1984; Streicher *et al.*, 1975; Tyler & Goldberg, 1976). Furthermore, P1 has been used to mediate transduction from *E. coli* to *Myxococcus xanthus*, despite the inability of the phage to replicate (O'Connor & Zusman, 1983).

P1 has been used to achieve transposon mutagenesis of various enteric bacteria, as well as the non-enterics, *M. xanthus* and *Vibrio harveyi*. A generalised method has been developed for enterics, and involves the superinfection of a P1CM lysogen with P1::Tn*5*. Transposition of Tn*5* can be selected due to the incompatibility of the two phages (Quinto *et al.*, 1984). However this method was not applicable to one strain of *Ecc* (Jayasawal *et al.*, 1985b).

Lambda

Generally, the host range of λ does not extend beyond *E. coli* K12. Methods for the selection of λ-sensitive mutants of other strains or species are not available. However, the basis of λ adsorbtion to *E. coli* K12 has been studied extensively (Randall-Hazelbauer & Schwartz, 1973; Schwartz & Le Minor, 1975). A single receptor protein, the *lamB* gene product, has been shown to facilitate λ adsorbtion and to be sited in the outer membrane (Silhavy *et al.*, 1983). The *lamB* gene has been cloned onto multicopy vectors, and introduced to a number of bacterial species (Clement *et al.*, 1982; Harkki & Palva, 1985; de Vries *et al.*, 1984). This approach has led to the construction of λ-sensitive derivatives of bacterial species such as *S. typhimurium* and *K. pneumoniae* although it failed for *P. aeruginosa* (de Vries *et al.*,

1984). Such derivatives do not support the growth of λ, but λ can replicate in *S. typhimurium* when the *nusA*$^{+}$ gene of *E. coli* is present (Friedman *et al.*, 1984; Harkki & Palva, 1984).

Nevertheless, in the absence of replication, the ability of λ to inject its DNA can be exploited for transposon mutagenesis. Deletion derivatives of λ are commonly used for the introduction of transposons to chromosomal or plasmid-borne genes of *E. coli* (Berg, D. & Berg, C., 1983; de Bruijn & Lupski, 1984; Kleckner *et al.*, 1977). They are λ-derivatives which are unable to form lysogens or to replicate in a *Sup*^{o} host (e.g. λ_{467}). Upon introduction to a *Sup*^{o} strain, they act as suicide vectors, allowing selection to be made for transposon insertion.

The advantage of λ suicide vectors over plasmid vehicles for transposon mutagenesis of a strain of interest are twofold. Firstly, many transposon-carrying λ-derivatives are available, allowing the use of any transposon of choice (Berg, D., & Berg, C., 1983; de Bruijn & Lupski, 1984; Kleckner *et al.*, 1977; Way *et al.*, 1984), including transposons developed as promoter probes (Way *et al.*, 1984). Secondly, phage infection takes place more rapidly than plasmid transfer, and allows the formation of random transposon-induced mutants without concomitant sibling formation. Finally, λ-sensitive bacteria should prove useful in other genetic manipulations apart from transposon mutagenesis, such as the direct introduction of cosmid libraries, which can be efficiently packaged *in vivo* by λ (Faelen *et al.*, 1985; Vollenweider *et al.*, 1980).

From the diversity of transposon delivery systems which are available, it may be inferred that no system is universally applicable. Table 4:3 shows the systems which are available for various bacterial species.

However, these delivery vehicles show great species and even strain-dependence (e.g. Zink *et al.*, 1984). Consequently, the development of a transposon mutagenesis system for a previously uncharacterised strain necesitates the investigation of a range of different techniques.

RESULTS AND DISCUSSION

4:4 Suicide plasmids in SCRI193

The inheritance of the three suicide plasmids pSP601, pSUP2021 and pJB4JI was studied in SCRI193. These plasmids were conjugated from *E. coli* into two antibiotic-resistance derivatives of SCRI193. The donors were counterselected and transconjugants were screened for resistance markers carried by the various transposons.

Preliminary experiments showed that SCRI193 had a high rate of sportaneous kanamycin resistance on LB or M9 medium containing 50 μg/ml kanamycin (1.3×10^{-6} per cell in both cases). This may be compared with a spontaneous Km^r frequency of 10^{-11} for *E. coli* K12 on Difco nutrient media (Apirion & Schlessinger, 1968). Fortunately, spontaneous Km^r of SCRI193 exhibited medium-dependence, and was greatly reduced on Oxoid nutrient media ($< 10^{-10}$ per cell). The explanation for this is not known, but Oxoid nutrient media was used in all subsequent experiments.

4:4:1 Results

pSP601

As shown in table 4:4:1, pSP601 transferred to KF1033 at a lower

TABLE 4:4:1 Introduction of suicide plasmids to SCRI193

Donor	Recipient	Selection	Transfer Frequency	Phenotype of Transconjugants
C600(pSP60)	KF1033	SmNxTc	7×10^{-5}	Sm^r Nx^r Tc^r Ap^r Km^r
C600(pSP601)	KF1033	SmNxTc	6×10^{-8}	Sm^r Nx^r Tc^r Ap^r Km^r
S17-1(pSUP2021)	KF1033	SmNxKm	1×10^{-6}	Sm^r Nx^r Km^r Cm^r Ap^r
J53*nal*(pJB4JI)	KF1033	SmKm	*2×10^{-6} †2×10^{-7}	Sm^r Km^r Gm^r Sp^r Sm^r Km^r Gm^s Sp^s

* small colonies; † large colonies

frequency (10^{-3}) than pSP60. When selection was made for the Ap^r, Km^r or Tc^r markers of pSP601 (i.e. Tn*1*, Tn*5* and Tn*1771* respectively), similar frequencies of transfer were obtained. Tc^r transconjugants for each plasmid were screened for the presence of non-selected antibiotic resistance markers. All pSP60 (95/95) and pSP601 (90/90) transconjugants were Sm^r Nx^r Ap^r Km^r Tc^r.

Thus, the presence of Mu reduced the transfer frequency of pSP60, but it did not seem to affect plasmid maintenance. All the KF1033(pSP601) recipients carried a full complement of plasmid markers, suggesting that the plasmid was maintained in SCRI193.

pSUP2021

pSUP2021 was mobilised to KF1033 at a reasonable frequency (10^{-6} TPR; table 4:4:1). Km^r transconjugants were screened for other plasmid markers, and were found to be Sm^r Nx^r Km^r Ap^r Cm^r (94/94). Agarose gel analysis confirmed that the KF1033(pSUP2021) transconjugants carried a plasmid that comigrated with pSUP2021 (data not shown).

At the time of this experiment it was not known whether ColE1-derived plasmids would replicate in SCRI193. This result suggested that the pBR325 replicon was maintained in this strain, and so was unable to serve as a suicide plasmid.

pJB4JI

The frequency of transfer of pJB4JI to KF1033 is shown in table 4:4:1. Following the mating, two distinct colony types appeared on the selection plates (Fig. 4:4:1a); "small" (1 mm diameter) colonies arose at ten times the frequency of "large" (3 mm diameter) colonies. Both

Figure 4:4:1a

Introduction of pJB4JI into KF1033.

Following a J53*nal*(pJB4JI) X KF1033 mating, cells were plated on NBA Sm Km, and grown at 30°C (36 h). Many "small" transconjugant colonies and 6 "large" transconjugant colonies can be seen.

Figure 4:4:1a

Introduction of pJB4JI into KF1033.

Following a J53*nal*(pJB4JI) X KF1033 mating, cells were plated on NBA Sm Km, and grown at 30°C (36 h). Many "small" transconjugant colonies and 6 "large" transconjugant colonies can be seen.

colony types were tested for coinheritance of plasmid markers. "Small" colonies exhibited a Sm^r Nx^r Km^r Gm^r Sp^r phenotype (26/26), whereas all "large" colonies (31/31) were Sm^r Nx^r Km^r Gm^s Sp^s. This suggested that the plasmid was maintained in the "small" but not the "large" colonies.

This phenomenon of differential colony size was studied further. Following initial subculture on selective media, both colony morphology types bred true. However, following repeated subculturing, a "small" colony (HC122) gave rise to several "large" colonies which had lost the Gm^r and Sp^r plasmid markers. One such spontaneous "large" colony was purified and designated HC124.

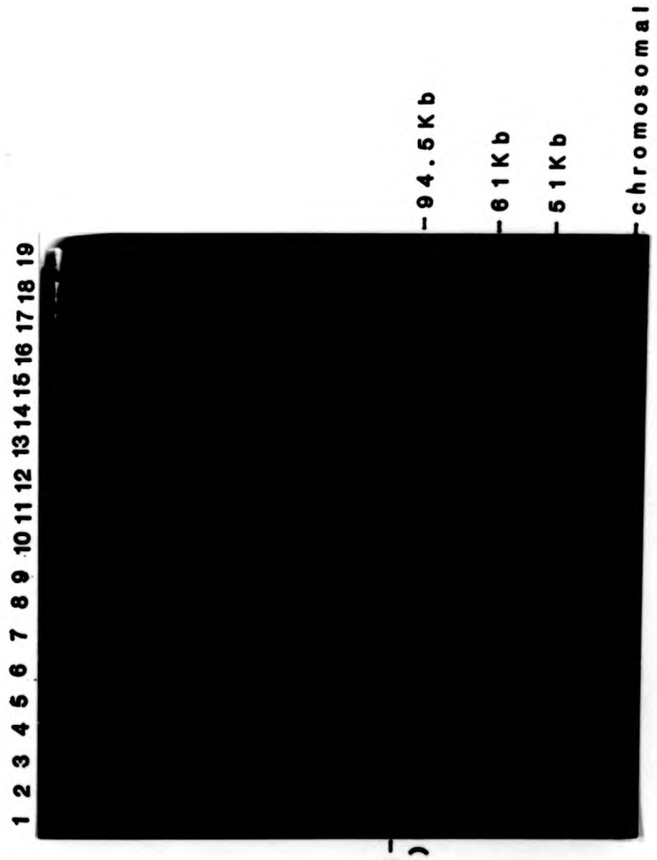
In order to prove that the "small" transconjugants carried an intact Tra^+ plasmid, HC122 was mated with QD*supF*. Exconjugants were plated on NA Km, and donors counterselected at 42°C. Putative QD*supF*(pJB4JI) transconjugants were detected at 10^{-7} TPR, and 10/17 were Sp^r. (The incomplete linkage between the transposon and plasmid markers may reflect zygotic induction of Mu following introduction to *E. coli*.) A "large" transconjugant (HC500) was also mated with QD*supF* to determine whether the Km-resistance could be transferred. No Km^r transconjugants were detected (frequency < 5×10^{-8} TPR).

Agarose gel electrophoresis data (Figure 4:4:1b), demonstrates that the "small" colonies carried a plasmid corresponding to pJB4JI, and the "large" colonies were plasmid-free. This Eckhardt gel shows pJB4JI carried by the donor *E. coli* strain (track 1), and by two "small" transconjugants (tracks 2 & 3). J53*nal*(pJB4JI) possesses a major band which corresponds to the closed-circular form of the 98 Kb plasmid (track 1). This track also contains a larger plasmid which has not been

Figure 4:4:1b

Analysis of pJB4JI in "small" and "large" KF1033 transconjugants.

Samples were run on a 0.7% Eckhardt gel as described. Track 1 = J53*nal* (pJB4JI); track 2 = HC122 ("small" transconjugant); track 3 = HC123 ("small" transconjugant); track 4 = HC124 (spontaneous "large" colony); tracks 5-9 are Tn*5*-induced mutants isolated from "large" colonies. Track 5 = HC503; track 6 = HC500; track 7 = HC505; track 8 = HC506; track 9 = HC504. Tracks 10-13 are wild-type strains; track 10 = KF1033; track 11 = KF1005; track 12 = SCRI193; track 13 = W3110. Tracks 14-19 are plasmid size markers; track 14 = KF1033(RP4-Km^S); track 15 = W3110(RP4-Km^S); track 16 & 17 = W3110(RP4-Km^S(::Tn5)); track 18 = C600(R751); track 19 = RB308.



identified, but may represent the open-circular form (Forbes, 1983), or the dimeric form of pJB4JI. The larger plasmid is absent from track 2 but appears in track 3, and the significance of this is unclear. Track 4 shows the plasmid profile of the "large" transconjugant (HC124) which arose spontaneously from HC122, and confirms it is plasmid-free. Tracks 5-9 represent a number of Tn*5*-induced mutants isolated from amongst the "large" colonies (see table 4:6:1). Once it was determined that the "large" transconjugants were plasmid-free, it was apparent that they must have arisen from Tn*5* transposition into the chromosome of SCRI193. Consequently, 1600 of these colonies were screened to obtain a variety of Tn*5*-induced auxotrophic, catabolic and enzymic mutants. Several colonies (10/1600) were auxotrophic (a frequency of 0.6%) and 3/450 colonies were catabolic mutants (0.7%) (see table 4:6:1). Only 1/1050 colonies was Pel$^-$, and this mutant (HC500) is described in section 4:6 and chapters 5 and 6.

4:4:2 Discussion

The conjugation data involving pSP60 and pSP601 demonstrates the effect of the Mu prophage on plasmid transfer (the only difference between the two plasmids being the presence of Mu on pSP601). Similar observations have been made previously; RP4::Mu *cts*62 transfers to SCRI193 at a lower frequency (10^{-4}) than RP4 (Forbes, 1983). This reduction in transfer frequency could arise from several factors including restriction of Mu DNA by SCRI193, zygotic induction, or the expression of a Mu function which affects plasmid maintenance.

As soon as these preliminary experiments suggested that pSP601 was maintained in SCRI193, work with this plasmid was discontinued. The maintenance of pSP601 contrasted with the behaviour of pJB4JI in this

strain. The two plasmids have several similarities in that both are based on R751 and carry Mu and transposons. However, little is known about the precise structure of pSP601, although unlike pJB4JI, it is able to give rise to viable Mu phage (Ali *et al.*, 1984). However, derivatives of pJB4JI which carry Tn*5* at different sites on the plasmid, outwith the Mu prophage, retain efficient suicide properties in *Caulobacter crescentus* (D. Hodgson, pers. comm.). It is possible that the presence of Tn*1*, Tn*1771* and Tn*402* interfere with the transposition of Tn*5* from pSP601.

Although the results involving pSUP2021 showed that this vector was unsuitable for transposon mutagenesis, they proved extremely useful in other work with SCRI193, because the stability of ColE1-derived cloning vectors (such as pAT153, pBR322 and pBR325) meant that they could be used subsequently in transformation and cloning experiments (see Chapters 3 and 6).

Fortunately, pJB4JI proved applicable as a suicide vector for SCRI193, because of its instability which was manifested by the appearance of two colony types amongst the transconjugants. Two explanations could be offered for this. Either the plasmid, pJB4JI, represents a metabolic load on the cell causing the formation of "small" colonies (e.g. Zund & Lusk, 1980), or the transposition of Tn*5* is responsible for an increase in growth rate, resulting in "large" colonies. The correct explanation is not known, but it should be noted that Tn*5* can confer a transient selective advantage upon *E. coli* cells subjected to carbon source limitation in chemostats (Biel & Hartl, 1983; Hartl *et al.*, 1983). Obviously, nutrient limitation is not relevant here, but it may be significant that Tn*5* transposition can enhance growth rate. It is

interesting to speculate upon the nature of the pJB4JI plasmids maintained in the "small" transconjugants. Perhaps these plasmids carried deletions of Mu DNA as reported previously (Casey *et al.*, 1983; Forbes, 1983). However, this is not apparent from physical analysis (Fig. 4:4:1b), although it is possible that small changes in mobility would not be detected on a gel of this type. Although the basis of the differing colony morphologies was not understood, they could be used to identify those KF1033 colonies likely to carry Tn*5*-induced mutations, i.e. although 90% of the transconjugants from a mating of J53(pJB4JI) with KF1033 maintained the plasmids, the Tn*5* "transposees" could be easily recognised.

The effect of the instability of pJB4JI upon colony morphology has not been reported previously. However, Zink *et al.* (1984) observed that pJB4JI was maintained in 100%, 81%, 73%, 41%, 36% and 22% of the transconjugant colonies of different *Eca* strains, and similar observations have been made in this laboratory (D. Cardy, pers. comm.; Hinton *et al.*, 1985c). All of this data demonstrates the extreme strain-dependence of pJB4JI maintenance.

4:5 The use of lambda as a suicide vector in SCRI193

Because of the advantages of λ-based transposon mutagenesis systems (section 4:3:3), attempts were made to apply this technology to SCRI193.

4:5:1 Results

pHCP2 is a pBR322-derivative containing a 3 Kb *Eco*RI-*Bgl*II fragment which carries the entire *LamB*$^+$ gene of *E. coli* (Clement *et al.*, 1982).

This plasmid was introduced to SCRI193 by transformation, and a single Ap^r colony was purified and designated HC131. In order to determine whether the LamB protein was functional in *Ecc*, culture of HC131 were infected with λ_{467}, and Km^r transductants selected. This was performed, in parallel, in *E. coli*, and the results are shown in table 4:5:1. The data may indicate that λ can adsorb and inject its DNA to HC131 at a similar efficiency to *E. coli*. However, because this assay for λ infection involves the transposition of Tn*5*, it is possible that λ adsorbs to HC131 more efficiently than to *E. coli*, but that Tn*5* transposes at a lower frequency than in *E. coli*, or *vice-versa*.

The ability of HC131 to propagate several λ derivatives was determined; λ^+, λcI_{857} and λ_{540} were spot-tested on lawns of HC131, but did not form plaques (see appendix 1:2:2).

After λ_{467} infection, 850 Km^r HC131 colonies were screened for altered nutritional and exoenzymic phenotypes. Nine auxotrophs were isolated and characterised (a frequency of *ca.* 1%), and two putative *Cel*::Tn*5* mutants were obtained (see section 4:6) (Hinton *et al.*, 1985c; Salmond, 1985). The genetical analysis of these mutants, presented in section 4:6:1 suggests that they were caused by single Tn*5* insertions, and demonstrates the utility of λ as a suicide vector in this strain.

More recent experiments in this laboratory have led to the isolation of a wide range of Tn*5*-induced extracellular enzyme mutants of HC131, and several other transposons have been used successfully (Salmond *et al.*, 1986). Moreover, the use of pHCP2 to render strains λ-sensitive has also proved applicable to *Eca* and *Echr* (Salmond *et al.*, 1986), as well as other *Erwinia* spp. (unpublished data).

TABLE 4:5:1 λ::Tn*5* infection of *Ecc* and *E. coli*

Strain	No. of Km^r transductants following λ_{467} infection
CSH26ΔF6(pBR322)	3.9×10^{-6}
SCRI193	$< 3 \times 10^{-10}$
HC131	4.4×10^{-6}

Transduction frequencies are expressed per singly-infected cell (as Wilson *et al.*, 1979).

4:5:2 Discussion

The frequency of auxotrophs found amongst the Km^r colonies generated by both the pJB4JI and the λ_{467} mutagenesis system was 1%. Similar frequencies have been reported in *Eca*, *Ecc*, *Echr* and *E. coli* (Chatterjee *et al.*, 1983; Shaw & Berg, 1979; Zink *et al.*, 1984).

Nevertheless, it is possible that the mutants generated by λ_{467} have arisen due to insertion of the complete λ::Tn*5* element into particular genes. Two lines of evidence suggest that the series of mutants HC513-HC523 were not caused by the λ_{467} prophage. Firstly, in the absence of *att*λ *in E. coli*, λ is known to insert preferentially into the *pro* genes (Shimada *et al.*, 1973). Secondly, the reversion analysis of these mutants is consistent with simple Tn*5* insertions. Thus, although it is unlikely that λ is directly involved this has not been proven.

The ability of λ to adsorb to HC131 suggests that the *lam*B protein is expressed and exported from SCRI193 in a manner analogous to *E. coli*. However, it has been reported that strains of *Lam*BΔ *E. coli* carrying the *lam*B gene in multicopy were not fully λ-sensitive due to the weak internal promoter between *mal*K and *lam*B (Harrki & Palva, 1985). Furthermore, it is known that *lam*B expression is dependent upon a positive regulator, the *mal*T gene product (Raibaud & Schwartz, 1984). Perhaps the expression of sufficient amounts of *Lam*B to facilitate λ adsorbtion by *Ecc*, suggests that this strain produces a protein analogous to *mal*T, as reported for *E. herbicola* (Raibaud & Schwartz, 1984). Recently it has been determined that adsorbtion of λ to HC131 does not require the presence of maltose as an inducer in growth medium (unpublished data), which questions a direct role of a *mal*T homologue.

Further experiments to analyse the fate of λ in SCRI193 are in progress in this laboratory, in an attempt to determine if the *nus*A gene can support λ replication in *Ecc*, as it does in *S. typhimurium* (Harrki & Palva, 1984). We also want to determine whether λ can lysogenise *Ecc*.

4:6 Analysis of Tn*5*-induced mutants of SCRI193

4:6:1 Results

The pJB4JI and λ-based transposon mutagenesis systems were used to isolate a variety of Tn*5*-induced mutants, which are listed in table 4:6:1. At least fourteen classes of mutants were obtained, suggesting that the transposon inserted non-specifically in SCRI193. One-third of the mutants isolated had some defect in cysteine biosynthesis. However, mutants HC500, HC501 and HC502 did not cross-feed each other. Similarly, cross-feeding was not observed between the uracil-requiring mutants, HC503 and HC504. All mutants except HC500, HC522 and HC523 were Pel^+ Cel^+ Prt^+ when tested on assay media. The results of a detailed study of the Pel phenotypes of HC500, HC501 and HC523 are presented in Chapter 5. All the mutants HC513-HC523 were streptomycin-sensitive, demonstrating that Tn*5* does not express this antibiotic resistance in *Ecc*. Confirmation that the mutant phenotypes were caused by Tn*5* insertion was obtained by reversion analysis. Sixteen auxotrophs reverted to prototrophs at frequencies of between 6×10^{-7} to 3×10^{-10} (table 4:6:1) Revertants were not detected for mutants HC502 or HC508. Reversion to prototrophy, and concomitant loss of Km^r was observed for nine of the sixteen mutants. However, revertants of seven mutants were often Km^r, indicating maintenance of Tn*5*.

TABLE 4:6:1 Tn*5*-induced mutants of SCRI193

Strain	Parent	Phenotype	Genotype	Reversion Frequency per cell	No. Km^S /total no. tested
HC500	KF1033	Cysteine-requirement Pel^- Cel^- Prt^-	*Cys*B::Tn*5*	$8x10^{-7}$	105/105
HC501	"	cysteine or thiosulphate requirement	*cys*-1::Tn*5*	$2x10^{-7}$	2/7
HC502	KF1005	thiosulphate-requirement	*cys*-2::Tn*5*	$<2x10^{-10}$	
HC503	KF1033	uracil-requirement	*ura*-1::Tn*5*	$1x10^{-9}$	23/23
HC504	"	uracil-requirement	*ura*-2::Tn*5*	$7x10^{-8}$	0/10
HC505	"	glutamate-requirement	*glt*-1::Tn*5*	$1x10^{-10}$	8/8
HC506	"	tryptophan-requirement	*trp*-1::Tn*5*	$3x10^{-10}$	4/4
HC507	"	uncharacterised-requirement	*zzz*-1::Tn*5*	ND	
HC508	"	" "	*zzz*-2::Tn*5*	$<2x10^{-10}$	
HC509	KF1005	arginine-requirement	*arg*-1::Tn*5*	$6x10^{-7}$	22/23
HC510	KF1033	mannitol-non-utilisation	*mnu*-1::Tn*5*	$4x10^{-8}$	1/15
HC511	KF1005	fructose-non-utilisation	*fnu*-1::Tn*5*	ND	
HC512	"	lactose-non-utilisation	*lac*-1::Tn*5*	ND	
HC513	HC131	serine-requirement	*ser*-1::Tn*5*	$9x10^{-9}$	28/28
HC514	"	uncharacterised requirement	*zzz*-1::Tn*5*	$2x10^{-8}$	0/3
HC515	"	methionine-requirement	*met*-1::Tn*5*	$6x10^{-9}$	8/25
HC516	"	cysteine or thiosulphate requirement	*cys*-3::Tn*5*	$3x10^{-9}$	0/8
HC517	"	uncharacterised requirement	*zzz*-4::Tn*5*	$1x10^{-8}$	0/13

Table 4:6:1 continued

Strain	Parent	Phenotype	Genotype	Reversion Frequency per cell	No. Km^s /total no. tested
HC518	"	uncharacterised requirement	*zzz*-5::Tn*5*	ND	
HC519	"	leucine-requirement	*leu*-1::Tn*5*	$1x10^{-9}$	3/3
HC520	"	uncharacterised requirement	*zzz*-6::Tn*5*	$1x10^{-9}$	9/9
HC521	"	uncharacterised requirement	*zzz*-7::Tn*5*	$3x10^{-9}$	12/12
HC522	"	Cel^- Pel^+ Prt^+	*cel*-1::Tn*5*	ND	
HC523	"	Cel^- Pel^+ Prt^+	*cel*-2::Tn*5*	ND	

HC500-512 were isolated with the pJB4JI system; HC513-523 were isolated following λ_{467} infection. ND = not determined

Very little difference was observed between the nature of the mutants obtained with the pJB4JI or λ_{467} systems. Both gave rise to a variety of mutants, which reverted at similar frequencies. Reversion was accompanied by loss of Km^r in about 60% of cases.

The mutant which appeared to be of most interest was HC500. As table 4:6:1 shows, the cysteine auxotroph had a Pel^- Cel^- Prt^- phenotype on assay plates. Reversion to prototrophy was accompanied by complete loss of Km^r. Furthermore, 83/83 of these prototrophic revertants were simultaneously Pel^+ Cel^+ and Prt^+. This proved that the Cys^- and Pel^- Cel^- Prt^- phenotypes were caused by a single Tn*5* insertion.

4:6:2 Discussion

The data shown in table 4:6:1 suggests that insertion of Tn*5* into the chromosome of SCRI193 is random. However, it seems to induce an excess of cysteine-requiring mutants (and other sulphur amino acid-requiring mutants), as has been observed in *E. coli* and *Caulobacter crescentus* (Ely & Croft, 1982; Shaw & Berg, 1979). Similar "hot- spots" for Tn*5* insertion have been reported in various genes including *arg*, *cys*, *his* and *met* of *R. melliloti* and *Yersinia pestis* (Ali *et al.*, 1984; Meade *et al.*, 1982; Zarenkov *et al.*, 1984). In contrast, no insertional hot-spots have been found for Tn*5* in *Echr*, *Ecc* or *Eca* (Chatterjee *et al.*, 1983; Zink *et al.*, 1984).

The biochemical nature of the mutants shown in table 4:6:1, appears to be fairly straightforward. It had been shown previously that mutations of SCRI193 that were analogous to well-defined lesions in *E. coli* could be isolated readily (Forbes, 1983). The precise phenotype of seven auxotrophic mutants remained uncharacterised by the pool plate method

employed. Mutants which require cysteine *or* thiosulphate (HC501 and HC516) have not been reported in *E. coli*, but have been isolated from *S. typhimurium* (Clowes, 1958). The *cel* mutants HC522 and HC523 appeared to be of great interest; such a class of mutant has not been isolated previously amongst soft rot erwinias (Andro *et al.*, 1984; Hinton *et al.*, 1985c; A. Kotoujansky, pers. comm.). Detailed characterisation of the Pel phenotype of HC523 is presented in Chapter 6.

The reversion analysis indicates that most of the nutritional mutants were caused by simple Tn*5* insertion. Revertants of two mutants were not obtained (HC502 and HC508), and this might be the result of the involvement of Mu sequences derived from pJB4JI (Meade *et al.*, 1982), or the inability of Tn*5* to excise precisely from certain sites on the SCRI193 genome (Zink *et al.*, 1984).

Prototrophic revertants which remained Km^r, may have arisen by extragenic suppression, or through the existence of a second copy of Tn*5* at another site on the chromosome (Shaw & Berg, 1979), or may reflect independent JSS0 transposition. Alternatively, spontaneous reinsertion of Tn5 can occur upon selection for precise excision (Zink *et al.*, 1984).

It was not considered necessary to confirm the nature of the Tn*5* mutants by physical analysis, because genetical data indicated that most were caused by single insertions. Rather than perform a detailed analysis of the pJB4JI and λ_{467} mutagenesis systems in SCRI193, it was decided to continue the study of a particular mutant at both the physiological and molecular genetic level. This work is presented in Chapters 5 and 6.

CHAPTER 5

PECTATE LYASE PRODUCTION BY SCRI193 AND HC500

5:1 Introduction

A discussion of the importance of extracellular enzymes in the pathogenicity of the soft rot erwinias has been presented previously (section 1:3). Obviously, the mechanism of exoenzyme secretion is currently of great interest to workers studying *Erwinia* pathogenicity. Furthermore it is apparent that the soft rot erwinias constitute an excellent model system for the study of protein secretion by Gram-negative bacteria (Pugsley & Schwartz, 1985). Consequently secretion by *Erwinia* spp. is being studied is a number of laboratories.

Recent work has implicated a common pathway involved in the secretion of Pel, Peh and Cel by *Ecc* and *Echr*. Secretion-defective (*out*) mutants have been isolated, and are unable to secrete the three enzymes or to macerate potato tissue (Andro *et al.*, 1984; Chatterjee *et al.*, 1985; Thurn & Chatterjee, 1985b). It is known that *Echr* possesses analogues of the *sec* genes of *E. coli* (A. Chatterjee, pers. comm.), suggesting that much of the genetic and biochemical data on the export of protein across the inner membrane of *E. coli* may be applicable to *Erwinia* spp. The range of techniques that are available for the study of the Pel, Peh and Cel enzymes, coupled with the molecular genetic methods which have been developed recently, promise to yield important data concerning a process essential for pathogenesis.

It was hoped that an approach similar to that outlined above could be used to analyse extracellular enzyme production by SCRI193. However, to study extracellular mutants of SCRI193, an understanding of the synthesis and localisation of exoenzymes in the wild-type strain was required. Consequently, the production of Pel by SCRI193 was studied in

detail. This information served as a point of reference with which to compare the mutant HC500, and is currently proving useful in the analysis of other secretion-defective mutants of SCRI193.

RESULTS AND DISCUSSION

5:2:1 Pel production by SCRI193

To determine the kinetics of Pel synthesis, and its location throughout the growth phase, SCRI193 was cultivated in MIM + 0.1% (w/v) yeast extract (YE), and a series of supernatants and whole cell lysates were assayed for enzyme activity. The thiobarbituric acid-based, spectrophotometric Pel assay provided reproducible, quantitative data and proved particularly suitable for processing a large number of samples (section 2:11:2).

Figure 5:2:1a and 5:2:1b show the pattern of secretion of Pel by SCRI193. Figure 5:2:1a demonstrates the synthesis of Pel from early exponential phase, and total culture activity was maximal in mid-exponential phase (A_{600}= 2.0). The relative amount of Pel in the supernatant to that present in the cell lysate reached its maximum at A_{600} = 1.5. At A_{600} > 2.0, approximately 90% of the total Pel activity of the culture was present in the supernatant.

The attainment of maximal Pel activity at mid-exponential phase may be explained in terms of the cessation of enzyme synthesis at this stage, and subsequent stability of the enzyme. Alternatively, it may reflect the establishment of an equilibrium betwen Pel synthesis and degradation. The transient intracellular pool of the enzyme reached a

Figure 5:2:1a

The production of Pel through the growth curve of SCRI193.

SCRI193 was cultivated in MIM + 0.1% (w/v) YE.

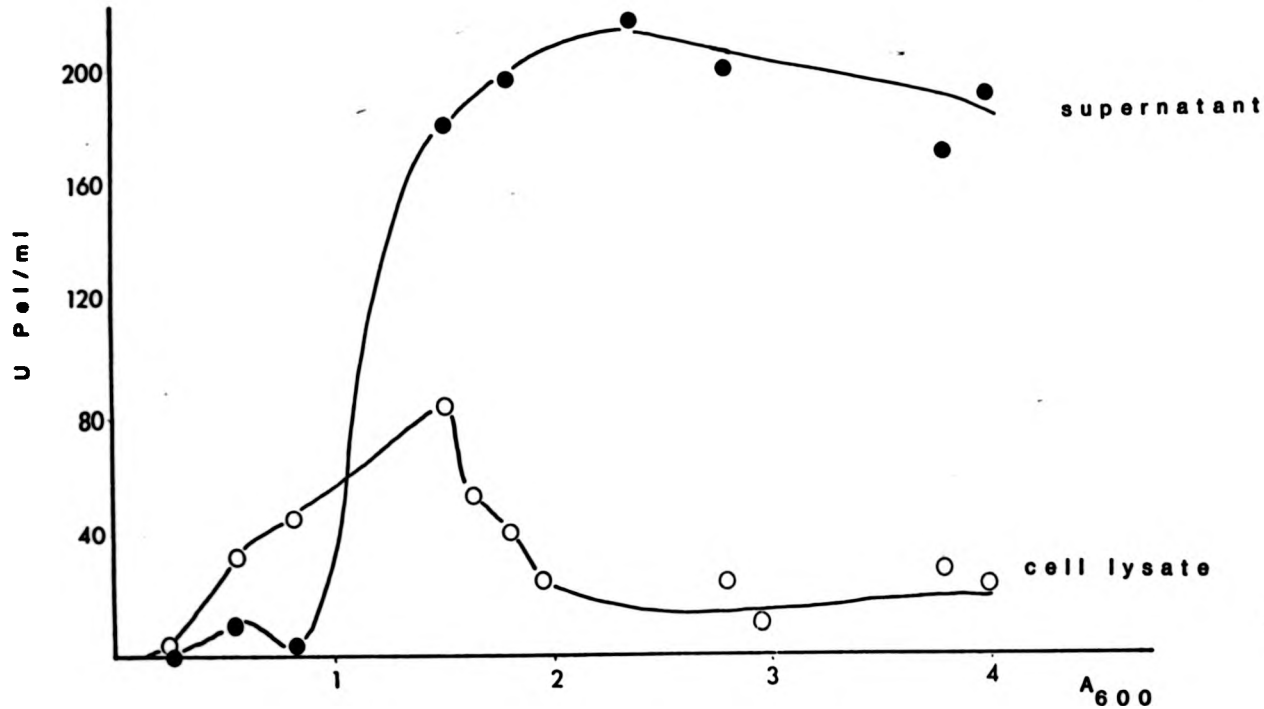
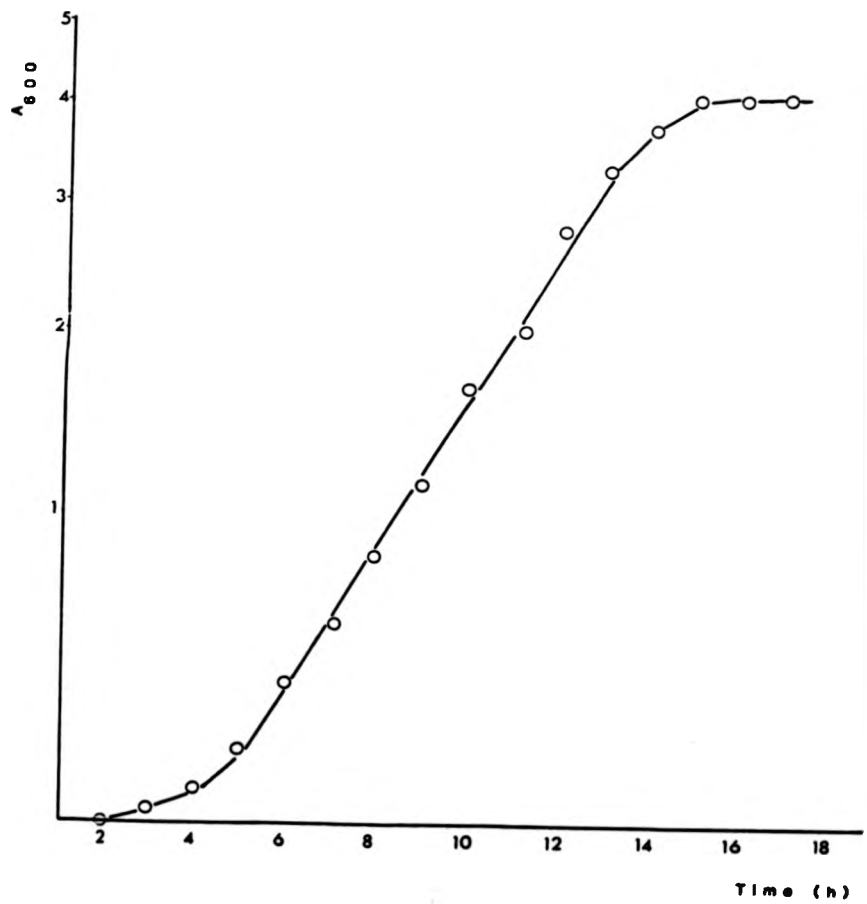


Figure 5:2:1b

A growth curve of SCRI193 cultivated in MIM + 0.1% (w/v) YE.

Samples were taken and used for determination of intracellular and extracellular Pel (Figure 5:2:1a).



peak at A_{600} = 1.5. At later stages in the growth curve, the size of the pool diminished, presumably reflecting active secretion of the newly-synthesised Pel. Although this experiment indicated that 90% of the Pel activity was extracellular, this level can vary from 90-97.5% for SCRI193 (table 5:4:5).

This experiment suggested that SCRI193 secretes Pel extracellularly by an active mechanism. The kinetics of Pel production by SCRI193 resembles those of *Echr* strain 3937j. In the latter case it was shown that the formation of Pel was not accompanied by other intracellular components such as β-galactosidase and β-lactamase in the culture supernatant, implicating a specific active transport mechanism for Pel export (Andro *et al.*, 1984). Recently, studies in this laboratory have confirmed that β-galactosidase is cytoplasmic and β-lactamase is periplasmic in SCRI193, whereas Pel is actively secreted (G. Salmond, pers. comm.).

5:2:2 Pel secretion by *Ecc* and *Echr*

It has been reported previously that *Ecc* secretes a great deal less Pel than *Echr*, and the relevant details are listed here. The percentage total activity of Pel reported in the supernatant was: 94% (*Echr*, EC16; Chatterjee *et al.*, 1979), 90% (*Echr*, 3937; Andro *et al.*, 1984), 90% (*Ecc*, ATCC 8061; Zucker & Hankin, 1970), 13-71% (*Ecc*, EC153; Chatterjee *et al.*, 1979; Moran & Starr, 1969; Starr *et al.*, 1977). The fact that EC153 secreted proportionally less Pel, and synthesised 90% less of the enzyme than EC16 has been considered an important difference between *Ecc* and *Echr* (Chatterjee *et al.*, 1979; Starr *et al.*, 1977). However, since strains ATCC 8061 (Zucker & Hankin, 1970) and SCRI193 (table 5:4:5) synthesise and secrete levels of Pel that are comparable to those

produced by *Echr* strains, it is likely that such differences are strain-dependent and not characteristic of particular species.

5:2:3 Development of a rapid and reproducible Pel assay

The secretory kinetics described in section 5:2:1 were used as a basis for the development of a routine Pel assay which could be applied to a large number of strains. Figure 5:2:1a shows that extracellular Pel activity stabilises at A_{600} > 2.0, for SCRI193. Consequently, strains to be assayed were grown to A_{600} ≈ 2.0 routinely, to ensure that maximal Pel activity had been attained.

It was appreciated that even during the growth of derivatives of SCRI193 in parallel under identical batch conditions, the physiological state of each culture would not be identical, and so could affect the level of Pel production. The only definitive way of comparing enzyme secretion and localisation by different strains, is to obtain data throughout the growth phase for each strain. However, a simple assay was necessary to facilitate the study of Pel produced by 26 strains (table 5:4:5, 6:5:1 & 7:5). Thus, although this assay method was crude, it was adopted because of practical considerations.

5:3 Study of the putative extracellular enzyme mutant HC500

At the time these experiments were done, HC500 was the only extracellular enzyme mutant of SCRI193 that had been isolated. Therefore, HC500 was studied in great detail in an attempt to discover the nature of the Pel$^-$ Cel$^-$ Prt$^-$ phenotype and its effect on pathogenesis. Recently, further work with the λ::Tn*5* mutagenesis system (section 4:5) has led to the isolation of a number of Tn*5*-induced extracellular enzyme mutants of HC131, including those with Pel$^-$ Cel$^-$,

Cel$^-$ Prt$^-$, Cel$^-$, Prt$^-$ and hyper-activity phenotypes (Salmond *et al.*, 1986).

5:3:1 Preliminary evidence concerning the HC500 phenotype

Following the observation of the Pel$^-$ Cel$^-$ Prt$^-$ phenotype of HC500 on assay media (section 4:6:1), it was important to determine whether other lesions in the cysteine biosynthetic pathway of SCRI193 affected extracellular enzyme production. The *cys* mutants KF1036, HC501, HC502, HC503 and HC516 all had Pel$^+$ Cel$^+$ Prt$^+$ phenotypes on assay plates (see section 5:4:5), suggesting that the HC500 lesion was of particular interest, i.e. the HC500 phenotype did not always arise from a decreased availability of cysteine within the cell but had a more complex basis.

This preliminary data indicated that HC500 could possess a cysteine biosynthetic lesion which directly affected extracellular enzyme production. In order to investigate the molecular basis of the HC500 phenotype, both the mutant allele and its wild-type homologue were cloned for further analysis (see Chapter 6).

Examples of the assay media, and the Pel$^-$ phenotype of HC500 are given in figures 5:3:1a and 5:3:1b. Figure 5:3:1a shows the Pel$^+$ Cel$^+$ Prt$^+$ phenotype of the wild-type strain SCRI193. Figure 5:3:1b illustrates that SCRI193 and KF1033 possess much Pel activity whereas HC500 produces very little of the enzyme.

Figure 5:3:1a

Colonies of SCRI193 grown on extracellular enzyme assay media.

The media are (left to right) Pel, Cel and Prt.

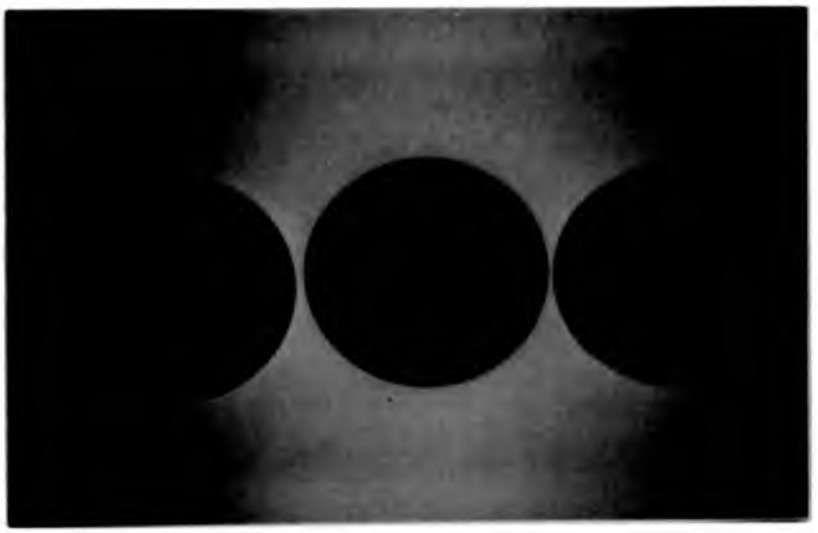


Figure 5:3:1b

Colonies of SCRI193 derivatives grown on Pel assay media.

The strains are (left to right) SCRI193, KF1033 and HC500.

5:4 Physiological analysis of the HC500 phenotype

5:4:1 Introduction

Pel, Cel and Prt assay plates have been widely used in the study of soft rot erwinias (Andro *et al.*, 1984; Chatterjee *et al.*, 1985a,b; C. Wandersman, pers. comm.), and the size of activity zones can be related to enzyme activity (Beraha & Garber, 1971). However, the use of such assay media when screening for extracellular enzyme mutants has some disadvantages. For example, since activity zone size is proportional to the amount of bacterial growth, the growth rate of particular mutants upon assay plates can affect their apparent enzyme activity. In addition, false negative results have been observed on Cel media (M. Chippaux, pers. comm.). Therefore, confirmation of extracellular enzyme phenotypes with appropriate spectrophotometric assays is essential. Consequently, concurrent with the molecular cloning of the *cys*::Tn*5* mutation (Chapter 6), physiological experiments were undertaken to investigate the production of extracellular enzymes by HC500. These involved the use of the spectrophotometric Pel assay essentially as described earlier (section 2:11:2).

5:4:2 The effect of nutrient concentration upon the HC500 phenotype

The Pel assay medium of Andro *et al.* (1984), which was used to demonstrate the Pel$^-$ phenotype of HC500, contained 0.1% (w/v) YE. Therefore HC500 and its parental strain KF1033 were grown in MIM containing 0.1% (w/v) YE, and the Pel activity in the supernatant was assayed. As Figure 5:4:2 shows, HC500 grew very poorly in this medium when compared with KF1033, and produced no Pel. To determine whether the reduced growth of HC500 reflected nutrient-limitation, HC500 and KF1033 were grown in MIM containing increasing amounts of YE (Fig.

5:4:2). The cell density of KF1033 cultures almost doubled with a ten-fold increase in YE concentration, whereas the amount of Pel exported remained between 206 and 234 U per ml of culture supernatant. In contrast, the cell density of the HC500 cultures increased by more than ten times with a ten-fold increase in YE concentration. Furthermore, the amount of Pel produced by HC500 rose dramatically from 0 (in media + 0.1% (w/v) YE) to 211 U Pel per ml culture supernatant, in media containing 1% (w/v) YE.

In summary, the data presented in Figure 5:4:2 suggests that the Pel$^-$ phenotype of HC500 in media containing 0.1% (w/v) YE reflects growth under limiting conditions. It shows that growth of HC500 under non-limiting conditions leads to similar levels of Pel export as in the parental strain.

5:4:3 The effect of cysteine upon the HC500 phenotype

As described in section 5:4:1, the Pel$^-$ phenotype of HC500 is physiologically remedied by growth in medium containing 1-2% (w/v) YE. However, the mechanism by which YE caused the effect was unknown. Bacteria grow faster in rich media than minimal media because they can take up amino acids and other nutrients rather than synthesise them *de novo*. Did YE cause its effect upon Pel production by serving as an undefined rich medium, or was a particular constituent important? To discover whether cysteine could cause similar effects upon Pel production by HC500, cultures were grown in media containing increasing levels of the amino acid, with and without YE (Fig. 5:4:3).

When cysteine was added to the MIM, it did not markedly affect the culture density or KF1033, or the level of Pel production (although Pel

Figure 5:4:2

The effect of nutrient concentration upon Pel production by KF1033 and HC500.

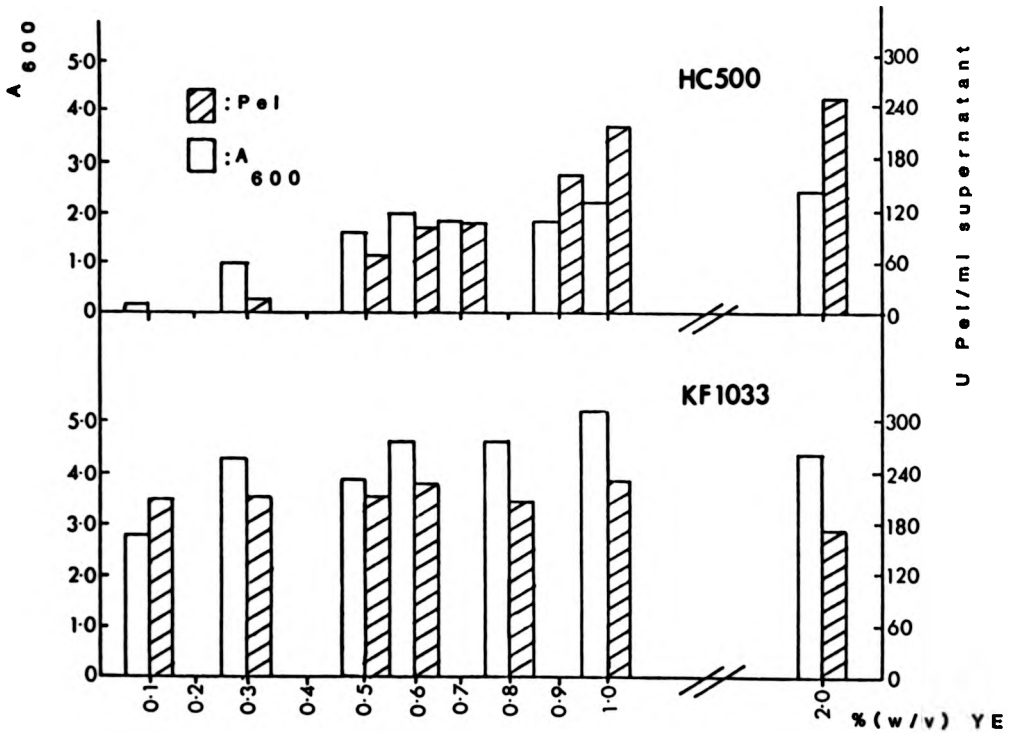
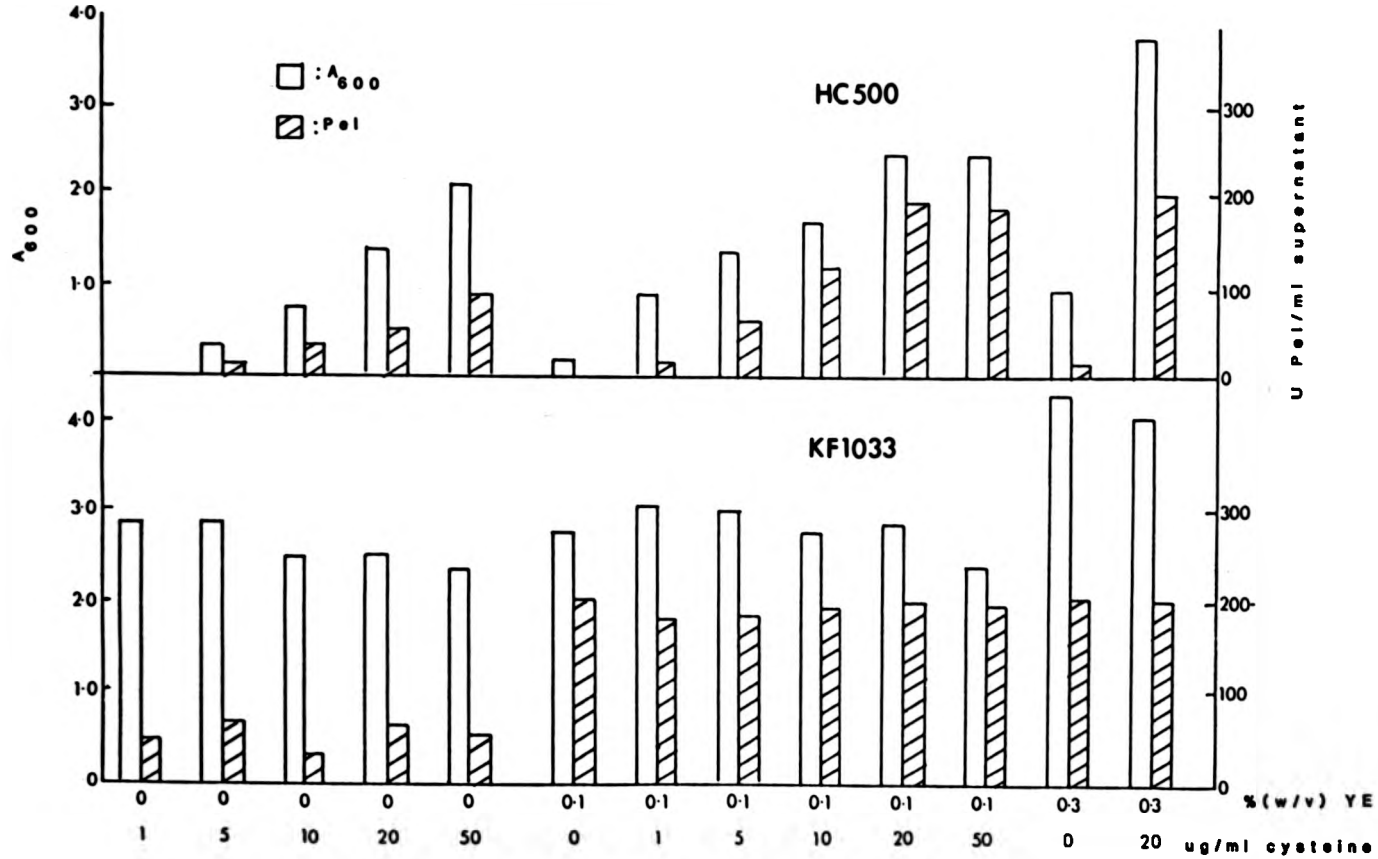


Figure 5:4:3

The effect of cysteine upon Pel production by KF1033 and HC500.



production did show some fluctuation). However, the culture density and level of Pel production by HC500 was substantially increased by the addition of cysteine. In the presence of 50 μg/ml cysteine, HC500 produced significantly more Pel per ml culture supernatant than did KF1033.

The addition of cysteine to MIM containing 0.1% (w/v) YE led to a slight decrease in KF1033 culture density, with little effect on the level of Pel production. In contrast the addition of 50 μg/ml cysteine caused a ten-fold increase in culture density of HC500, and a level of Pel production comparable to that of KF1033. Media containing 0.3% (w/v) YE and 0.3% (w/v) YE + 20 μg/ml cysteine gave a similar effect upon Pel production to that seen in media containing 0.1% (w/v) YE.

To determine the nature of the effect of cysteine, 20 μg/ml of the amino acid was added to stationary phase cultures of KF1033 and HC500 (grown in MIM + 0.3% (w/v) YE, 30°C, 20 h), and incubated at 30°C with shaking for 2 h before Pel assay (table 5:4:3). This had no effect upon culture density or the level of Pel production by KF1033. However the addition of cysteine to stationary phase HC500 caused a doubling in culture density, but had little effect upon the level of Pel production, when compared with KF1033. The apparent increase in Pel production by HC500 in this experiment, may be attributed to inaccuracy of the assay at low levels of Pel activity.

The data presented in this section suggests that cysteine has an indirect effect upon Pel production by HC500. The addition of cysteine to media appears to remove growth-limitation, and allows HC500 to attain

TABLE 5:4:3 The effect of cysteine upon a stationary-phase HC500 culture

Media	KF1033		HC500	
	A_{600}	Pel activity in supernatant (u/ml)	A_{600}	Pel activity in supernatant (u/ml)
MIM + 0.3% (w/v) YE	4.28	212	1.0	12
Culture grown to stationary phase in MIM + 0.3% (w/v) YE (30°C, 20h, 150 rpm) 20 µg/ml cysteine added followed by a further 2h incubation (30°C, 150 rpm)	3.82	205	2.30	18

similar culture densities and levels of Pel production as its parental strain. Table 5:4:3 shows the addition of cysteine to a stationary phase culture of HC500 did not restore Pel activity to parental levels. However, it seems that the cysteine-limited HC500 cells had resumed growth as soon as additional cysteine was added, causing a sharp increase in culture density. This would suggest that longer incubation, or addition of more cysteine, would lead to a density comparable to that of KF1033, as well as some Pel production. Although the nature of the effect of cysteine-limitation upon Pel production is not clear, it is apparent that cysteine acts indirectly, rather than causing direct renaturation of Pel to its active form.

5:4:4 Growth characteristics of HC500 under nutrient-limiting conditions

As mentioned earlier, growth of HC500 in MIM + 0.3% (w/v) YE appeared to be nutrient-limited. To determine the growth kinetics of HC500 and its parent, KF1033, in this media, standard growth curves were constructed. Figure 5:4:4 shows that KF1033 exhibits distinct lag, exponential and stationary phases of growth, with a doubling time of 140 min. In contrast, HC500 grew at a considerably slower rate (doubling time = 281 min), suggesting growth limitation in this media.

5:4:5 Is the HC500 phenotype caused by a secretory or biosynthetic defect?

To determine whether production of extracellular enzymes by HC500 was affected at the secretory or biosynthetic level, a range of strains was grown in media containing 0.3% (w/v) YE, and the levels of Pel and its localisation were determined. Table 5:4:5 shows little difference between the levels and location of Pel produced by SCRI193, 3237jLD and

Figure 5:4:4

Growth curves of KF1033 and HC500 cultivated in MIM + 0.3% (w/v) YE.

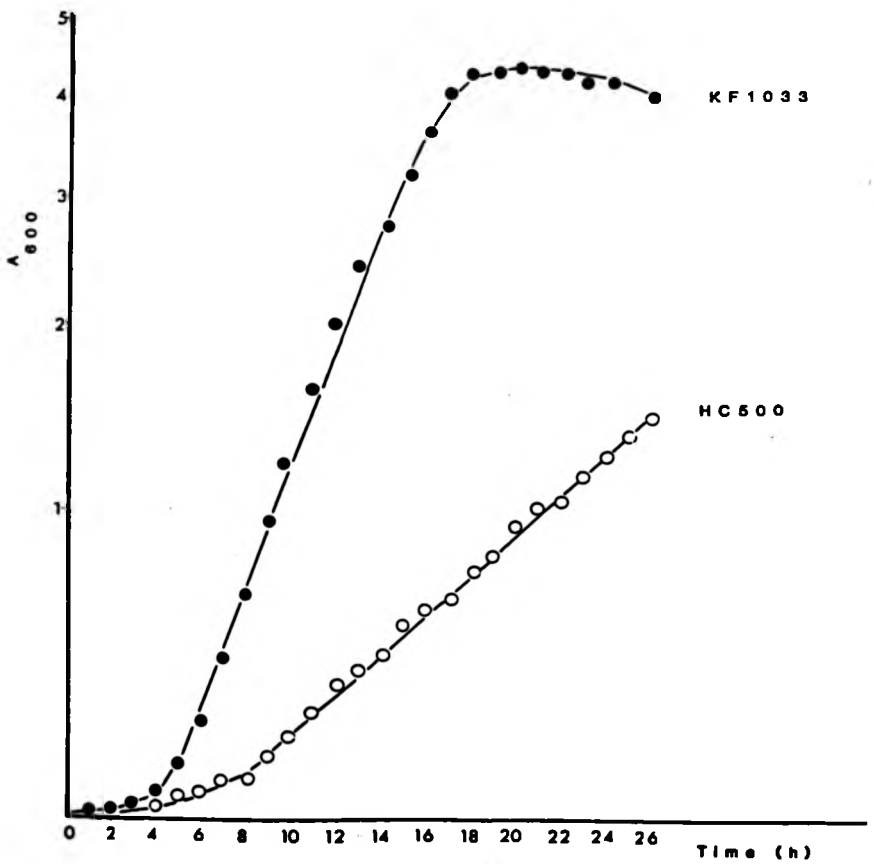


TABLE 5:4:5 Pel production by *Ecc* and *Echr* strains

Strain	Total Pel activity in Pel/mg protein	Total activity as % of SCRI193	% of total activity in cell lysate	% of total activity in supernatant
1066	1129	152	52	48
3937jLD	847	114	2	98
B374	608	82	6	94
SCRI193	743	100	6	94
KF1005	711	96	6	94
KF1033	460	62	7	93
HC500	10	1.4	31	69
KF1036(*cys*)	671	90	3	97
HC501(*cys*-1::Tn*5*)	397	53	3	97
HC523(*cel*::Tn*5*)	568	76	4	96

Assays were performed as described in section 2:11:2.

B374, as discussed in section 5:2:2. The *Echr* strain 1066 has been selected as a high asparaginase-producing strain (Gilbert *et al.*, 1985), and exhibits anomalous Pel localisation. The remaining strains in table 5:4:5 are derived from SCRI193; KF1005 (Sm^r SCRI193) produces similar levels of Pel as its parent. However, KF1033 (Nx^r KF1005) produces 40% less Pel than its parent, although localisation is not affected. It seems likely that the difference in Pel activity between KF1005 and KF1033 can be attributed to the Nx^r mutation, and similar effects have been observed in *E. coli* and *Echr*. It has been proposed that mutations in *gyrA* (the major Nx^r locus) cause lower levels of superhelicity which interferes with the expression of many operons including *bgl* (Dinardo *et al.*, 1982) and *Clb* from *Echr* (Barras *et al.*, 1984a).

The addition of 0.3% (w/v) YE to the media used for this experiment was chosen to allow some growth of HC500 but the production of only 11 U/ml of extracellular Pel (Fig. 5:4:2). It was hoped that the Pel localisation data obtained from HC500 grown under these conditions would show whether the level of intracellular Pel was affected. The data presented in table 5:4:5 indicates that HC500 produces only 2% as much Pel as its parent (KF1033). Although HC500 appears to have relatively more intracellular Pel than KF1033, this may reflect inaccuracy of the assay at such low levels of the enzyme. Therefore, the inability of HC500 to produce Pel under nutrient-limited conditions does not result from a block in enzyme secretion, with a concomitant build-up of enzyme within the cell, as was observed for the *out* mutants of *Echr* (Andro *et al.*, 1984). Rather, the HC500 phenotype reflects a lack of synthesis of the active enzyme.

Table 5:4:5 also gives Pel data for two *cys* mutants. KF1036 and HC503

were derived from KF1005 and KF1033 respectively, and their levels of Pel production resemble their parents. This supports the preliminary plate assay evidence of section 5:3:1, and confirms the observation that Pel production by SCRI193 is not affected by other *cys* lesions. Finally, the *cel* mutant HC523 (derived from HC131) produces 24% less Pel than SCRI193, but enzyme localisation is not affected.

5:4:6 Pathogenicity of HC500

The ability of HC500 and other strains to macerate potato tuber tissues was tested by an established method (Collmer *et al.*, 1985). The data presented in table 5:4:6 shows a high standard deviation, which reflects the inherent inaccuracy of this method (Collmer *et al.*, 1985). SCRI193 appears to cause nearly twice as much maceration as its derivatives (although these figures show a large standard deviation). Time was not available to confirm this result. However, there is not a great difference between the amount of maceration caused by KF1005, KF1033 and HC500. The data concerning the pPEL1 clone in *E. coli* is discussed in section 7:5:1.

The pathogenicity data demonstrates that the Tn*5*-induced mutation of HC500 does not impair its ability to cause soft rot *in planta*. This suggests that growth of HC500 in potato tubers is not cysteine-limited.

5:5 The basis of the extracellular enzyme phenotype of HC500

The data presented in this chapter suggests that the Pel$^-$ phenotype of HC500 results from growth under limiting conditions in media containing less than 1% (w/v) YE. The analysis of Pel production by SCRI193

TABLE 5:4:6 Pathogenicity of SCRI193 derivatives and *E. coli* clones

Strain	Wet weight (g) of macerated tissue at sites injected with 8×10^7 cfu of bacteria
SCRI193	12.4 ± 8.1
KF1005	6.6 ± 2.2
KF1033	6.7 ± 0.9
HC500	4.8 ± 0.5
HB101(pBR322)	0
HB101(pPEL1)	5.1 ± 1.9

Results are mean values and standard deviations from three injection sites.

(section 5:2:1) showed that Pel was synthesised and secreted during rapid exponential growth (doubling time = 109 min). However, the growth characteristics of HC500 in media containing 0.3% (w/v) YE (section 5:4:4) demonstrate that this strain did not grow at a comparable rate but was subject to some limitation. The addition of increasing YE and/or cysteine levels to the medium (sections 5:4:2 & 5:4:3) led to higher culture densities and increasing levels of Pel production. Finally, analysis of Pel localisation by HC500 suggested that under low nutrient conditions, enzyme did not accumulate intracellularly (section 5:4:5).

The following explanation can be offered for the results presented above. HC500 requires *ca*. 50 μg/ml of cysteine to attain a rapid growth rate and to produce high levels of Pel. In medium containing reduced levels of cysteine, HC500 grows slowly, and so does not synthesise Pel. Evidence presented in Chapter 6 proves that this effect is not caused simply by cysteine-limitation but by sulphur-limitation which arises from the pleiotropic nature of the *cys* lesion of HC500. Studies with HC500 in a sulphur/cysteine-limited chemostat would allow the detailed analysis of the exact relationship between Pel synthesis and sulphur limitation.

CHAPTER 6

GENE CLONING IN SCRI193

6:1 Introduction

The results presented in Chapter 5 suggested that the Tn*5*-induced mutation of HC500 could be involved in extracellular enzyme synthesis. In order to further characterise this mutation, the Tn*5* element and flanking sequences were cloned from HC500, and the corresponding wild-type gene isolated. These clones were used to determine the genetic nature of the cysteine auxotrophy of HC500. This section describes the cloning, subcloning and subsequent manipulations of *cys*::Tn*5* and *cys*$^+$ from HC500.

RESULTS AND DISCUSSION

6:2 Isolation of pJH1

Figure 6:2a shows the strategy used to clone the Tn*5* insertion from HC500. pBR322 and HC500 chromosomal DNA were digested with *Eco*RI. These DNA molecules were then ligated together in a ratio of 1:6, and used to transform *E. coli* HB101. 4.2 μg of religated DNA gave rise to two Ap^rKm^r colonies, which were purified. These colonies were designated HB101(pJH1) and HB101(pJH2).

Restriction mapping of pJH1 and pJH2 confirmed that they carried the transposon by demonstrating the presence of the internal *Bgl*II and *Hin*dIII restriction fragments of Tn*5* (de Bruijn & Lupski, 1984). Detailed mapping proved that pJH1 and JH2 were identical, carrying a 17.7 Kb insert in similar orientation (Figs. 6:2b and 6:2c).

Figure 6:2a

The strategy employed to construct pJH1 and its derivative pJH3.

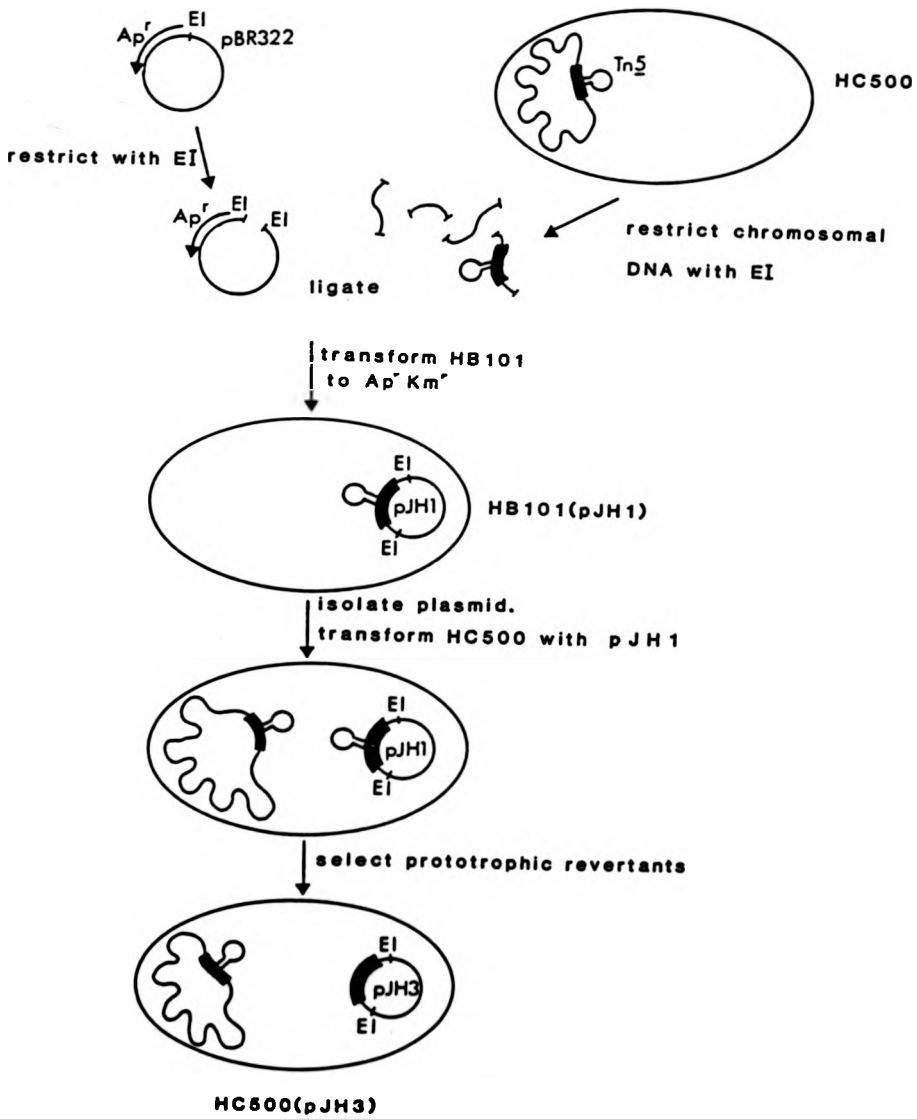


Figure 6:2b

Restriction map of the chromosomal insert of pJH1.

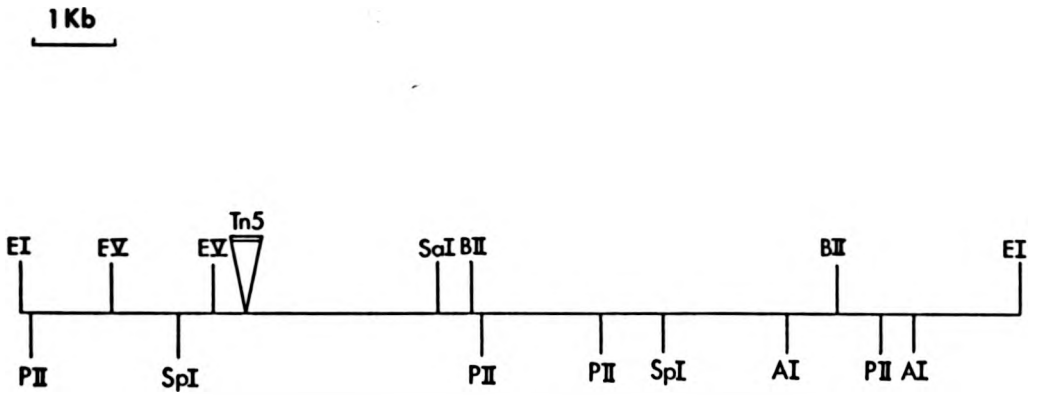
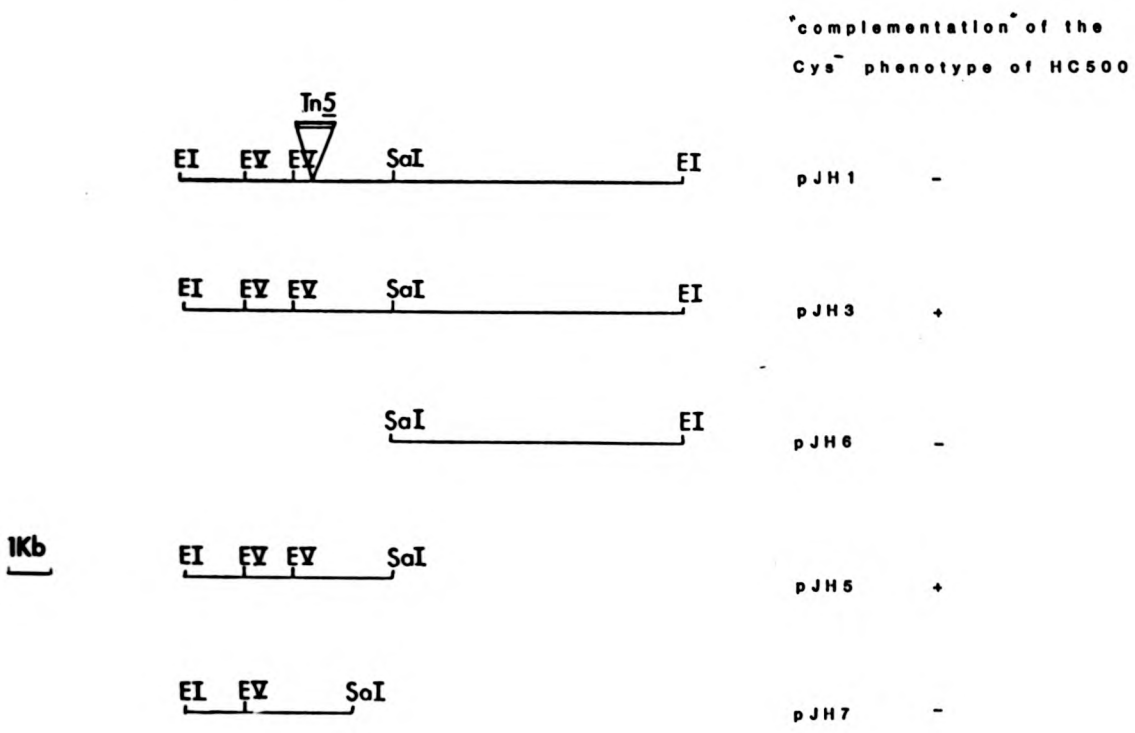


Figure 6:2c

Partial restriction maps of the chromosomal inserts of pJH1, pJH3, pJH5, pJH6 and pJH7.



6:3 Construction of pJH3

As mentioned earlier, secondary transposition events can complicate the molecular analysis of Tn*5*-induced mutations (section 4:2). Thus, following the isolation of pJH1, it was necessary to prove that the chromosomal insert which contained Tn*5* corresponded to the lesion of HC500. This was achieved by using pJH1 to obtain the corresponding Tn*5*-free clone, which carried a fully-functional wild-type gene, and "complemented" the HC500 phenotype.

This clone was used to characterise the lesion of HC500 in more detail, and to yield information concerning the physical and genetical organisation of the wild-type gene.

One method of isolating the fully-functional gene would be to construct a new gene library of of wild-type DNA, and to use pJH1 as a probe. Colony hybridisation techniques could be used to detect the wild-type gene by sequence homology (Maniatis *et al.*, 1982). However, in this case, a novel approach was adopted which proved to be less time-consuming. This relied on a well-characterised property of transposable elements; precise excision (section 4:2).

In *E. coli*, Tn*5* elements can excise precisely from their site of insertion at a frequency of between 10^{-6} and 10^{-7} (Berg, D. & Berg, C., 1983), leaving an entire fully-functional gene. Such excision events can give rise to revertants of Tn*5*-induced mutants, following direct selection. The strategy used to select for precise excision of Tn*5* from pJH1 is summarised in Figure 6:2a and has been published (Hinton *et al.*, 1985c). HC500(pJH1) was constructed by transformation, and the presence

of the plasmid was confirmed physically. This strain was grown overnight under non-selective conditions, and approx. 10^9 cells were plated on minimal agar. Prototrophic revertants arose at a frequency of 1.7×10^{-6} per cell. These revertants could have arisen due to excision of the Tn*5* element from either the chromosome or the plasmid (assuming the lesion of HC500 could be complemented *in trans*). Because pBR322 probably exists as multiple copies in SCRI193, the majority of the revertants should result from excision of Tn*5* from pJH1. The fact that the reversion frequency of HC500(pJH1) is twice that of HC500 (table 4:6:1) confirms this.

Following the isolation of plasmid DNA from twelve prototrophic revertants, it was determined that they had all lost a 5.7 Kb fragment, corresponding to the Tn*5* element. This was confirmed by restriction analysis which showed that the *Bgl*II and *Hin*dIII internal fragments of Tn*5* were now absent.

One of these plasmids was designated pJH3, and was reintroduced to HC500 by transformation. Ap^r transformants became simultaneously Cys^+, Pel^+, Cel^+ and Prt^+. More detailed data concerning the effect of this plasmid on the phenotype of HC500 is presented later (section 6:5).

6:4 Subcloning of pJH3

After a restriction map of pJH3 had been constructed (Fig. 6:2c), experiments were continued to localise the gene of interest within the 11.9 Kb insert. Chromosomal fragments were subcloned into pBR322 and deletion derivatives were constructed. Although all subcloning was performed in HC500 (see section 3:4:2), all plasmids were routinely

maintained in a *rec*A derivative of *E. coli* (DH1), to prevent rearrangements.

6:4:1 Construction of pJH5 and pJH6

As shown in Figure 6:2c, the chromosomal insert in pJH3 carries a unique *Sal* I site; following a *Sal*I-*Eco*RI digestion, the 5 Kb and 6.9 Kb fragments were prepared and subcloned into *Sal*I-*Eco*RI-cut pBR322. The resulting plasmids were designated pJH5 and pJH6 respectively (see Fig. 6:2c). pJH5 was found to "complement" the HC500 phenotype (section 6:5), and this subclone was characterised further.

6:4:2 Construction of pJH7

As can be seen in Figure 6:2c, pJH5 contains two *Eco*RV sites. In order to obtain more information regarding the position of the gene of interest, these sites were used to construct a deletion derivative of the plasmid. pJH5 was restricted with *Eco*RV, religated in a large volume (to promote intramolecular recircularisation) and introduced to HC500. The resultant plasmid had a deletion of 1.2 Kb, and was designated pJH7.

Complete restriction maps of pJH1 and its derivatives were constructed following detailed analysis of each plasmid (Fig. 6:2c). The effect of these plasmids on the HC500 phenotype is described in the next section.

6:5 Localisation of the cys^+ gene of pJH3

"Complementation" tests with HC500 carrying pJH1 and its derivatives were performed upon assay media, and the data are presented in table 6:5. As

TABLE 6:5 Plate tests of HC500 containing various plasmids

Strain	Phenotype			
	Cys	Pel	Cel	Prt
SCRI193	+	+	+	+
KF1033	+	+	+	+
HC500	-	-	-	-
HC500(pBR322)	-	-	-	ND
HC500(pJH1)	-	-	-	-
HC500(pJH3)	+	+	+	+
HC500(pJH5)	+	+	+	+
HC500(pJH6)	-	-	-	-
HC500(pJH7)	-	-	-	-

ND = not determined

Phenotypes were determined on appropriate minimal and assay media.

implied earlier, pJH1 did not affect the HC500 phenotypes, but pJH3 restored HC500 to wild-type. Restriction data showed that the Tn*5* element of pJH1 was inserted 2.7 Kb into the 5 Kb *Sal*I-*Eco*RI fragment of pJH3 (Fig. 6:2c). This suggested that the *cys*$^+$ gene lay wholly or partially upon this *Sal*I-*Eco*RI fragment. Subcloning of the two *Sal*I-*Eco*RI fragments confirmed this; pJH5 "complemented" the HC500 lesion, but pJH6 did not. The deletion constructed in pJH7 lay 400 bp to one side of the site of Tn*5* insertion, and this inactivated the *cys*$^+$ gene.

These data suggested that part of the *cys*$^+$ gene of pJH3 lay between the point of Tn*5* insertion and the right-hand *Eco*RV site of the plasmid. Further discussion concerning the precise location of this gene appears in section 6:8.

6:5:1 "Complementation" of the Pel$^-$ phenotype of HC500 by pJH1 and its derivatives

In order to obtain quantitative, as well as qualitative data concerning the "complementation" of the HC500 phenotype, Pel assays were performed upon strains carrying the plasmids listed in Figure 6:2c. Table 6:5:1 shows the results of Pel assays on several HC500 derivatives. It should be noted that the Pel assays were only performed once for the cell lysates and twice for the culture supernatants (as described in section 5:4:5), and so some variability may reflect experimental error rather than intrinsic differences between strains.

As reported previously (Keen *et al.*, 1984), the presence of the antibiotics Ap, Km, Sm or Tc did not influence the levels of Pel activity. The plasmids pBR322, pJH1, pJH6 and pJH7 did not affect the level of Pel synthesis by HC500, confirming the qualitative data of

TABLE 6:5:1 Pel production by HC500 and other strains containing pJH1-derived plasmids

Strain	Total Pel activity in Pel/mg protein	Total activity as % of SCRI193	% of total activity in cell lysate	% of total activity in supernatant
SCRI193	743	100	6	94
KF1033	460	62	7	93
HC500	10	1.3	31	69
HC500(pBR322)	7	0.9	20	80
HC500(pJH1)	9	1.2	25	75
HC500(pJH3)	185	25	3	97
HC500(pJH5)	336	45	3	97
HC500(pJH6)	7	0.9	0	100
HC500(pJH7)	4	0.5	33	67
SCRI193(pJH1)	503	68	3	97
SCRI193(pJH3)	683	92	3	97
KF1036(pJH3)	705	95	3	97

Assays were performed as described in section 2:11:2.

table 6:5. As mentioned earlier (section 5:4:5), the discrepancies in the cellular location of Pel in these strains probably reflects the insensitivity of this assay system at low levels of Pel. The two plasmids thought to carry the *cys*$^+$ gene "complemented" Pel synthesis by HC500 to varying degrees; HC500(pJH3) produced 40% as much Pel as the parental strain KF1033. However HC500(pJH5) produced 73% as much Pel as KF1033. This might reflect different levels of complementation by pJH3 and pJH5, for which a number of explanations could be offered: perhaps pJH5 is present at a higher copy number than pJH3, or a regulatory element is present on the 6.9 Kb *Sal*I-*Eco*RI fragment of pJH3. It is possible that the differences in Pel production betweeen HC500(pJH3) and HC500(pJH5) could be explained by differential effects of the plasmids upon growth rates of these strains.

In an attempt to discover whether pJH1 or pJH3 affected Pel synthesis by SCRI193 itself, the plasmids were introduced to the strain by transformation. Table 6:5:1 shows that SCRI193(pJH1) synthesises 32% less Pel than the plasmid-free strain. Interestingly, pJH3 has little effect on Pel production by SCRI193. It seems that the presence of Tn5 upon pJH1 causes a marked affect on Pel production for reasons that are not understood, but this should be confirmed by a detailed analysis, as described in section 5:2:1. Nevertheless, because pJH3 appears to have little or no effect on Pel production, it suggests that the presence of the *cys*$^+$ gene product in multi-copy is not deleterious to the cell.

Earlier, it had been shown that the *cys* mutation of KF1036 did not affect Pel production (table 5:4:5). pJH3 was introduced to this strain, and had no effect on the *cys* phenotype or the level of Pel production (table 6:5:1).

6:6 Genetic Identification of the *cys* mutation of HC500

As detailed in section 1:6, a number of workers have complemented mutations of *E. coli* with *Erwinia* genes, reflecting the close relatedness of the two species. Such heterogeneric complementation was used to characterise the HC500 lesion.

The plasmid pJH3 has already been shown to carry the *cys*$^+$ gene which "complemented" the HC500 phenotype. This plasmid was introduced to ten cysteine-requiring mutants of *E. coli* K12, by transformation. Ap^r transformants were screened for "complementation" of the *cys* defect. The results (table 6:6a) show that only the *cysB* lesion of strain CB64 was complemented by pJH3.

To confirm this result, four other pJH1 derivatives were introduced to CB64, and the transformants screened for complementation of the Cys$^-$ phenotype. Table 6:6b shows the results of this experiment, and reveals an identical "complementation" pattern for HC500 and CB64. This suggests that the HC500 and CB64 *Cys*$^-$ phenotypes are "complemented" by the same gene, and that HC500 carries a lesion in a gene analagous to *cysB* of *E. coli*. Discussion on this, and the implications for the HC500 phenotype is presented in section 6:8.

6.7 Gene product identification

Following the presumptive identification of the HC500 lesion, experiments were undertaken to identify the *cysB*$^+$ gene product of SCRI193. This was achieved by comparison of the gene products of pJH1,

TABLE 6:6a "Complementation" of *E. coli cys* mutations by pJH3

Strain	Mutation	"Complementation" of Cys- phenotype by pJH3
DG37	*Cys*A	-
CB64	*Cys*B	+
JM81A	*Cys*C	-
JM221	*Cys*D	-
JM15	*Cys*E	-
AT2455	*Cys*G	-
JM96	*Cys*H	-
JM246	*Cys*I	-
AT2427	*Cys*J	-
UQ818	*Cys*S	-

Phenotypes were determined on appropriately supplemented minimal media.

TABLE 6:6b "Complementation" of CB64 and HC500 by pJH1-derived plasmids

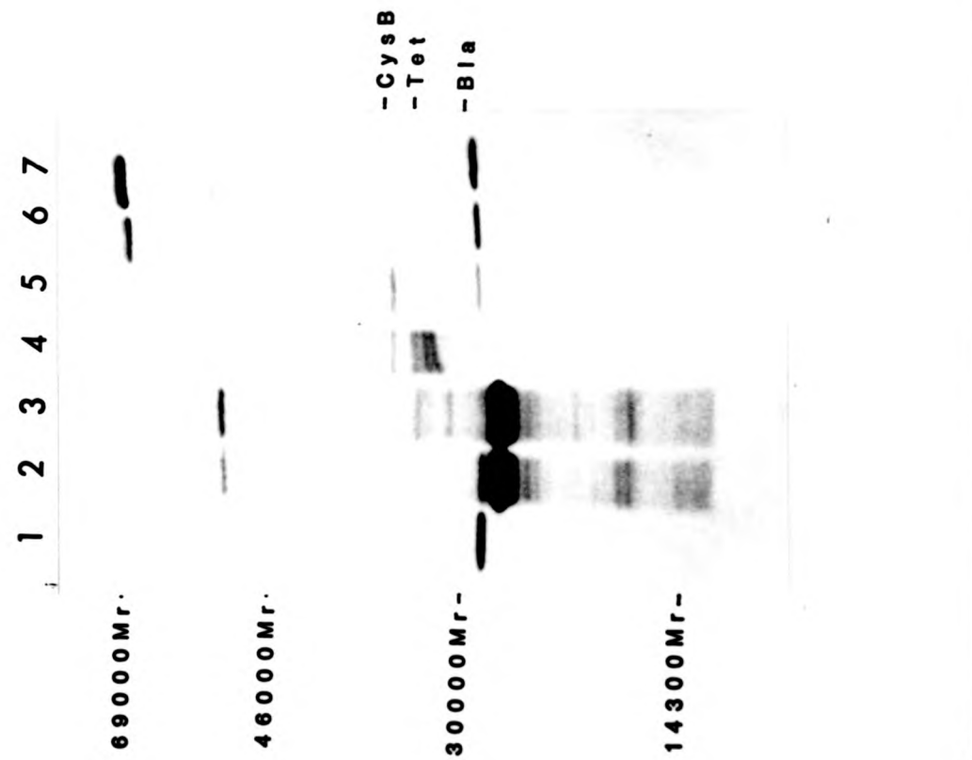
	Cys⁻ Phenotype	
Plasmid	HC500	CB64
pJH1	-	-
pJH3	+	+
pJH5	+	+
pJH6	-	-
pJH7	-	-

Phenotypes were determined on appropriately supplemented minimal media.

Figure 6:7

Identification of the *Ecc cysB* gene product.

^{35}S-labelled "maxicell" proteins were prepared as described, and analysed by 10-30% gradient SDS-PAGE. The autoradiogram was exposed for 3 d. Track 1 = pBR322; track 2 = pJH10 (pBR322::Tn*5*); track 3 = pJH1; track 4 = pJH3; track 5 = pJH5; track 6 = pPEL1; track 7 = pPEL2.



pJH3 and pJH5.

A modified "maxicell" method was used, as detailed in section 2:27. The Tn*5*-carrying derivative of pBR322 which was used as a control, was constructed by a standard procedure, and designated pJH10 (section 2:8:1). Plasmid-carrying strains were labelled with ^{35}S-methionine, and the proteins were analysed on a 10-30% gradient SDS-polyacrylamide gel. The autoradiogram is shown in Figure 6:7. Protein sizes were estimated by comparison with ^{14}C-labelled molecular weight markers (section 2:26).

The parental plasmid, pBR322, encoded β-lactamase (Bla: Mr 28,000) and the *tet* gene product (Mr 33,000). In addition to these, pJH10 produced three novel Tn*5*-encoded proteins; Kanamycin acetyltransferase (Mr 27,500; Genilloud *et al.*, 1984), and the products of IS50L and IS50R (Mr 58,000 and Mr 54,000; Rothstein *et al.*, 1980). The Mr 49,000 protein encoded by IS50L was not observed, but this protein is rarely apparent in maxicells (Rossetti *et al.*, 1984).

In addition to the pJH10-coded polypeptides, pJH1 produced a protein of Mr 31,500. pJH3 encoded Bla, Tet and three other proteins: Mr 36,000, Mr 33,500 and Mr 32,500. pJH5 only produced Bla and the Mr 36,000 protein. The two smaller proteins produced by pJH3 (Mr 33,500 and 32,500), and not by pJH5 are presumably encoded by the larger SalI-EcoRI fragment of the plasmid. The Tn*5* insertion in pJH1 inactivates the *cysB*$^+$ gene, and prevents the synthesis of the Mr 36,000 protein of pJH3. The Mr 31,500 protein produced by pJH1 may be a truncated form of the *cysB*$^+$ gene product (de Bruijn & Lupski, 1984). It should be noted that pJH5 encodes no Tet protein, because part of the *tet* gene had been deleted in its construction (the gene products of pPEL1 and pPEL2 are

discussed in section 7:4). The data presented here allows the unequivocal identification of the *cysB*$^+$ gene product as the Mr 36,000 protein encoded by pJH5.

6:8 Is the lesion of HC500 directly analogous to *cysB*?

A number of lines of evidence suggest that the genetic lesion of HC500 is in a single gene, analogous to *cysB* of *E. coli*:

(a) The mutant phenotype of HC500 and CY64 is "complemented" by a *cys*$^+$ gene carried by pJH3 and pJH5.

(b) The *cys*$^+$ gene is inactivated by a single Tn*5* insertion.

(c) Deletion of the 1.2 Kb *Eco*RV fragment of pJH5 leads to inactivation of this gene.

(d) The synthesis of the *cys*$^+$ gene product (Mr 36,000) was abolished by the insertion of the Tn*5* element, which was accompanied by the appearance of a novel protein of Mr 31,500. This may be a truncated form of the Mr 36,000 protein, suggesting that the Tn*5* is inserted promoter distal in the *cys*$^+$ gene.

Thus all the available evidence is consistent with the existence of a *cysB* mutation in HC500. However it is possible that the data presented above could be explained by the presence of two *cys*$^+$ genes which are cotranscribed, rather than a single gene. One gene could "complement" HC500, and the other could complement CB64, indicating that the HC500 mutation was not analogous to *cysB* of *E. coli*. If this was the case, the Tn*5* insertion and the 1.2 Kb deletion data may be explained in terms of polarity. The 1.2 Kb deletion could have affected both genes either

by removing portions of each, or by deleting part of the promoter-proximal gene and so causing a frame shift that prevented faithful translation of the promoter-distal gene.

Three pieces of circumstantial evidence suggests that the involvement of two genes is unlikely. Firstly, *cysB* is known to be a single cistron in both *E. coli* and *S. typhimurium*, mapping 16 minutes away from the closest *cys* gene (Cheney & Kredich, 1975; Tully & Yudkin, 1977). Secondly, the *cysB* gene products of these two species are similar in size to the Mr 36,000 protein produced by pJH5 (see section 6:9). Thirdly, the chromosomal insert of pJH5 only encodes a single protein in *E. coli* (this in itself is equivocal, because some gene products are not observed in the maxicell system (Ito *et al.*, 1984)).

In order to prove unequivocally that HC500 carries a mutation analogous to *cysB*, a clone carrying the functional gene from *E. coli* should be introduced to the strain. If this *cysB*$^+$ clones complemented the HC500 phenotype, it would establish that a single gene is involved.

In summary, a formal possibility exists that two cotranscribed genes are present on pJH5. Nevertheless, it is likely that the HC500 lesion is indeed analogous to *cysB* of *E. coli*, and the implications of this are discussed in section 6:10.

6:9 *cysB* in *E. coli* and *S. typhimurium*

In *E. coli* and *S. typhimurium*, the induction of the cysteine biosynthetic pathway is controlled by a positive activator, the *cysB*

gene product (Jones-Mortimer, 1968b; Kredich, 1971), which has been shown to act at the level of transcription (Fimmel & Loughlin, 1977; Jagura *et al.*, 1978; Raibaud & Schwartz, 1984). Consequently, *cysB* mutants have a pleitropic phenotype, usually lacking all of the five cysteine biosynthetic enzymes (Boronat *et al.*, 1984; Jagura *et al.*, 1978; Jones-Mortimer, 1968a). the *cysB* gene maps close to the *trp* operon and adjacent to *top* in both *E. coli* and *S. typhimurium*, unlinked to all other *cys* genes (Bachmann, 1983; Sanderson & Roth, 1983).

The *cysB* gene product of *E. coli* is known to be autoregulated (Jagura-Burdzy & Hulanicka, 1981), and has been identified in phage infection experiments as a protein of Mr 39,000 (Mascarenhas & Yudkin, 1980). Two-dimensional PAGE was used to identify the *cysB* product of *S. typhimurium* as a Mr 34,500 protein (Baptist *et al.*, 1982). The *cysB*$^+$ gene from *E. coli* and *S. typhimurium* has been cloned, and the direction of transcription has been determined (Jagura-Burdzy & Hulanicka, 1981; Jagura-Burdzy & Kredich, 1983; Wang & Becherer, 1983).

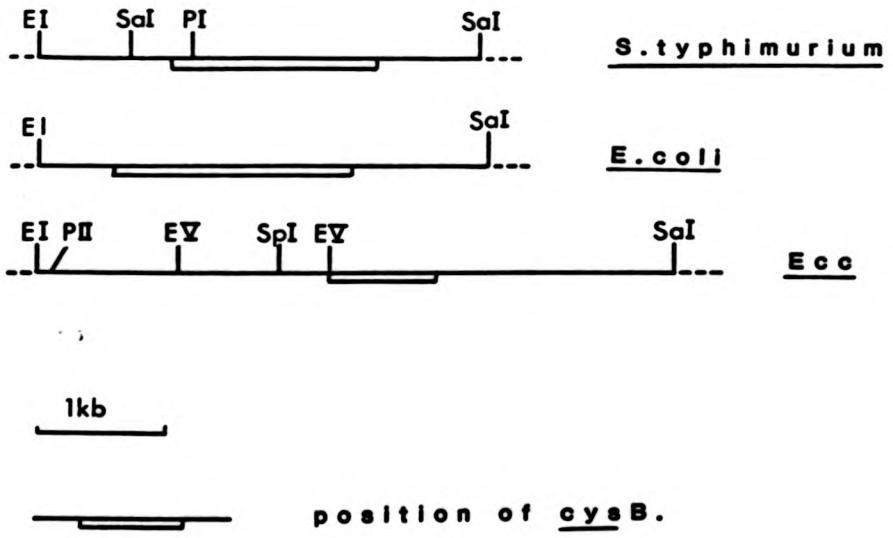
6:10 Comparison of *cysB* from *Ecc*, *E. coli* and *S. typhimurium*

The *cysB* gene products of *Ecc*, *E. coli* and *S. typhimurium* are broadly similar (Mr 36,000, Mr 39,000 and Mr 34,000 respectively). However, comparison of restriction maps of the *cysB*$^+$-containing fragments from the three species (Fig. 6:10) shows little similarity (Jagura-Burdzy & Kredich, 1983; Wang & Becherer, 1983). The *cysB*$^+$ genes are all located on *Sal*I or *Sal*I-*Eco*RI fragments, but the three maps have no restriction sites in common, within the gene itself. Likewise, when the restriction map of *ars*C (the regulatory protein of arabinose utilisation) from *Ecc*

Figure 6:10

A comparison of the restriction maps of *cysB* from *S. typhimurium* (Jagura-Burdzy & Kredich, 1983), *E. coli* (Wang & Becherer, 1983) and *Ecc* SCRI193, aligned with respect to *Eco*RI.

The positions of the seven restriction sites *Eco*RI (EI), *Eco*RV (EV), *Hin*dIII (HIII), PstI (PI), *Pvu*II (PII), *Sal*I (SaI) and *Sph*I (SpI) were considered.



was compared with those of *E. coli* and *S. typhimurium* no conservation of restriction maps was observed (Lei *et al.*, 1985b), suggesting that neither *ara*C or *cys*B have been highly conserved within the Enterobacteriaceae. This contrasts with the conservation of restriction maps found for the *rec*A homologues of *Ecc* and other enteric bacteria (Keener *et al.*, 1984; Zink *et al.*, 1985). However, it is dangerous to assess homology simply on the basis of restriction maps, since these can be markedly affected by a few base changes in the DNA itself. Homology can only be determined accurately by direct comparison of nucleotide sequence data.

6.11 The *cys*B mutation and its implications for extracellular enzyme production by HC500

As mentioned in section 6.9, mutants of *E. coli* and *S. typhimurium* which lack the *cys*B gene product cannot produce any of the enzymes involved in sulphate assimilation. The addition of exogenous cysteine to such a mutant serves only to satisfy its auxotrophic requirement and does not directly affect the synthesis of the sulphate assimilatory enzymes. This has important implications for the HC500 phenotype which has been characterised and described in chapter 5. The addition of exogenous cysteine to HC500 allowed the cells to produce Pel in the absence of a functional CysB protein (section 5:4:3). Therefore, although the CysB protein can serve as an activator of *cys* gene expression, there cannot be a direct link between the *cys*B gene product and extracellular enzyme production at the genetic level.

In addition, because *cys*B mutants are unable to assimilate exogenous sulphate, they require a rich medium which satisfies their sulphur-

requirement in the form of sulphur-containing amino acids that can be taken up directly. It is therefore likely that the Pel$^-$ Cel$^-$ Prt$^-$ phenotype of HC500 is a secondary effect of growth under sulphate-limitation. The effect of this limitation upon the growth rate of HC500 can be seen in Figure 5:4:4. Other cysteine auxotrophs grow well in MIM to produce Pel (section 5:4:5), suggesting that they were not sulphate-limited.

The data that has been presented suggests that the HC500 phenotype can be explained in terms of growth under sulphate-limitation. To confirm that the *cysB* lesion affects growth on MIM it would be interesting to study the growth characteristics of a *cysB*-deficient *E. coli* mutant on this media and compare this with the growth of HC500.

The data shown in table 6:5 suggests that the multicopy *cysB*$^+$ gene can "complement" the extracellular enzyme defect of HC500. Presumably, this reflects the Cys$^+$ phenotype of HC500(pJH3) and HC500(pJH5) which leads to increased growth rates in MIM + 0.3% (w/v) YE. It would be useful to repeat the experiments described earlier (sections 5:4:2, 5:4:3, and 5:4:4) to determine the precise effect of pJH3 and pJH5 upon the physiology of HC500.

In summary, although HC500 appeared initially to be an interesting mutant, suggesting a direct link between cysteine biosynthesis and extracellular enzyme synthesis, it now appears that the extracellular enzyme deficiency was a secondary effect of the *cysB* mutation. Nevertheless, all the molecular genetic technology that has been described for the analysis of the *cysB* gene in SCRI193 can be applied to the study of other genes of interest, and such work is now continuing in

this laboratory. Tn*5* insertions which affect the secretion of Pel, Cel and Prt have been isolated (Salmond *et al.*, 1986), and are currently being studied at the physiological and molecular genetic level.

CHAPTER 7

CLONING OF Pol^{+} FROM SCRI193

7:1 Introduction

As described in section 1:6, a number of papers describing the cloning of *pel*$^+$ genes from *Erwinia* spp. have been published recently. This work showed that the *Pel*$^+$ phenotype was expressed in *E. coli*, and could be detected by simple plate assays. Consequently, the cloning of *pel*$^+$ from an uncharacterised *Ecc* strain appeared to be straight-forward, and did not require the use of specific cloning vectors or the use of antibody. Because of the interest in Pel as an important pathogenicity factor (section 1:3), and as a major extracellular enzyme secreted by *Ecc*, attempts were made to clone *pel*$^+$ genes from a library of SCRI193. It was anticipated that cloned *pel*$^+$ genes would prove useful in future work on the genetics of secretion by SCRI193 (Kotoujansky *et al.*, 1985; Lei *et al.*, 1985a). The cloning strategy and the analysis of the Pel$^+$ clones are described in this chapter.

RESULTS AND DISCUSSION

7:2 Gene bank construction and cloning of a *pel*$^+$ gene

A *Hin*dIII gene library of HC500 was constructed in the vector pBR322, as described in Materials and Methods. Upon introduction of 4 μg religated DNA to HB101, 5 x 10^4 Ap^r transformants were obtained, and some were screened for Tc^s to determine the proportion of recombinants. Plasmid DNA was isolated from twelve Tc^s colonies and restricted with *Hin*dIII to determine the mean insert size. A proportion of the transformants (20%) carried inserts of chromosomal DNA, with an average size of 6.0 Kb. Assuming the genome size of SCRI193 is similar to *E. coli*, the Clarke &

Carbon (1976) equation suggests a 99.99% probability that this library is representative, carrying at least one copy of each gene on the HC500 chromosome.

Subsequently, the library was stored and screened for the presence of Pel^+ recombinants as described in section 2:24. This led to the isolation of two plasmids designated pPEL1 and pPEL2, which were studied at a molecular and physiological level.

7:3 Restriction mapping of pPEL1 and pPEL2

Restriction maps of the two clones are presented in Figure 7:3. They show that both pPEL1 and pPEL2 carry identical (6.3 Kb) chromosomal fragments, in opposite orientations. Comparison with restriction maps of other Pel^+ clones (referenced in section 1:6) reveals no obvious similarity.

7:4 Identification of the pPEL1 and pPEL2 gene products

Although *pel*$^+$ genes have been cloned in several laboratories, in no case have the gene products been positively identified using the *in vivo* gene expression systems of *E. coli* (A. Chatterjee, A. Collmer, A. Kotoujansky, pers. comm.). As described previously, the "maxicell" system was used to identify the proteins encoded by pPEL1 and pPEL2 (Fig. 6:7). Both the plasmids expressed a major protein of Mr 66,000 and a minor protein of Mr 53,500. It is possible that the major proteins encoded by the two plasmids are slightly different in size, but this awaits confirmation by analysis in a linear gel system. In addition, pPEL2 produced a small amount of the *tet* product (Mr 33,000).

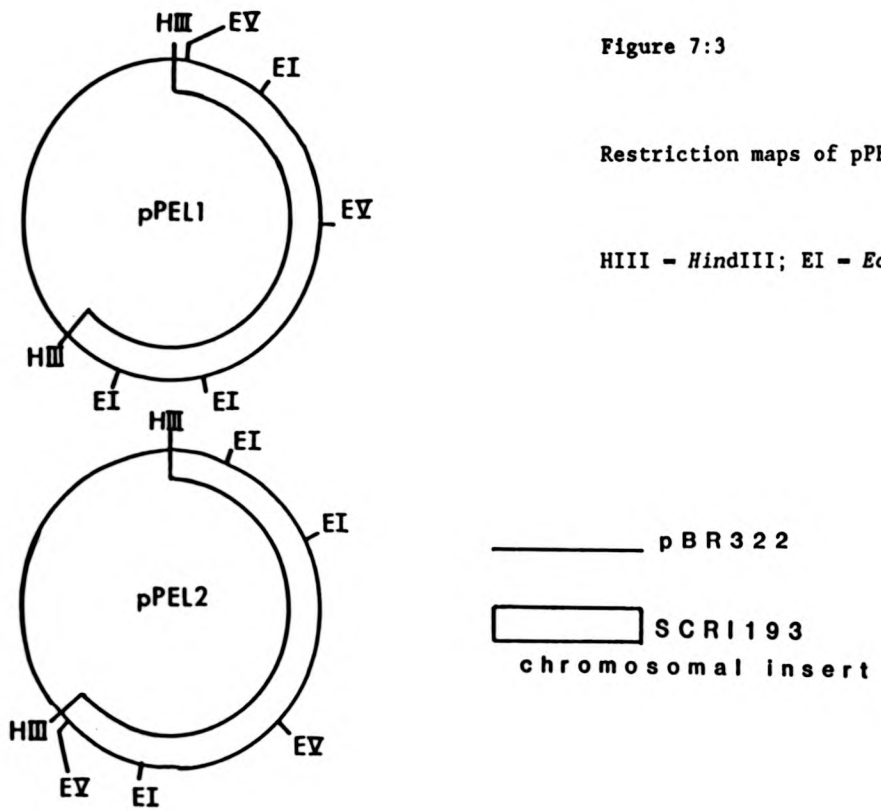


Figure 7:3

Restriction maps of pPEL1 and pPEL2.

HIII = *Hin*dIII; EI = *Eco*RI; EV = *Eco*RV.

7:4:1 Discussion

Analysis of the protein encoded by genes for secreted enzymes, generally reveals the presence of a full-length gene product, and its processed form which has a lower molecular weight, e.g. analysis of the Bla gene product in minicells reveals two proteins. The processed form of Bla is Mr 26,000, and the gene product itself is Mr 28,000 (Boronat *et al.*, 1984). Post-translational processing of an exported protein involves the removal of the signal peptide upon its translocation (Pugsley & Schwartz, 1985). It seems likely that similar processing accompanies export and/or secretion of the *pel*$^+$ gene product, but this has yet to be proved.

The Mr 53,500 protein encoded by pPEL1 and pPEL2 may represent a processed form of the Mr 66,000 polypeptide. Alternatively, the Mr 66,000 protein may be associated with a slightly larger protein which remains poorly resolved on this gradient gel system, again reflecting processing. However, since further analysis of these plasmids has not been performed, it is not certain which of the Mr 66,000 and the Mr 53,500 proteins are the true *pel*$^+$ gene product(s).

As mentioned in section 1:3:7:2, Pel enzymes produced by different *Erwinia* spp. have molecular weights varying from Mr 33,000 to Mr 44,000. The molecular weight of Pel produced by SCRI193 has not been unequivocally determined. However, a preliminary experiment which involved the analysis of proteins produced by cells subjected to repression or induction of Pel, identified three putative Pel proteins of Mr 40,000-44,000 (data not shown), which would be consistent with other published data. Therefore it is possible that neither the Mr 66,000 nor the Mr 53,500 protein of pPEL1 and pPEL2 represents the

form of the enzyme secreted by SCRI193, but this awaits further experimentation.

7:5 Analysis of the Pel$^+$ phenotype in *E. coli*

HB101 and CSH26ΔF6 derivatives carrying pPEL1 & pPEL2 were cultured under various conditions and assayed for the production of Pel (table 7:5). As expected, HB101(pBR322) produced no detectable Pel. However, HB101(pPEL2) synthesised 54% as much Pel as SCRI193. A significant proportion (45%) of the enzyme expressed by this clone was detected in the culture supernatant. In addition, HB101(pPEL2) was grown up in the presence of glucose, and in the absence of PGA. Glucose appeared to reduce Pel synthesis by almost 30%, and reduced the amount of Pel in the culture supernatant by 60%. The culture grown in the absence of PGA produced 12% more Pel than the culture grown in standard minimal induction media, and the enzyme localisation was unaffected.

The level of Pel synthesis and the proportion of the enzyme which was extracellular, was reduced in the CSH26ΔF6 derivatives. CSH26ΔF6(pPEL1) and CSH26ΔF6(pPEL2) only synthesised between 22 and 24% as much Pel as SCRI193, less than half as much as the HB101 derivatives. Furthermore, approx. 25% of the enzyme was extracellular, again a much smaller proportion than for the HB101 derivatives. Finally, a derivative of SCRI193 carrying pPEL1 produced a comparable amount of Pel to the wild-type strain, and enzyme localisation was unaffected.

To determine whether pectolytic *E. coli* clones could macerate potato tissue, stab inoculation tests were performed (table 5:4:6). HB101(pPEL1) caused substantial maceration under these experimental conditions whereas HB101(pBR322) did not. Therefore, soft rot-induction

TABLE 7:5 Pel production encoded by pPEL1 and pPEL2

Strain	Media	Total Pel activity in Pel/mg protein	Total activity as % of SCRI193	% of total activity in cell lysate	% of total activity in supernatant
SCRI193	MIM + 0.3% YE	743	100	6	94
SCRI193(pPEL1)	" "	760	102	3	97
HB101(pBR322)	" "	0	0	-	-
HB101(pPEL2)	" "	407	55	55	45
HB101(pPEL2)	" " (+ 0.2% glucose)	291	39	82	18
HB101(pPEL2)	" " (-0.2% PGA)	461	62	50	50
CSH26ΔF6(pPEL1)	MIM + 0.3% YE	177	24	77	23
CSH26ΔF6(pPEL2)	" "	163	22	74	26

Assays were performed as described in section 2:11:2.

by HB101(pPEL1) results from its ability to produce Pel.

7:5 Discussion

pPEL1 and pPEL2 expressed similar levels of Pel in CSH26ΔF6, suggesting that the expression of this enzyme was unaffected by the orientation of the 6.3 Kb fragment in pBR322. When pPEL2 was introduced to HB101, it encoded significantly more Pel than in CSH26ΔF6. Moreover, HB101(pPEL2) produced more extracellular Pel than CSH26ΔF6(pPEL2). The disparities in the levels of Pel production probably reflect the different genetic backgrounds of the two strains.

In other laboratories, *E. coli* carrying Pel^+ genes from *Ecc* produce varying amounts of Pel, ranging from 30% to 370% as much enzyme as the wild-type strain (Lei *et al.*, 1985a; Zink & Chatterjee, 1985). Thus the amount of Pel produced by pPEl1 and pPEL2 is comparable with levels observed for clones that have been isolated previously. Obviously it is of great interest to determine if Pel is actively secreted by *E. coli*, or if it appears in the supernatant by leakage from the periplasm or by cell lysis. HB101 has been shown to leak large amounts of Pel from its periplasm, nonspecifically (Collmer *et al.*, 1985). In order to determine the nature of Pel production by these clones it is necessary to perform comparative assays with known cytoplasmic and periplasmic enzymes. It has been reported that Pel expression from recombinant plasmids in *E. coli* is catabolite repressible, to varying degrees (Collmer *et al.*, 1985; Keen *et al.*, 1984). Furthermore, Pel^+ clones isolated from *Echr* have proved to be non-inducible in *E. coli*, and PGA has been observed to reduce the Pel activity of some clones (Collmer *et al.*, 1985; Keen *et al.*, 1984). Thus, the effect of glucose upon Pel synthesis by HB101(pPEL2) agrees with published data, but the cause of

the reduced level of the enzyme in the supernatant is not clear.

All the data concerning Pel expression encoded by pPEL1 and pPEL2 agrees with other published work. However, the effect of introducing Pel^+ clones to *Erwinia* strains, on multicopy vectors has not been described previously. Because SCRI193(pPEL1) produced similar levels of Pel to the wild-type strain, it suggests that Pel synthesis in *Ecc* is fairly tightly regulated.

The ability of HB101(pPEL1) to induce maceration of potato tissue agrees with published data concerning pectolytic *E. coli* clones, and confirms the central role of Pel in pathogenesis (Collmer *et al.*, 1985; Keen *et al.*, 1984; Lei *et al.*, 1985a; Zink & Chatterjee, 1985).

7:6 Conclusion

The cloning of extracellular enzyme structural genes from various *Erwinia* strains and species has proved to be relatively simple (section 1:6). The work presented here confirms that these techniques may be successfully applied to SCRI193. Indeed, recent work in another laboratory has led to the cloning of one peh^+ gene, one Pem^+ gene and four pel^+ genes from SCRI193, which may be differentiated by restriction mapping, as well as by IEF of culture supernatants (G. Plastow, pers. comm.). SCRI193 produces three Pel isozymes; two major forms (pI 9.3 and pI 9.0), and one minor form (pI ≈ 7.5) (G. Plastow, pers. comm.), which may differ from other *Ecc* strains (Ried & Collmer, 1985b). The pel^+ gene of pPEL2 encodes an isozyme of pI 9.0 (G. Plastow, pers. comm.).

The gene product data presented in section 7:4 is novel, but meaningful interpretation of this awaits further physiological work on SCRI193 and *E. coli* strains carrying pPEL1, as well as detailed molecular characterisation of the clones. It would be interesting to sequence the *pel*$^+$ and *peh*$^+$ genes of SCRI193 that have now been cloned, and look for domains of homology which might be common to secreted proteins.

7:7 Final comments

The work described in this thesis demonstrates that *Ecc* strain SCRI193 is amenable to molecular genetic manipulation.

(1) The pJB4JI-based or λ-based transposon mutagenesis systems may now be used to isolate a variety of mutants of interest. Recent work with the λ system has shown that it may be used to introduce a range of transposons, and allow the application of gene fusion technology to *Ecc* (Salmond *et al.*, 1986; Way *et al.*, 1984).

(2) The transformation system described in this thesis facilitates the introduction of native and *in vitro*-manipulated plasmids to *Ecc*. the ability to perform one-step cloning experiments in *Ecc* should prove valuable in the future.

(3) The application of molecular cloning technology to the cloning of *pel*$^+$ genes and Tn*5* elements has been successful. The work presented in this thesis demonstrates that experiments in *Ecc* genetics are now limited only by imagination, not by the lack of technology.

Ongoing research at Warwick involves the use of SCRI193 as a model system for protein secretion. In addition, the technology described earlier has now been applied to *Eca*, and is being used in the analysis of the pathogenesis of blackleg disease.

APPENDIX 1

ALTERNATIVE GENETIC APPROACHES IN SCRI193

A1:1 Introduction

In addition to the molecular genetic methods which were applied to SCRI193 (Chapters 3, 4, 6 and 7), preliminary experiments involving other genetic systems were undertaken. Although much of this work did not prove successful, it is included here for completeness, showing that a comprehensive genetic analysis of SCRI193 had been attempted. It is hoped that the data presented here concerning the search for bacteriophage for SCRI193 will highlight the potential difficulties that may be encountered by future workers.

A1:2 Attempts to isolate a transducing phage for SCRI193

A1:2:1 Why search for transducing phages?

Transducing phages are of great value for determining genetic linkage and in bacterial strain construction (Ely & Johnson, 1977; Masters, 1985). Although three generalised transducing phages are available for *E. coli* K12 (Masters, 1985), their restricted host range limits their use with other bacterial species (see Section 4:3:4 for a discussion of P1).

It is possible to isolate phages for particular strains from sewage, culture supernatants or other sources. Generalised transducing phages have been isolated for the phytopathogens *P. syringae* pv. *syringae* and *Echr* (Chatterjee & Brown, 1980; Nordeen & Currier, 1983; Resibois *et al.*, 1984). ϕEC2 has been used to transduce markers between *Echr* strains B374 and 3937j, and to demonstrate linkage between *thr* and *car* in strain B374 (Resibois *et al.*, 1984). A detailed analysis of the

physical and transducing properties of ϕEC2 has been undertaken, and Tn9-carrying derivatives have been constructed (Resibois *et al.*, 1984; E. Schoonejans, pers. comm.). Subsequently, ϕEC2 has been used for strain construction and analysis of mutants of B374 (Hugouvieux-Cotte-Pattat & Robert-Baudouy, 1985b; van Gijsegem *et al.*, 1985a).

Thus the utility of a transducing phage for SCRI193 is apparent. A number of standard procedures were used in an attempt to isolate phage for SCRI193 from a variety of sources.

A1:2:2 Practical approaches

Sensitivity of SCRI193 to coliphages and erwinia phages

The phages P1CM, Mu *cts*62, λ^+, λ_{540} and T4GT7 (10^9 pfu) were spot-tested on lawns of SCRI193. The P1CM and Mu *cts*62 lysates were made by thermal-induction to ensure random G-loop orientation. No plaques or zones of lysis were observed. In addition high-titre lysates of the six virulent phages isolated by E. Jones (M. Pérombelon, pers. comm.) were prepared on *Ecc* strain C466. Following spot-testing of SCRI193 with 10^9 pfu of the phages ϕ73, ϕ91, ϕ113, ϕ301, ϕE19 and ϕE21, no phages were observed.

Enrichment for SCRI193 phages

Three phage sources were used for attempted enrichment of SCRI193 phage. First, a "post-filtration" sewage sample (filter-sterilised) from Finham sewage works. Second, soil taken from an *Ecc*-infected potato field (from M. Pérombelon). Third, river water concentrates prepared by the method of Seeley *et al.* (1979), by J. Nicolson. Spot-testing of water concentrate and sewage sample upon lawns of SCRI193 yielded no plaques. Portions of the water concentrate, sewage and soil samples were added to

log-phase SCRI193 cultures and enrichments carried out as Adams (1955). Again these procedures yielded no SCRI193 phage. However, the water concentrates were used by M. Perombelon to isolate phages for 11 of 35 *Ecc* strains tested, demonstrating the presence of *Ecc* phages.

Mitomycin C induction

Mitomycin C is commonly used to induce phage or bacteriocin production (e.g. Chatterjee & Brown, 1980). Following the screening of 50 *Ecc* strains for mitomycin C-sensitivity, 25 sensitive strains were selected (data not shown). These strains were grown to log phase, mitomycin C added to 1 μg/ml, and incubated for a further 24 h. Strain SCRI101 produced a bacteriocin which caused a zone of lysis on an SCRI193 lawn. However, no strains produced phage that plated on SCRI193.

More recently, 70 *Ecs* strains were grown and induced with 1 μg/ml mitomycin C, and supernatants were spot-tested on SCRI193. Again, no plaques were observed (data not shown).

Conclusion

The data in this section is presented to demonstrate the difficulty in isolating phages for SCRI193. The reason that phages were not isolated is not known, and presumably reflects their rarity in the environment.

Attempts to isolate phages from sewage for the *lamB*$^{+}$ derivative of SCRI193 (HC131) were made, since several phages require the *lamB* receptor protein (Charbit & Hofnung, 1985). However, no phages were isolated (G. Salmond, pers. comm.).

A1:3 Observations on the use of Hfr-mediated genetic transfer to SCRI193

Chatterjee & Starr (1980) reported that it had not been possible to achieve Hfr-mediated chromosomal gene transfer from *E. coli* to *Echr*, although it was possible to transfer F' plasmids to several strains. Attempts were made to use an Hfr system to transfer a Tn*10* marker to SCRI193.

The strains JC10240 (Csonka & Clark, 1980) and MCL31 (Lorence & Rupert, 1983) are derived from the Hfr strain KL16, and carry a *srl*::Tn*10* mutation. They are used to transfer *rec*A mutations between *E. coli* strains. JC10240 and MC131 were mated with KF1005 (section 2:5), and Sm^r Tc^r transconjugants were selected. No transconjugants were obtained despite repeated attempts ($< 3 \times 10^9$ in both cases).

It is known that F' plasids are transferred to SCRI193 (Forbes, 1983). The reason for the absence of Hfr-mediated gene transfer is not known, but this observation resembles the findings for strains of *Echr*.

A1:4 Isolation of nonsense-suppressor mutants of SCRI193

A1:4:1 Introduction

The analysis of various biological functions in *E. coli* has been aided by the use of nonsense mutants. Such mutants have been used to study bacteriophage replication (Mindich *et al.*, 1982), operon function (Zipser, 1969) and other aspects of bacterial gene expression (Oeschger, 1980). The use of nonsense-suppressor mutants has been extended to a

wide range of organisms including *Bacillus subtilis*, *Pseudomonas* spp., *Salmonella typhimurium* and *Saccharomyces cerevisiae* (Bossi, 1985; Mindich *et al.*, 1976, 1982).

To enable the isolation of nonsense-mutations in any genes of interest (e.g. *out*, *pel*, *cel* or *prt*), *sup*$^+$ derivatives of SCRI193 are required. A method developed for the isolation of nonsense-suppressing mutants of *Pseudomonas* spp. proved successful in SCRI193 (Hinton *et al.*, 1985b).

A1:4:2 Rationale

The procedure for the isolation of nonsense suppression mutants involves the use of the *incP* plasmid pLM2 (Mindich *et al.*, 1976). This is a Km^r derivative of the broad host-range plasmid RP1, which carries amber mutations in both the Ap^r and Tc^r genes. pLM2 produces a Km^r Ap^s Tc^s phenotype in a *Sup*^{o} strain, and a Km^r Ap^r Tc^r phenotype in a *Sup*$^+$ strain, allowing positive selection of amber-suppressing mutants.

RESULTS AND DISCUSSION

A1:4:3 Isolation of *Sup*$^+$ SCRI193

pLM2 was transferred from OV2 to KF1033 at a frequency of 3×10^{-2}. Transconjugants proved to be Sm^r Km^r Ap^s Tc^s (14/14), suggesting that there was no endogenous amber suppression in KF1033. One transconjugant was designated HC105, and to ensure that pLM2 had not been substantially altered, the plasmid was re-transferred to *E. coli*. Following patch mating of HC105 with QD*sup*F, transconjugants were selected at 42^{o}C, and shown to be Km^r Ap^r Tc^r (10/10), confirming that the plasmid retained amber mutations in the Ap and Tc genes. One of these QD*sup*F(pLM2)

transconjugants was purified and retained.

To obtain amber-suppressing derivatives, strain HC105 was mutagenised with EMS to 25% survival, and plated on NBA Tc. Putative Sup^+, Tc^r mutant colonies arose at a frequency of 10^{-8} per cell. When two colonies (HC106 and HC107) were restreaked, they were shown to be not only Sm^r Km^r Tc^r, but also Ap^r. This simultaneous resistance to Tc and Ap suggests that HC106 and HC107 arose from an amber suppression mutation, rather than from structural gene reversion of the mutant Ap^r_{am} and Tc^r_{am} genes of pLM2.

The genotype of pLM2 carried by HC106 and HC107 was confirmed by patch mating the plasmid into the *Sup*^{o} strain W3110. Transconjugants were counter-selected at 42^{o}C and possessed a Km^r Ap^s Tc^s phenotype (4/4), proving that pLM2 still carried the two amber mutations.

Experiments have shown that the Ap^r phenotype of HC106 and HC107 is temperature-sensitive (ts). These strains are Ap^r at 30^{o}C, but Ap^s at 37^{o}C. The Ap^{ts} phenotype is probably the result of the synthesis of a ts β-lactamase, as has been reported in other *sup*$^+$ strains (Donachie *et al.*, 1979; Mindich *et al.*, 1976).

A1:4:4 Confirmation of the Sup^+ phenotype

The two putative amber suppressor mutants of KF1033 were tested for sensitivity to an amber mutant of the plasmids-specific phage PRD1. The mutant, PRD1 *sus*-2 is only able to replicate in a host carrying an amber suppressor (Mindich *et al.*, 1976).

Spot tests were performed on lawns of different bacterial strains and

the results are presented in table A1:4. These results show that wild-type PRD1 only plates on strains which carry pLM2. However, PRD1 *sus*-2 only plates on a plasmid-containing strain that also carries a suppressor (e.g. QD*sup*F(pLM2)). Although PRD1 *sus*-2 fails to plate on HC105, it does plate on the two putative amber suppressor derivatives of KF1033, HC106 and HC107. This result corroborates the data of section 8:2:3, and proves that two nonsense suppressor mutants of SCRI193 have been isolated.

A1:4:5 Future experiments

The availability of amber-suppressing derivatives of SCRI193 should facilitate the isolation of amber mutations in genes of interest (Oeschger, 1980). A further round of mutagenesis could be used to isolate ts suppressor derivatives of HC106 or HC107 (as Oeschger, 1980). Such derivatives should allow the study of amber extracellular enzyme mutants and other biochemical mutants, in temperature-shift experiments (as Oeschger & Woods, 1976). For example, amber mutations in extracellular enzyme secretion could be studied in a sup^{ts} background, to determine the role of particular gene products in secretion, as has been done for *E. coli* (Oliver, 1985).

Table A1:4 Sensitivity of various strains to plasmid-specific phages

	Phage	
Bacterial Host	PRD1	PRD1-*sus*-2
QD*supF*	-	-
QD*supF*(pLM2)	+	+
W3110(pLM2)	+	-
KF1033	+	-
HC105	+	-
HC106	+	+
HC107	+	+

+, sensitivity; -, resistance.

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Efficient Transformation of *Erwinia carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica*

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We used a modified version of the method of Hanahan (D. Hanahan, J. Mol. Biol. 166:557–580, 1983) to transform *Erwinia carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* with the plasmids pBR322, pBR325, and pAT153. The transformation frequency ranged from 1×10^2 to 4×10^4 colonies per μg of plasmid DNA. The nature of these transformants was confirmed by plasmid analysis. ColE1-based plasmids make potentially useful cloning vectors for the study of genes involved in the pathogenesis of this species.

Erwinia carotovora subsp. *carotovora* is responsible for the soft rot of a number of economically important crops, including potatoes, celery, carrots, green peppers, and cucumbers. The host range of *E. carotovora* subsp. *atroseptica* is confined to potatoes, in which it is the causal agent of blackleg in the field and soft rot of tubers in stores. Various aspects of the epidemiology of the disease caused by these phytopathogens are understood, but no control method is currently available (18).

Genetic and molecular techniques are now being used to study the basis of pathogenicity of these subspecies (17, 20). The development of an efficient transformation system for strains SCRI193 (Ecc193) and SCRI31 (Eca31) is an important step in this approach. This permits inter- and intrageneric transfer of native and recombinant plasmids and facilitates direct shotgun cloning into *Erwinia*.

Transformation procedures have been reported for other *Erwinia* spp.: *E. amylovora* (D. W. Bauer and S. V. Beer, Phytopathology 73:1342, 1983) and *E. herbicola* (13; S. E. Lindow and B. J. Staskawicz, Phytopathology, 71:237, 1981). However, the only method described for the transformation of *E. carotovora* subsp. *carotovora* is inefficient (P. M. Berman, M. S. Mount, and G. H. Lacy, Phytopathology 73:1342, 1983). Extrapolation from the data of Berman et al. gives a frequency of less than 1 transformant per μg of pBR322 DNA (given a transformation mix containing 10^8 cells).

The basis of the induction of competence in gram-negative bacterial cells is poorly understood (3). Consequently, the development of a transformation system in a new species requires an empirical approach.

The bacterial strains and plasmids used in this study are listed in Table 1. pBR322 and pBR325 DNA and restriction enzymes were obtained from Bethesda Research Laboratories. Restrictions were performed as described previously (14), with the addition of 4 mM spermidine (Sigma Chemical Co.). This was required to prevent breakdown of Ecc193-derived plasmids due to the action of nonspecific endonucleases. pKT210 was purified in this laboratory, and pAT153 was a gift from M. Richardson. Preliminary work involved attempts to transform SCRI193 with pKT210 and pBR322 DNA by a number of published methods (7–9, 14–16; Bauer and Beer, Phytopathology 73:1342). Of the seven methods, only those of Morrison (15) and Maniatis et al. (14) (modified by substitution of 150 mM $CaCl_2$ for 100 mM $CaCl_2$) yielded any transformants. Both methods gave a frequency of 4 transformants per μg of pKT210 DNA. All transformants were Sm^r Cm^r and carried a plasmid which comigrated with authentic pKT210 (plasmid screens were performed by a scaled-down adaptation of the method of Hansen and Olsen [11]). No indigenous plasmids have been detected in Ecc193 or Ecc31 (data not shown).

A more efficient transformation system has recently been developed for both Ecc193 and Eca31 based on the method of Hanahan (10), but with several modifications. *Erwinia* cultures were routinely grown at 30°C. Samples of competent cells (200 μl) were treated with 100 ng of plasmid DNA, incubated on ice for 30 min, and then heat-shocked at 42°C for 1 min without agitation. After 100 min of expression time (30°C), transformants were selected on LM agar (10) containing 35 μg of ampicillin per ml.

Frequencies of transformation of Ecc193 and Eca31 with various plasmids are shown in Table 2. A number of Ap^r transformants were screened for the coinheritance of other plasmid-borne antibiotic resistance markers. In all cases, 100% linkage was observed (data not shown).

The effect of various components in the transformation system upon the transformation efficiency of Ecc193 was studied. Growth of cells at 37°C, instead of 30°C, before transformation doubled the transformation efficiency. Removal of dithiothreitol or dimethylsulfoxide from the buffer reduced the transformation efficiency by 10- and 3-fold, respectively. Incorporation of a freeze-thaw step before the heat-shock reduced the transformation efficiency by threefold. Addition of a 30-min incubation on ice after the heat-shock reduced transformation efficiency by fourfold. Storing cells in transformation buffer at 4°C for 18 h before addition of dithiothreitol, dimethylsulfoxide, and plasmid DNA reduced the transformation frequency by 30-fold (data not shown) (the last three modifications were found to increase the transformation efficiency of *E. amylovora* [Bauer and Beer, Phytopathology 73:1342]).

The plasmids carried by the transformants were analyzed by a modification of the boiling method (12). Plasmid bands were found in both Ecc193 and Eca31 transformants, which comigrated with the appropriate monomeric forms of the three control plasmids (data not shown). Transformants possessed similar pathogenic, pectolytic, and biochemical properties as the parental strain (data not shown).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant details	Source or reference
E. carotovora subsp. *carotovora* SCRI193	(Formerly SR44)	17
E. carotovora subsp *atroseptica* SCRI31		Laboratory collection
E. coli K-12 DH1	*recA hsdR*	10
pAT153	Ap^r Tc^r	19
pBR322	Ap^r Tc^r	5
pBR325	Ap^r Tc^r Cm^r	4
pKT210	Cm^r Sm^r	1

Plasmids were isolated from DH1(pBR322) and Ecc193 (pBR322) transformants (6) and compared. These plasmids were run with commercial standards by using a Tris-acetate gel system. The pBR322 obtained from Bethesda Research Laboratories was in dimeric form (as was the pBR325 from the same source [data not shown]). When this pBR322 was transformed into DH1, it continued to replicate as a dimer. However, when the same plasmid was transformed to Ecc193, it assumed a monomeric conformation. When plasmid DNA was isolated from Ecc193(pBR322) and transformed into DH1, it continued to replicate as a monomer, as confirmed by comparison with a monomeric sample of pBR322 (data not shown).

In *Escherichia coli*, the *recA* gene product is required to resolve a multimeric plasmid to its monomeric form (2). This is demonstrated by the fact that dimeric plasmids continue to replicate as dimers in DH1. The observation that dimeric pBR322 is resolved to a monomer upon introduction to Ecc193 suggests that this strain possesses a system analogous to that of *recA* in *E. coli*.

Plasmid isolated from Ecc193(pBR322) and Eca31 (pBR322) gives a 4.3-kilobase linear fragment after digestion with *Hin*dIII, which comigrates with that of commercial pBR322 and pBR322 isolated from DH1. Further evidence that the plasmids carried by Ecc193(pBR322) and DH1(pBR322) are similar was obtained by digesting DNA from each, in parallel, with *Hae*III and *Sau*3A (two enzymes which cut pBR322 to give 22 fragments). After electrophoresis on a 3% gel, the same number and size of restriction fragments were produced from both plasmids (data not shown).

These results show that Ecc193 can be efficiently transformed with pBR322 and that this plasmid is probably unaltered in this strain. The utility of transformation in recombinant DNA manipulations involving Ecc193 and Eca31 is apparent. This system has already been used to introduce recombinant plasmids to Ecc193, and to achieve direct complementation of a transposon-induced mutation in this strain (unpublished data).

TABLE 2. Transformation frequencies of the plasmids pAT153, pBR322, and pBR325 in Ecc193 and Eca31

Strain	Plasmid	Frequency (Ap^r transformants per μg DNA)	
		Range	Mean
SCRI193	pBR325		4×10^{4a}
SCRI193	pBR322	1.3×10^3 to 2.6×10^3	2.0×10^3
SCRI193	pAT153	1.0×10^3 to 2.2×10^3	1.7×10^3
SCRI31	pBR322	1×10^2 to 1×10^3	7.0×10^2

[a] Result of a single experiment.

The technique of Hanahan, which was successfully applied here, may prove applicable to a wide range of gram-negative bacteria which have previously proved recalcitrant to transformation.

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Nonsense-suppressor mutants of *Erwinia carotovora* subsp. *carotovora*

(*Erwinia*; pLM2; mutagenesis)

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1. SUMMARY

A promiscuous plasmid (pLM2) carrying amber mutations in two antibiotic-resistance genes was transferred to a derivative of *Erwinia carotovora* subsp. *carotovora* strain SCRI193. Following mutagenesis, two putative amber-suppressing mutants of this strain were isolated. The genotype of these mutants was confirmed by use of rep_{am} plasmid-specific phage. This constitutes the first isolation of amber-suppressing mutants in *Erwinia* spp.

2. INTRODUCTION

The analysis of various biological functions in *Escherichia coli* has been aided by the use of nonsense mutants. Such mutants have been used to study bacteriophage replication [1,2], operon function [3], and the expression of essential bacterial genes [4–6]. Genetical identification of some nonsense mutants is possible by use of 'suppressors': genes which code for mutant tRNA molecules which do not recognise stop codons as translation termination signals but insert an amino acid, and thereby phenotypically suppress the amber mutation. Nonsense suppressor mutants of this type have been isolated in a range of organisms, including *E. coli* [7,8], *Bacillus subtilis* [9], *Pseudomonas* [10], *Salmonella* [2] and *Saccharomyces* [8], and these suppressing strains have been used to further isolate nonsense mutants with a variety of phenotypes.

Erwinia carotovora subsp. *carotovora (Ecc)* is an important plant pathogen which causes soft rot of a variety of crops such as potato and green pepper. Methods of genetic analysis already exist for this subspecies, and include Tn*5* mutagenesis [11; unpublished data], chromosomal gene transfer [18], and transformation [12]. As another approach, we wish to isolate nonsense mutants defective in various genes, particularly those which code for proteins involved in pathogenicity (e.g., pectate lyase [13]). To isolate nonsense mutants, we need an *Ecc* strain which carries a nonsense suppressor. A method had previously been developed for the isolation of nonsense suppressor mutants of *Pseudomonas aeruginosa* and *P. pseudoalcaligenes*, although it was unsuccessful for the isolation of similar mutants of the phytopathogen *P. phaseolicola* [10]. This report concerns the application of this method, and the isolation of amber-suppressing mutants of *Ecc*.

3. MATERIALS AND METHODS

3.1. Strains and media

The bacterial and phage strains used are listed

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in Table 1. The medium used throughout was Oxoid nutrient broth (NB), solidified, where appropriate, with 1.5% Difco 'Bacto' agar (NBA). Streptomycin (Sm), kanamycin (Km), ampicillin (Ap) and tetracycline (Tc) were used as required at 100 μg/ml, 50 μg/ml, 50 μg/ml and 20 μg/ml, respectively.

3.2. Bacterial conjugation

When plasmid transfer frequencies were determined, donor and recipient cells were grown in static liquid culture overnight, mixed in eppendorf tubes (ratio 1 : 10), and concentrated by centrifugation. The mating mixtures were carefully resuspended in 50 μl of NB and spotted onto filters (pore size 0.2 μm). Filters were incubated on NBA medium at 30°C for 16 h. Cells were resuspended in NB, and aliquots spread on selective plates.

Patch matings were performed by mixing loopfuls of donor and recipient cells on NBA plates, incubating at 30°C overnight, and streaking onto selective media.

3.3. Mutagenesis

A 10-ml overnight culture of HC105 was treated with 200 μl of ethyl methane sulphonate (EMS), shaken vigorously to dissolve the mutagen, and maintained at 30°C for 2 h. Cells were centrifuged and resuspended in NB, and aliquots were spread on selective meda. Viable counts were taken before and after mutagenesis to quantify survival.

3.4. Phage preparation

Lysates of the PRD1 wild-type and amber phages were prepared on lawns of PSA by the confluent lysis technique. Titres were also determined on this host. Spot tests were performed with 10^6 plaque-forming units on lawns of the various strains (Table 2).

4. RESULTS AND DISCUSSION

The procedure for the isolation of nonsense suppressor mutants [10] involves the use of the

Table 1

Bacterial and phage strains

Strain	Relevant markers	Phenotype of pLM2	Source/ comments
E. coli			
QD8sup3	*sup*$^+$	–	[14]
HE111	QD8sup3 (pLM2)	Km^r Ap^r Tc^r	This study
W3110	*sup*^{0}	–	Laboratory collection
HE125	W3110 (pLM2)	Km^r Ap^r Tc^r	This study
QS103	*supF*(Ts), (pLM2)	Km^r Ap^{ts} Tc^{ts}	[5]
Ecc			
KF1033	SCR1193 Sm^r Nal^r	–	Prototrophic; pathogenic [12,15]
HC105	KF1033 (pLM2)	Km^r Ap^r Tc^r	This study
HC106	HC105 *sup*-1	Km^r Ap^{ts} Tc^r	This study
HC107	HC105 *sup*-2	Km^r Ap^{ts} Tc^r	This study
S. typhimurium			
PSA	*sup*$^+$ LT2 (pLM2)	Km^r Ap^r Tc^r	[2]
Phage			
PRD1	wild-type	–	[10]
PRD1 *sus*-2	replication-amber	–	[10]

Table 2

Sensitivity of various strains to plasmid-specific phage

Phage	Bacterial host						
	QDSup3	HE111	HE125	KF1033	HC105	HC106	HC107
PRD1	–	+	+	–	+	+	+
PRD1*sus*2	–	+	–	–	–	+	+

+, sensitivity; –, resistance.

incP plasmid pLM2. This is a Km^r derivative of the broad-host-range plasmid RP1, which carries amber mutations in both the Ap^r and Tc^r genes. pLM2 produces a Km^r Ap^s Tc^s phenotype in a non-suppressing (*sup*^{0}) strain, and a Km^r Ap^r Tc^r phenotype in a suppressing (*sup*$^+$) strain, allowing positive selection of amber-suppressing mutants.

4.1. Transfer of pLM2 to Erwinia

Strain GS103 was mated with KF1033, and selection made for Sm^r Km^r transconjugants. These arose at a frequency of 3×10^{-2} per recipient cell. Transconjugants (14/14) were shown to be Sm^r, Km^r, Ap^s, Tc^s, suggesting that there was no endogenous amber suppressor in KF1033. One transconjugant was designated HC105. To ensure that pLM2 had not been substantially altered in this strain, the plasmid was patch-mated to *E. coli* QDSup3. Transconjugants were selected on NBA Km at 42°C (KF1033 is unable to grow above 39°C). Transconjugants (10/10) were screened and shown to be Km^r Ap^r Tc^r, suggesting that the pLM2 from HC105 still carried amber mutations in the Ap and Tc genes. One of these transconjugants was designated HE111.

4.2. Isolation of sup$^+$ Ecc

Strain HC105 was mutagenised with EMS to 25% survival, and plated on NBATc. Putative Sup$^+$ Tc^r mutant colonies arose at a frequency of 10^{-8}. When 2 colonies (HC106 and HC107) were restreaked, they were shown to be not only Sm^r Km^r Tc^r but also Ap^r. This simultaneous resistance to Tc and Ap suggests that HC106 and HC107 arose from an amber suppressor mutation, rather than from reversion mutation of the mutant Ap$^r_{am}$ and Tc$^r_{am}$ genes of pLM2.

The genotype of pLM2 in HC106 and HC107 was confirmed by patch-mating the plasmid into the *sup*^{0} strain W3110. Transconjugants (4/4) selected on NBAKm at 42°C were shown to be Km^r Ap^s Tc^s, proving that pLM2 still carried the two amber mutations.

Experiments have shown that the Ap^r phenotype of HC106 and HC107 is temperature-sensitive(ts). These strains are Ap^r at 30°C, but Ap^s at 37°C. The Ap^{ts} phenotype is probably the result of the synthesis of a ts β-lactamase. Similar findings have been reported in other suppressing strains carrying pLM2 [5,10].

4.3. Confirmation of sup$^+$ phenotype

The two putative amber suppressor mutants of KF1033 were tested for sensitivity to an amber mutant of the plasmid-specific phage PRD1. The mutant, PRD1 *sus*-2 was isolated by Mindich [10], and is only able to replicate in a host carrying an amber suppressor.

Spot tests were performed on lawns of different bacterial strains and the results are presented in Table 2. These show that wild-type PRD1 only plates on strains which carry pLM2. However, PRD1 *sus*-2 only plates on a plasmid-containing strain that also carries a suppressor (e.g. HE111). Although PRD1 *sus*-2 fails to plate on HC105, it does plate on the two putative amber suppressor derivatives of KF1033, HC106 and HC107.

This result confirms the antibiotic resistance data and suggests that we have isolated two nonsense suppressor mutants of *Ecc*.

4.4. Future uses of sup$^+$ Erwinia *strains*

We have cloned various *Ecc* genes on pBR322 (unpublished) and can transform these plasmids directly into *Ecc* [12]. By localised mutagenesis of naked plasmid DNA [16], we should be able to

generate amber mutations in these cloned genes. These amber mutations will be suppressed when transformed into a *sup*$^+$ strain, but not a *sup*^{0} strain. Isogenic plasmids which differ only in an amber mutation in the gene of interest could be used to identify the respective protein product by standard techniques [17].

It should also be possible to isolate ts amber-suppressing *Ecc* strains by another round of mutagenesis on HC106 or HC107, and screening for Km^r Tc^r at 25°C, and Km^r Tc^s at 37°C. Such *sup*^{ts} strains will be used to isolate amber pathogenicity mutants. These would have a pathogenic phenotype at 25°C but not at 37°C. To confirm that these were amber mutants, and ts missense mutants, a reversion analysis could be performed: amongst spontaneous Tc^r revertants selected at 37°C some should simultaneously become pathogenic due to reversion from *sup*^{ts} to *sup*$^+$.

Having amber pathogenicity mutations in a *sup*^{ts} background would allow us to study the synthesis of the pathogenicity protein(s) in temperature-shift experiments.

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