The isolation of novel *Erwinia* phages and their use in the study of bacterial phytopathogenicity

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Contents

Summary

A number of bacteriophages were isolated on the "soft rot" phytopathogens *Erwinia carotovora* subsp. *atroseptica* SCRI1043 and *Erwinia carotovora* subsp. *carotovora* SCRI193. Several of these phages were used to obtain phage resistant mutants of SCRI1043, in order to investigate the role of the bacterial cell surface in virulence. While a number of phenotypic properties relating to pathogenicity and virulence of this strain have already been uncovered, little is known about the role of the cell surface in virulence. It was hoped that the use of phages would allow selection of mutants altered in both cell surface and virulence.

Two phage resistant mutants, A5/22 and A5/8, exhibited reduced virulence when inoculated into potato plants, and were investigated further. Both mutants showed pleiotropic phenotypes. As well as reduced virulence and phage resistance, these mutants showed a number of other phenotypic alterations including, a reduction in the production of plant cell wall degrading enzymes, increased sensitivity to surface active agents, alterations in lipopolysaccharide and outer membrane protein profiles and reduced motility. A5/22 also exhibited bacteriostasis in the presence of galactose. Mutant A5/22 was more severely affected in its virulence than A5/8, which reflected in its greater deviation from the wild type phenotype. While no one phenotypic alteration could be directly associated with the reduced virulence of either mutant, a combination of several phenotypes may have been responsible.

The phages isolated in this study were the first reported for these strains of *Erwinia*, and were therefore characterised under a number of criteria. All phages were grouped on the basis of structural morphology, restriction endonuclease digestion and host range. This is the first detailed characterisation of phages for *Erwinia carotovora* subsp. *atroseptica*.

All isolated phages were tested for generalised transduction, a method of molecular genetic analysis so far unavailable to *Erwinia carotovora* subsp. *atroseptica* SCRI1043 and *Erwinia carotovora* subsp. *carotovora* SCRI193. Two phages, ϕKP and ϕM1, were capable of generalised transduction in SCRI193 and SCRI1043 respectively. Both these phages were characterised and transducing frequencies improved. ϕM1 is the first transducing phage reported *for Erwinia carotovora* subsp. *atroseptica* and ϕKP is only the second *for Erwinia carotovora* subsp. *carotovora*. Both phages are now being used extensively in the laboratory.

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Acknowledgements

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I acknowledge the financial support of the SERC and SCRI.

Declaration

The results of this thesis have been produced by myself, except for the isolation of a number of bacteriophages, ie. S21 to S75. The thesis itself has also been composed by myself with specific reference to other work where appropriate.

Ian Toth

Abbreviations

CAP = catabolite activator protein

Cel = cellulase

CP = clear plaquing

CPS = capsular polysaccharide

DOC = deoxycholate

Eca = *Erwinia carotovora* subsp. *atroseptica*

Ecc = *Erwinia carotovora* subsp. *carotovora*

Echr = *Erwinia chrysanthemi*

EDTA = ethyl diamine tetraacetic acid

EPS = extracellular polysaccharide

Gal = galactose

GalE = UDP-galactose-4-epimerase

GalK = galactokinase

GalT = galactose-1-phosphate-uridyl-transferase

GalU = UDP-glucose-pyro-phosphorylase

HR = hypersensitivity reaction

LPS = lipopolysaccharide

MDO = membrane derived oligosaccharide

MM = minimal medium

OBR-cellulose = ostazin brilliant red-hydroxyethyl cellulose

OD = optical density

OMP = outer memebrane protein

PC = phenotype conversion

Pem = pectin methylesterase

PG = polygalacturonase

PGA = polygalacturonic acid

PL = pectate lyase

PNL = pectin lyase

Prt = protease

RE = restriction endonuclease

Rvi- = reduced virulence

SDS = sodium dodecyl sulphate

TP = turbid plaquing

U. V. = ultraviolet

CHAPTER 1

INTRODUCTION

1 Chapter 1

1.1 The pathogenesis of pectolytic erwinias

1.1.1 *Erwinia* taxonomy

The genus *Erwinia* is composed of Gram-negative non spore-forming, facultative anaerobes belonging to the family Enterobacteriaceae. As well as being pathogenic to plants, members of this genus also include both human and animal pathogens. In the current edition of "Bergey's Manual" (Lelliot and Dickey 1984) fourteen distinct species within this genus have been recognized including the "soft rot" erwinias. Of the six soft rot erwinias listed, three are of substantial economic importance, *Erwinia carotovora* subsp. *carotovora* (*Ecc*), *Erwinia carotovora* subsp. *atroseptica* (*Eca*) and *Erwinia chrysanthemi* (*Echr*). *Ecc* and *Eca* by biochemical (Table A1), serological and DNA reassociation studies are found to be more closely related to each other than to *Echr*. Monoclonal antibodies specific to the lipopolysaccharide of *Eca* allow its differentiation from other pectolytic erwinias (Halk and De Boer 1985).

1.1.2 Soft rot erwinias as phytopathogens

The soft rot erwinias produce a large amount of extracellular enzymes which appear to play a major part in pathogenicity (throughout this thesis pathogenicity will be defined as the ability to produce disease by a bacterium, and virulence will be defined as the relative pathogenicity of a bacterium). Throughout the world these erwinias are responsible for large crop losses, and such losses totalled around $50-$100 million annually around 1980 (Perombelon and Kelman 1980). The infection of potatoes by soft rot erwinias is of great concern to the potato industry,

Test	*Echr*	*Ecc*	*Eca*
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	+	-	-

Table A1. Biochemical differentiation of the soft rot erwinias. From Lelliot and Dicky (1984).

particularly in relation to seed production and certification (Perombelon and Kelman 1987).

1.1.3 Aetiology and epidemiology of soft rot disease

A great deal of attention has been given to the aetiology and epidemiology of soft rot disease. Although these organisms appear to have little host specificity they do have different host ranges, this probably reflect differences in the temperature tolerence and geographical location of the pathogen (Perombelon 1985).

Echr infects a wide range of tropical and sub-tropical crops, as well as greenhouse-grown crops in temperate regions. Host plants include African violet (*Saintpaulia ionantha*), often used as a model system for pathogenicity experiments, carnation, leopold lily and the field crops, maize, pineapple and potato. *Echr* has also been found in potato infections in temperate regions, ie. Holland and France (Perombelon pers. comm.), although in temperate regions this is much less common and has not been reported in Britain.

Ecc has probably the widest host range, causing soft rot in a number of crops from both temperate and sub-tropical regions. Such crops include brussel sprout, carrot, celery, cucumber, green pepper, turnip and potato. *Ecc* can readily be isolated from a variety of environmental sources including air, rivers, lakes, oceans and soil (McCarter-Zorner *et al.* 1984). *Ecc* isolated from such crops include a large number of different serogroups (De Boer *et al.* 1979, 1985).

Eca appears to be restricted almost exclusively to potato in temperate regions. It is rarely isolated from the environment (McCarter-Zorner *et al.* 1984) and until recently little work on its pathogenicity was carried out. *Eca* isolated from potatoes

in temperate regions usually belong to one serogroup (serogroup I) (De Boer *et al.* 1979, 1985).

In the U.K. the largest crop losses caused by the soft rot erwinias are in potato, a large proportion of which are caused by *Ecc* and *Eca*, leading to soft rotting of tubers in storage. *Eca* is of interest to workers in temperate regions because in such climates, as well as causing soft rot of stored tubers, it also causes a systemic infection in the field called blackleg (stem rot) (Fig. A1). Blackleg can result from infection by *Eca*, *Ecc* or *Echr* depending on their geographic location (Perombelon and Kelman 1987), although there is still some doubt as to the ability of *Ecc* to cause this disease (Perombelon 1985). Attempts to create a blackleg type disease by inoculation of *Ecc* into *Erwinia*-free seed tubers has failed to show such symptoms (Perombelon 1985). Since more than one *Erwinia* species is often present in commercial seed (Perombelon 1985) it is not necessarily the case that the isolation of *Ecc* from a stem means that *Ecc* plays a part in the disease. It is possible that infection is initiated by *Eca* or *Echr* and *Ecc* then invades the damaged tissue. If this is confirmed it would suggest that pathogenicity determinants of the erwinias are more complex than presently thought (Perombelon 1985).

The soft rot erwinias move passively into potato tubers via the soil, from rotting mother tubers and other sources (Maher *et al.* 1986). If large numbers of pathogen are present in rotting mother tubers the vascular system of the plant is invaded and bacteria move into the stem. They remain there in a latent state until activated by conditions still unspecified. Blackleg usually develops in only one of several systemically infected stems and disease development may be simply due to a large influx of bacteria into the stem (Perombelon and Kelman 1987). Under anaerobic conditions $< 10^2$ cells per micro site (a localized site of

Plate 1

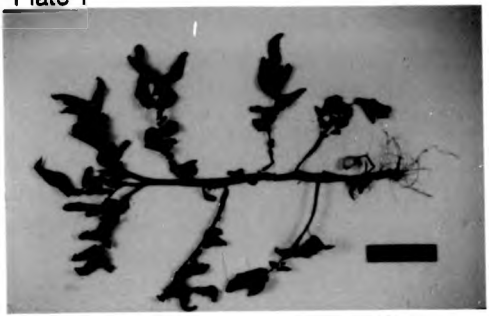


Plate 2

Figure A1
Plate 1 - blackleg of a potato plant (Maris Bard) caused by Eca SCRI1043 infection.
Plate 2 - soft rot of a potato tuber (Maris Bard) caused by Ecc SCRI193 infection.

bacterial propagation) is sufficient to start a lesion, whereas under aerobic conditions the number needed may be as high as 10^8 (De Boer and Kelman 1978). Since natural contamination rarely exceeds 10^2 per micro site, a lowering of the partial pressure of oxygen (p O_2) seems to be an important prerequisite for disease development. It has been suggested that at low p O_2 plant resistance mechanisms, including the oxidation of phenolic compounds and the production of antibacterial phytoalexins, may be impaired (Perombelon 1985). Symptoms of blackleg vary between a black/dark brown basal stem rot in wet conditions to a general wilting of leaves and plant desiccation in dryer conditions, the extent of disease being dependent on the number of invading bacteria and the environmental conditions (Aleck and Harrison 1978; Perombelon 1985).

A disease similar to blackleg can result under wet conditions from infection external to the crop via exposed stems and leaves and not, as in the case of blackleg, from the mother tuber. This disease (aerial stem rot) does not originate from the point of attachment to the tuber. The economic importance of aerial stem rot is relatively low compared to blackleg since seed crops are rarely affected (Perombelon and Kelman 1987).

The soft rot erwinias can spread laterally in a number of ways including wind-borne, water-borne, soil-borne and insect-borne dispersal, but the most important method of spread, from an economic point of view, is via contaminated seed potatoes (Harrison and Brewer 1982).

The disease-causing capacity of these erwinias seems to be temperature dependent (Graham and Dowson 1960; Perombelon 1985). *Eca* predominates at temperatures $<22°C$ whereas at higher temperatures *Ecc* predominates ($>22°C$). *Echr* is almost always found in countries only where ambient temperatures are $>25°C$. Exceptions to this are the temperate *Echr* strains which infect potatoes in Holland and France

(Perombelon pers. comm.). The exact role of temperature on the ability to cause disease is not clear, but it appears to reflect both variations in growth rate and the rate of extracellular enzyme production (Lanham *et al.* 1991; Salmond pers.comm.). In order to understand disease development it is important first to understand the nature of latency. Oligosaccharides released from the cell wall of pectic polymers by a pectolytic enzyme produced by *Erwinia* can trigger the production of antimicrobial phytoalexins under aerobic conditions (Davis *et al.* 1984). A state of equilibrium may therefore exist between bacterial growth and phytoalexin production under certain conditions resulting in latency (Perombelon 1985). Altered environmental conditions could therefore allow this balance to be disrupted and progressive infection to ensue.

Like blackleg, soft rot symptoms involve the degradation of parenchymous tissue, in this case in the tuber (Fig. A1). During lifting of the tubers at harvest, damage can occur. This, together with entry via lenticels, can allow the passage of bacteria into the tuber where it can remain dormant (latent state) (Perombelon 1982, 1985) until environmental conditions, which favour a rot, occur within the potato store. Such conditions include specific temperatures, high humidity and low oxygen tension (Perombelon and Kelman 1980). Attempts to control soft rot disease have had limited success. Such methods include tight controls on temperature and humidity during storage, segregation of infected tubers and in some cases heat treatment of newly harvested tubers (Robinson and Foster 1987). All these methods create problems for the production of large numbers of tubers which are stored for up to 10 months. Heat treatment and removal of contaminated tubers are important in the reduction of contaminated seed tubers which, if they were to go back into the field, could perpetuate the cycle.

Although the soft rot erwinias are plant pathogens of considerable importance they can be considered as opportunistic pathogens, since they enter the plant passively

and remain there until favourable conditions ensue. Other pectolytic bacteria are commonly isolated from tubers eg. *Pseudomonas* and *Clostridium* yet the erwinias are almost always responsible for disease symptoms. It may be that the erwinias are simply able to grow faster and produce more pectolytic enzymes than other bacteria under these conditions (Perombelon and Kelman 1980). It is not clear whether the ability of erwinias to produce and secrete certain pectolytic enzymes has to be accompanied by some other characters for pathogenesis (Bateman and Basham 1976) but this seems very likely, otherwise other pectolytic bacteria would also be significant potato pathogens. Only the soft rot erwinias are able to cause stem rotting ie. blackleg, even though other pectolytic bacteria are able to move into the stem via the vascular system (Perombelon 1985). Other factors involved in the pathogenesis of the soft rot erwinias have been investigated and will be discussed in subsequent Sections.

1.1.4 The plant cell wall

The cells of higher plants are surrounded by a wall which encases the plasmalemma and cell protoplast. The cell wall of dicotyledonous plants is divided into three structures, the middle lamella, the primary cell wall and in some cases a secondary cell wall (Fig A2). The middle lamella is composed predominantly of D-galacturonans (pectic polymers), which are also an important constituent of the primary cell wall. D-galacturonan 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 respectively (Chesson 1980). This latter structure is known as pectic acid or polygalacturonic acid (PGA). Arabinogalactan side chains are covalently linked to the rhamnogalacturonan backbone, accounting for the structural properties of the polymer (Cooper 1983). The middle lamella is seen as a "cementing" layer which

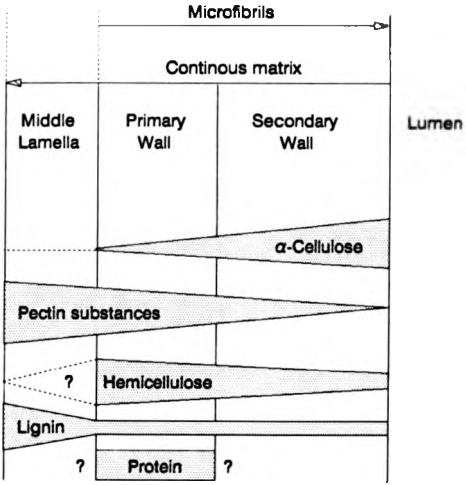


Figure A2. A diagrammatic representation of the plant cell wall (modified from Bateman and Basham 1976)

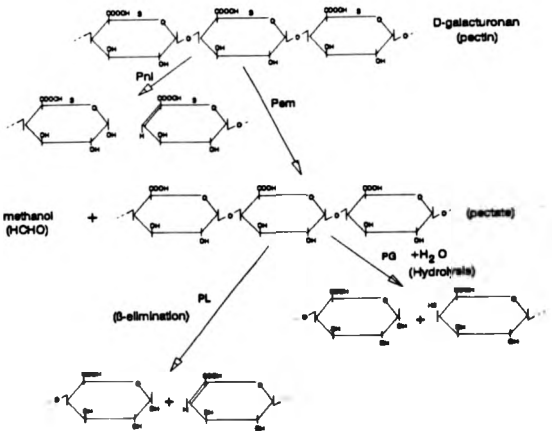
holds cells together to form a coherent tissue structure (Keegstra *et al.* 1973).

The primary cell wall is thought to be a tight mesh of cellulose fibres ensheathed in hemicellulose polymers, that are interconnected by galacturonan chains (McNeil *et al* 1984). The cellulose fibres containing both amorphous and crystalline regions are bounded by the hemicelluloses xyloglucan and arabinoxylan. Cross-linking of these structures with glycoproteins may also occur, giving added rigidity to the wall structure (Bateman and Basham 1976; Chesson 1980; Keegstra *et al.* 1973).

The secondary cell wall structure is less conserved than that of the primary cell wall and varies from plant to plant. Compared to the primary cell wall it contains more cellulose fibres arranged in parallel arrays, with less hemicellose and galacturonan polymers (Keegstra *et al.* 1973).

1.1.5 Invasion by soft rot erwinias

The ability of the soft rot bacteria to attack the middle lamella and primary cell wall causing disintegration of plant tissue, results primarily from their ability to form extracellular pectolytic enzymes which attack the galacturonan component leading to breakdown of the polymers by hydrolysis (hydrolases) or ß-elimination (lyases) (Fig. A3) (Bateman and Basham 1976; Chesson 1980). Following the action of the pectic enzymes, other cell wall polymers become accessible to enzymic degradation. The simultaneous action of the degradative enzymes solubilizes the middle lamella and destabilizes the structural integrity of the cell walls. The cell wall is no longer able to support the cytoplasmic membrane under conditions of osmotic shock leading to membrane damage and cell death (Bateman and Basham 1976). The release of nutrients then stimulates the growth of the invading bacteria.



FigureA3 The mode of action of four pectolytic enzymes; pectin lyase (Pnl), Pectin methylesterase (Pem), endo-polygalacturonase (PG) and endo-pectate lyase (PL) . Adapted from Hinton (1985).

Since *Ecc* and *Eca* do not appear to show cultivar specificity the study of host specificity using these bacteria would be of little value, unlike the situation with phytopathogenic pseudomonads (Daniels *et al.* 1988). However their role as examples of primitive pathogens is of interest. Studies of *Erwinia*/potato interactions could yield information concerning the mechanisms by which the bacteria overcome general host resistance. In contrast to the *Ecc* and *Eca* species, some limited host specificity has been shown between *Echr* and certain ornamental plants (Dickey 1981; Lelliot and Dickey 1984).

In the following section details of the genetic analysis of pathogenesis of the soft rot erwinias will be discussed, followed by a section on pathogenicity factors in other phytopathogenic bacteria.

1.2 Genetic analysis of pathogenicity in the soft rot erwinias : General approach

Two approaches have been used in the search for pathogenicity determinants in phytopathogenic bacteria. These approaches will be summarised below and explained in detail in Sections 1.2.1 and 1.2.2.

1.2.a Random mutagenesis

One approach to the analysis of bacterial plant pathogenicity has been described by Daniels *et al.* (1984a) who advocated the "black box" strategy involving the use of random transposon mutagenesis (using suicide vectors to allow their passage into the cell) for the generation of reduced virulence mutants. This method of mutagenesis allows inserts into the bacterial genome to be easily monitored, but it also creates insertions that can be precisely located. The aim of such an approach is to identify

any determinants of virulence, with no assumptions as to the nature of these determinants. The black box approach has been applied to several plant pathogens (Daniels *et al.* 1988) including *Eca* (Hinton *et al.* 1989a), *Ecc* (Handa *et al.* 1987; Jayaswal *et al.* 1984), *Xanthomonas* (Dow *et al.* 1987; Shaw *et al.* 1988), *Pseudomonas* (Anderson and Mills 1985; Boucher *et al.* 1985; Lingren *et al.* 1986; Malik *et al.* 1987; Somlyai *et al.* 1986) and *Agrobacterium* (Thomashaw *et al.* 1987). A similar strategy using bacteriophage Mu has been used to isolate reduced virulence mutants of *E.amylovora* (Bauer and Beer 1987) and *E.stewartii* (McCammon *et al.* 1985).

1.2.b Targeted approach

In contrast to taking a random approach, attempts are often made to determine whether or not specific putative virulence factors are involved in pathogenicity eg. pectolytic enzymes, cell surface components and plant inducible genes. In such cases more direct approaches have been used, and will be described below.

1.2bi Role of enzymes: Method of analysis

Pectolytic enzyme structural genes have been cloned from a number of *Echr*, *Ecc* and *Eca* strains (see Section 1.2.2.1.1) by exploiting the expression of these genes in *E.coli*. Once cloned into *E.coli* the role of an individual enzyme in pathogenicity can be crudely assessed by inoculation of the recombinant *E.coli* into plants or tubers. Insertion mutagenesis using Mu-*lacZ* or Tn*phoA* allows expression from individual promotors to be studied, and once mutated the genes can be replaced in the *Erwinia* chromosome by marker exchange mutagenesis (reverse genetics). Reverse genetics involves a) cloning the *Erwinia* gene in *E.coli*, b) making a fusion/insertion into the gene, c) transferring this back into *Erwinia* on an unstable

replicon and d) screening for loss of the plasmid while retaining selection for the fusion/insertion in the chromosome (Salmond 1987). This technique allows *Erwinia* strains deleted in one or more enzyme structural genes to be assessed for pathogenicity. This reverse genetics strategy is required because direct insertion mutagenesis would not be possible in cases where a number of similar isozymes were produced, since mutagenesis of one gene would be masked by synthesis of isozymes from the others.

The identification of genes involved in secretion and regulation is more straight forward, involving direct insertion mutagenesis by Tn*5* (Reverchon and Robert-Baudouy 1987; Thurn and Chatterjee 1985) Tn*10*, Tn10-*lacZ* (Murata *et al.* 1990), Mu-*lacZ* (Hugouvieux-Cotte-Pattat and Robert-Baudouy 1989; Jingwei *et al.* 1987), Tn*phoA* (Coleman *et al.* 1991; Ji *et al.* 1989) and chemical mutagenesis (Hugouvieux-Cotte-Pattat *et al.* 1986).

1.2.bii Role of the cell surface: Approach

The role of the cell surface in pathogenicity has been studied using mutants selected as spontaneously resistant to bacteriocins and phages. Using such techniques Schoonejans *et al.* (1987) isolated mutants of *Echr* altered in lipopolysaccharide (LPS) structure and Expert and Toussaint (1985) isolated mutants of *Echr* altered in three outer membrane proteins, later found to be involved in iron uptake (Expert *et al.* 1987; Franza *et al.* 1991). Mutations leading to alterations in both these structures were found to affect virulence on *Saintpaulia* plants. A similar technique has been used to isolate EPS (extracellular polysaccharide) deficient mutants (which may lack the enzyme UDP-Gal-4-epimerase [GalE]) (Billing 1984) and LPS deficient mutants (Billing 1987) of *E.amylovora*. This technique has been used to isolate T4 resistant mutants of *Ecc* and *Eca* deficient in LPS, although the resultant mutants were not affected in virulence (Pirhonen *et al.* 1988). More recently

Pirhonen *et al.* (1991) have obtained T4 resistant mutants affected in LPS structure, galactose utilization (may lack the enzyme UDP-G-pyro-phosphorylase [GalU]) and virulence. Phage resistant mutants of *Xanthomonas oryzae* have also been found to be affected in virulence (Goto 1972), and Ugalde *et al.* (1986), working on phage resistant mutants of *Rhizobium meliloti*, found a correlation between the lack of galactosyltransferase enzyme (GalT), altered LPS and reduced nodulation.

1.2.biii Role of plant inducible genes: Approach

Echr genes induced by plant extract have been studied by insertion mutagenesis using phage Mu containing a promoterless neomycin phosphotransferase gene (*npt*), which on promoter expression leads to kanamycin resistance (Beaulieu and Van Gijsegem 1990). Mutants containing inserts within plant inducible genes were selected for by their acquired resistance to kanamycin on addition of *Saintpaulia* plant extract. Using this technique reduced virulence mutants altered in cation uptake, galacturonate degradation and the production of the pectolytic enzyme acidic pectate lyase (acidic-PL) have been isolated. The function of other plant inducible genes are still unknown.

1.2.1 Random mutagenesis approach: Results summary

Hinton *et al.* (1989a) used random Tn*5* insertion mutagenesis on *Eca* SCRI1043 to obtain a number of mutants which exhibited reduced virulence on potato plants (cultivar Maris Bard). A total of 3425 kanamycin resistant colonies were inoculated into miniplants (micropropagated potato plants) of which 9 exhibited reduced virulence (Rvi-). Five of these mutants were auxotrophic, one was a growth rate mutant and three were enzyme mutants (reduced in both PL and polygalacturonase (PG) synthesis and their secretion). The gene responsible for the latter phenotype

was designated *pep* (*p*ectolytic *e*nzyme *p*roduction). Hinton *et al.* (1989a) concluded that either a) virulence in *Eca* simply reflects its ability to multiply and produce large amounts of pectolytic enzymes *in planta*, or b) other bacterial functions do exist but were not detected by the above mutagenesis and screening procedures. This would suggest that other factors apart from bacterial growth and enzyme production is involved in the induction of blackleg disease. Recently more Tn*5*-induced *Eca* mutants have been identified on potato, which exhibit an Rvi$^-$ phenotype. Analysis of these *Eca* mutants suggests that factors other than enzymes may play a role in pathogenicity (Pirhonen *et al.* 1991) (see Section 1.3.3).

Jayaswal *et al.* (1984) used phage Mu d1 mutagenesis of *Ecc* AH2 to obtain 4,700 ampicillin resistant colonies, of which 63 independent isolates were severely impaired in their ability to cause potato tuber decay. Of these mutants 16 were auxotrophic, 44 were impaired in the secretion of PL and PG (these differed from the *pep* mutants of Hinton *et al.* (1989a) which were affected in both synthesis and secretion of PL and PG), 4 were impaired in virulence but no other observable phenotype, and 1 was defective in its ability to form UDPG-pyrophosphorylase (an enzyme involved in galactose metabolism and coded by the gene *galU*). The latter mutant termed AH1028 was unable to utilize several carbon sources, including galactose (Gal), lactose, galacturonate and polygalacturonate (Jayaswal *et al.* 1985a). The mutant was also altered in its LPS core structure, although little significance was placed on this phenotype in virulence. Secondary mutants of AH1028 able to grow on all carbohydrates tested except Gal, became resistant to Gal but were unable to grow on it. When tested on potato tubers these secondary mutants were as virulent as the wild type strain. This evidence suggests a corrulation between Gal utilization and the ability to cause tuber rot.

E.coli mutants defective in the *galU* or *galT* genes of the Gal regulon have led to Gal-induced bacteriostasis, whereas mutants defective in *galE* have shown Gal-

induced lysis (Fukasawa and Nikaido 1961; Yarmolinsky *et al.* 1959). In addition *galU* mutations in *E.coli* have been shown to affect flagella formation (Komeda *et al.* 1977) and cause the synthesis of LPS deficient in hexoses (Fukasawa *et al.* 1962; Sundararajan *et al.* 1962). These results suggest that mutations in the Gal regulon affect a number of other phenotypes in addition to virulence and growth deficiencies.

1.2.2 Targeted approach: Main results

It is clear that the major pathogenicity determinants of the soft rot erwinias are pectolytic enzymes, although other factors do have a role in virulence (see Section 1.2.2.2). In this section our current knowledge on the production, regulation and action of degradative enzymes will be summarized, together with the role of the bacterial cell surface and plant-inducible genes in pathogenicity.

1.2.2.1 Enzymes

Pectolytic enzymes are produced by a wide range of phytopathogenic bacteria (Kotoujansky 1987) and are probably the main pathogenic determinants in these species. Investigations into the production of such enzymes are extensive and include studies of their structure, secretion and regulation. The majority of research on degradative enzymes has been carried out on the soft rot erwinias and this section will therefore concentrate on the erwinias (Table 2a).

1.2.2.1.1 Pectinases

To depolymerize and catabolize pectin, the soft rot erwinias produce a series of enzymes (Table A2). These enzymes are discussed below.

1.2.2.1.1.a Pectate lyase

Ecc, *Eca* and *Echr* produce pectate lyase (PL). PL cuts the α-1,4-glycosidic bonds at random within the pectate (an unmethylated form of pectin), and partially esterified pectin, chain. Its mechanism is a ß-elimination generating an oligomer with a 4,5 unsaturated galacturonosyl at the nonreducing end (Fig. A3) (Nasuno and Starr 1966).

Using isoelectric focusing in columns Garibaldi and Bateman (1971) and Pupillo *et al.* (1976) separated four PL isozymes from cultural filtrates of *Echr*. The more recent technique of isoelectric focusing in thin polyacrylamide gels has led to the discovery of a fifth PL and several minor ones (Ried and Collmer 1986). The five major PL isozymes of *Echr* are PLa, PLb, PLc, PLd and PLe and are grouped into acidic (PLa), neutral (PLb and PLc) and alkaline (PLd and PLe) isozymes with pIs of approximately 4.0 to 5.0, 7.0 to 8.5 and 9.0 to 10 respectively. The alkaline PLs seem to attack pectin more randomly than do the neutral ones (Barras *et al.* 1987; Collmer *et al.* 1982). All PLs are Ca^{2+} dependent, with a pH optimum for their activity between 7.9 and 9.8 (Quantick *et al.* 1983).

In *Ecc* and *Eca* PL isozymes are also produced (Hinton 1989b; Quantick *et al.* 1983; Ried and Collmer 1986). Hinton *et al.* (1989b) characterised four PLs of *Ecc* SCRI193 (PLa/b/c/d with pIs 7.2, 8.2, 10.3 and 11.0 respectively). Of these PLa and PLb were periplasmic while PLc and PLd were extracellular. On characterisation of two of these, PLb and PLc, they found a high degree of

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-PG	"	demethylated galacturonan	"	3.2.1.15
exo-poly-galacturonase	exo-PG	"	"	Terminal (exo)	3.2.1.82
endo-pectate lyase	endo-PL	"	"	Random	4.2.2.2
exo-pectate-lyase	exo-PL	cell-bound	"	Terminal	4.2.2.9
oligogalact-uronate lyase	OGL	"	demethylated oligomers	"	4.2.2.6

Table A2. Mode of action of pectolytic enzymes produced by the soft rot erwinias. From Hinton (1985).

homology with PLs from other *Erwinia* species. Recently a detailed study of the pectic enzymes of *Eca* SR-8 was undertaken (George *et al.* 1991), revealing that the PL profile of *Eca* is much more complex than previously realized. *Eca* SR-8 produced six endoPLs, four of which were located in the extracellular fraction (pIs 10.2, 9.6, 9.2 and 8.9) and two which were found in both the extracellular fraction and the cytoplasm (pIs 9.5 and 9.4). An exoPL was also found in the periplasm.

Organisation of PL genes

PL-encoding genes have been cloned from several strains of *Echr* (Collmer *et al.* 1985; Keen *et al.* 1984; Kotoujansky *et al.* 1985, 1987; Reverchon *et al.* 1985, 1986; Schoedel and Collmer 1986; Van Gijsegem *et al.* 1985), *Ecc* (Hinton *et al.* 1989b; Plastow *et al.* 1986; Roberts *et al.* 1986a; Roberts *et al.* 1986b; Willis *et al.* 1987; Zink and Chatterjee 1985) and *Eca* (Lei *et al.* 1985a), They have been identified by their expression in *E.coli* on enzyme activity detection media (Collmer *et al.* 1985; Keen *et al.* 1984; Kotoujansky *et al.* 1985) or by immunoscreening (Lei *et al.* 1985a; Nikaido *et al.* 1985; Ward and DeBoer 1989). By *in vitro* deletions and insertion mutagenesis, clones from *Echr* B374 and 3937 have been analysed (Diolez and Coleno 1985; Diolez 1986; Kotoujansky *et al.* 1987; Reverchon *et al.* 1986). Both strains possess five *pel* genes (*pel*A to *pelE*) which encode the major PL isozymes PLa to PLe (Kotoujansky *et al.* 1987; Reverchon *et al.* 1986). These genes are found in two clusters on the chromosome, one including *pel*B and *pelC* and the other *pelA*, *pelD* and *pelE* (Kotoujansky *et al.* 1987; Van Gijsegem *et al.* 1985). In *Echr* 3937 a pectin methylesterase gene (*pme*) (encoding an additional pectolytic enzyme responsible for removing methoxyl groups from pectin to form pectate, Plastow 1988) has been found adjacent to the *pelADE* cluster (Kotoujansky *et al.* 1987). Each *pel* gene is an individual transcription unit, and in each case the direction of transcription was determined by analysis of transcriptional

and translational fusions between the *pel* genes and ß-galactosidase or neomycin phosphotransferase genes (Diolez and Coleno 1985; Diolez *et al.* 1986; Reverchon and Robert-Baudouy 1987; Reverchon *et al.* 1986). DNA-DNA hybridization and nucleotide sequence analysis between *pelB* and *pelC* of *Echr* (encoding the neutral PLs, PLb and PLc) show over 80% DNA homology (Keen and Tamaki 1986). *pelA* (encoding the acidic PL, PLa), *pelD* and *pelE* (encoding the alkaline PLs, PLd and PLe) show 58-90% sequence homology (Keen and Tamaki 1986; Reverchon *et al* 1990; Tamaki *et al.* 1988). Sequence homology has been found between *pelB* and *pelE* and between *pelA* and *pelC* of *Echr*, but in only two short regions of conserved amino acids (Hinton 1989b; Tamaki *et al.* 1988). It seems likely therefore that the alkaline PL genes and the neutral PL genes have appeared by duplication of two ancestral genes. Cloning of *pel* genes in *Echr* has allowed the nature of the minor bands found in isoelectric focusing gels to be clarified. The minor bands are probably derived from the major PL isozymes by posttranslational modifications (Kotoujansky *et al.* 1987; Vergnet-Ballas *et al.* 1986).

From *Ecc*71 four *pel* genes encoding different PLs have been isolated of which *pel2* and *pel3* are clustered (Willis *et al.* 1987; Zink and Chatterjee 1985). Plastow *et al.* (1986) isolated four *pel* genes of *Ecc*, two of which were linked and encoded major PLs, the other two probably represent minor PLs (PLa and PLb) undetected in the *Ecc* culture filtrate. Like *Echr* therefore the PLs of *Ecc* are encoded by multiple genes.

The PLs of *Eca*, until recently (George *et al.* 1991), showed an identical pattern of PL isozymes to that of *Ecc* SCRI193 (Hinton 1989b). Using a *pel* gene of *Ecc* strain EC14 as a probe, an *Eca* library was screened which led to the identification of an alkaline PL *of Eca* (Roberts *et al.* 1986b). Lei *et al.* (1985a) identified a 7kb fragment containing at least two genes that coded for PLs from *Eca* (pIs of 9.1 and 9.4), and Allen *et al.* (1987) isolated an endo-PL gene (encoding a PL of pI 9.2)

from an *Eca* gene library. Recent work by George *et al.* (1991) has shown that *Eca* strain SR-8 produces six endo-PLs with pIs of 10.2, 9.6, 9.5, 9.4, 9.2 and 8.9, suggesting that this subspecies of *Erwinia carotovora* is much more complex than previously realized. All the PLs were found in the extracellular fraction, with those of pIs of 9.5 and 9.4 also being present in the periplasm.

From data obtained so far it is clear that all the soft rot erwinias secrete alkaline PL isozymes. EC16 however appears to produce only one such isozyme (Barras *et al.* 1987; Thurn and Chatterjee 1987) because it has a truncated gene for the other, normally secreted, PL.

1.2.2.1.1.b Polygalacturonases

Endo-polygalacturonase (PG) activity is found in *Ecc* and *Eca* but not in *Echr* (George *et al.* 1991; Lei *et al.* 1985b; Plastow *et al.* 1986; Ried and Collmer 1986; Roberts *et al.* 1986a; Roberts *et al.* 1986b; Zink and Chatterjee 1985). Endo-PG hydrolyzes internal α-1,4-glycosidic bonds in pectate polymers (Nasuno and Starr 1966) generating oligomers of galacturonic acid ranging from dimers to multimers (Roberts *et al.* 1986b; Willis *et al.* 1987). *Echr*, however, does possess an exo-PG (Brooks *et al.* 1990; He and Collmer 1990), which, using marker exchange mutagenesis, was shown to play a significant role in utilization of polygalacturonate and induction of PL (He and Collmer 1990). More recent work, using PL^- mutants of *Echr* EC16, suggests that this enzyme (encoded by the *pehX* gene) does not contribute to the macerating activity of *Echr* (Brooks *et al.* 1990). Unlike the PLs, PG does not require Ca^{2+} for activity. PG is constitutively expressed in *Ecc* (Chatterjee *et al.* 1981), its synthesis is not induced by pectate and is not affected by catabolite repression (Hinton *et al.* 1990). PG encoding genes (*peh*) have been

cloned from three different strains of *Erwinia carotovora* (Saarilhati *et al.* 1990a; Willis *et al* 1987; Zink and Chatterjee 1985), including SCRI193 (Plastow *et al.* 1986) and one strain of *Eca* (Lei *et al* 1985b). In EC14 and *Ecc*17 the *peh* gene has been shown to be linked to a *pel* gene (Roberts *et al.* 1986b; Willis *et al.* 1987). The two activities have been separated by insertion mutagenesis (Willis *et al.* 1987). Unlike the exo-PG of *Echr*, the purified endo-PG of *Ecc* and *Eca* are capable of causing potato tuber maceration (Lei *et al.* 1985b; Willis *et al.* 1987). The *peh* gene of *Ecc* SCRI193 shows 86% sequence homology to that of other *Ecc peh* genes (Hinton *et al.* 1990). Recent work by George *et al.* (1991) has shown that *Eca* produces two PGs, one of pI 10.7 located in the extracellular fraction, and one of pI 3.9 located mainly in the extracellular fraction with a small amount also occuring in the periplasm.

1.2.2.1.1.e Pectin lyase

Most soft-rot erwinias produce endo-pectin lyase when induced by the DNA damaging agents mitomycin C, nalidixic acid and ultraviolet light (Itoh *et al.* 1980; Tsuyumu and Chatterjee 1984). Pectin lyase (PNL) cleaves pectin or methylesterified PGA, but is not active on PGA (Itoh *et al.* 1982; Kamimiya *et al.* 1974). PNLs from several strains of *Ecc* have been isolated which have optimum activity at a pH around 8.0., a pI of 9.6 and are immunologically identical (Itoh *et al.* 1982). PNLs from several strains of *Echr* and *Ecc* were recognised by antibodies raised to an *Echr* PNL (Tsuyumu and Funakubo 1985). PNLs are immunologically distinct from PLs (Itoh *et al.* 1982) but *pnlA* has a 20% sequence homology with *pelB* of *Eca*, a 25.1% homology with *pelA* of *Ecu* and a 28.5 % homology with *pelE* from *Echr* 3937 (Chatterjee *et al.* 1991).

1.2.2.1.2 Regulation of pectinase synthesis

Polygalacturonate (PGA) and its degradation products are inducers of PL activity in the soft rot erwinias, as are plants (Garibaldi and Bateman 1971; Pupillo *et al.* 1976) and some plant extracts (Tomizawa *et al.* 1970; Tsuyumu 1977). Other factors also play a part in regulating pectolytic enzyme production, for example the production of PLs *by Eca* SCRI1043 are reduced 3-6 fold at 30.5°C compared to 27°C (Lanham *et al.* 1991; Salmond pers.comm.). This temperature dependent production almost certainly has an affect on the pathogenicity and world wide distribution of the species. The current knowledge of regulation is given below.

1.2.2.1.2.a Inducers

Induction levels of the *pel* genes in *Echr* 3937 and B374 have been studied using *pel-lacZ* fusions in Lac$^-$ strains (Diolez and Coleno 1985; Hugouvieux-Cotte-Pattat *et al.* 1986; Hugouvieux-Cotte-Pattat and Robert-Baudouy 1985; Reverchon and Robert-Baudouy 1987). Expression of the ß-galactosidase gene under the *pel* promoter allows transcription from that promoter to be quantified. Levels of transcription from the *pel* genes induced by PGA can vary. PLa/d/e synthesis in B374 varies between three and six fold (Reverchon and Robert-Baudouy 1987) while PLc in 3937 is increased by 37 fold on induction (Diolez and Coleno 1985). On induction of the *pel* genes all other genes in the pectinolysis pathway (Fig. A4) are also induced with intermediates in this pathway being the true inducers. These intermediates have been identified as 2-keto-3-deoxygluconate (KDG) (Hugouvieux-Cotte-Pattat and Robert-Baudouy 1989), 2,5-diketo-3-deoxyglutarate (DKII) and possibly 5-keto-4-deoxyuronate (DKI) (Condemine *et al.* 1986).

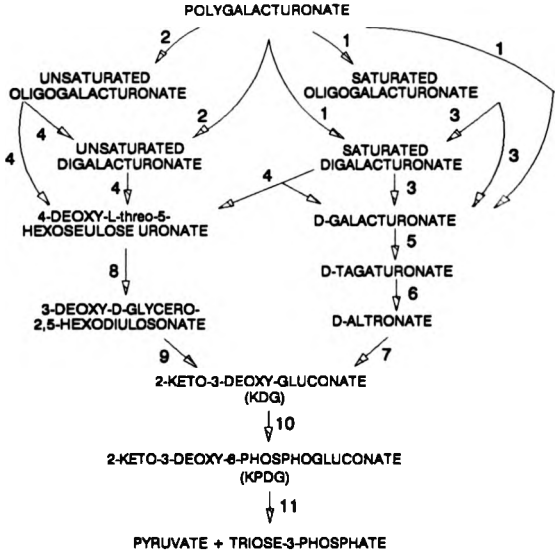


Figure A4. Major pathways of pectate catabolism in bacteria. Enzymes for the catabolic steps are: 1) polygalacturonase, 2) pectate lyase, 3) oligogalacturonate hydrolase, 4) oligogalacturonate lyase, 5) uronate isomerase, 6) altronate oxidoreductase, 7) altronate hydrolase, 8) 4-deoxy-L-threo-5-hexoseulose 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. 1985)

1.2.2.1.2.b Genetic control of regulation

1.2.2.1.2.bi Catabolite repression

The synthesis of PL isozymes in *Ecc* and *Echr* is reduced in the presence of a more easily catabolized carbon source than PGA, ie. synthesis is subject to catabolite repression (Fig A5) (Moran and Starr 1969). The breakdown products of PGA are also able to regulate PL production leading to a "self-catabolite" repression (Collmer and Bateman 1981; Tsuyumu 1979). Such an effect is supported by the production of mutants deficient in cyclic-AMP which have reduced PL synthesis (Mount *et al.* 1979), and the fact that the addition of cAMP to *Ecc* suppresses the action of repression (Hubbard *et al.* 1977). Further indirect support for the existence of catabolite repression includes the presence of *a cya* gene in *Echr*, encoding adenylate cyclase (Hedegaard and Danchin 1985). In the *cri* mutant of *Echr* B374 (Hugouvieux-Cotte-Pattat *et al.* 1986) PL synthesis was less sensitive to the repression observed in the presence of glucose in the wild type strain, as were cellulase and protease (Reverchon *et al.* 1990). The *cri* gene therefore seems to act at the level of catabolite repression. Expression from *pelB* and *pelC* were more sensitive to the effects of this mutation than were *pel*A, *pelD* and *pelE* (Hugouvieux-Cotte-Pattat *et al.* 1986; Tsuyumu and Chatterjee 1984). Sequence homology to the catabolite activator protein (CAP) target site of *E.coli* has been found in the promoter regions of *pelB* and *pelE* of *Echr* and *pelC* of *Ecc* SCRI193 (Hinton 1989b). In addition to catabolite repression, PL regulation is affected by a host of regulatory genes. Below is a summary of the regulatory genes of *Echr* isolated to date.

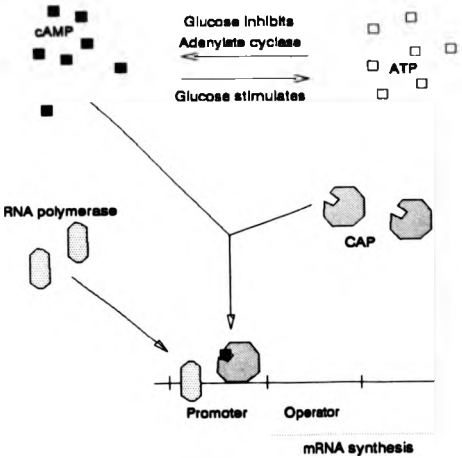


Figure45 Diagrammatic representation of the role of cyclic adenosine monophosphate (cAMP) and the catabolite activator protein (CAP) in catabolite repression. In the case of catabolite-repressible enzymes, binding of RNA polymerase only occurs if the CAP protein has first bound. This allosteric protein only binds if it has first bound cAMP which is produced from ATP by the enzyme adenylate cyclase. The presence of glucose either inhibits cAMP formation or stimulates its breakdown. In the presence of glucose a deficiency of cAMP occurs preventing CAP binding and in turn preventing RNA polymerase binding.

1.2.2.1.2.bii Negative regulation

kdgR and negative control of the pectinolytic pathway

The *kdgR* gene of *Echr* 3937 is a negative regulator of the 2-keto-3-deoxygluconate transport system (Fig. A4) (Condemine and Robert-Boudouy 1987; Reverchon *et al.* 1991). When inactivated by transposon mutagenesis (*kdgR*::Tn*5*) it leads to constitutive expression of all genes in the pectinolysis pathway, *pelD* and *pelE* being more strongly affected than *pelA*, *pelB* and *pelC* (Condomine and Robert-Baudouy 1987; Reverchon and Robert-Baudouy 1987).

pecS and negative control of PL synthesis

Using Mu*lac* insertions in *Echr* 3937 Reverchon *et al.* (1990) isolated a *pecS* regulatory mutant with derepressed PL levels, which remained PGA inducible. No other genes involved in pectinolysis were effected. In contrast to *kdgR*, *pecS* mutations did not affect expression of other genes in pectinolysis. Electrofocusing of PL isozymes revealed that PLb and PLc strongly increased in *pecS* mutants whereas PLa, PLd and PLe were not or were weakly affected by this mutation. *pecS* therefore appears to be a second negative regulatory gene whose product specifically represses *pel* genes.

gpiR and negative control of PL synthesis

Wild type *Echr* B374 synthesizes PLs at the end of log phase but chemically-induced mutants affected in the *gpiR* gene, synthesize PLs constitutively throughout the growth phase (Hugouvieux-Cotte-Pattat *et al.* 1986; Reverchon and Robert-Baudouy 1987). This probably results from the loss of a control mechanism regulating the temporal activation of PL and cellulase (Reverchon *et al.* 1990).

Unlike *kdgR* mutants, only *pel* genes in the pectinolysis pathway are affected, particularly *pelD* and *pelE*, while *pelA* expression is hardly affected (Reverchon and Robert-Baudouy 1987). Mutations in *gpiR* also enhance cellulase gene expression in *Echr* B374 (Hugouvieux-Cotte-Pattat *et al.* 1986). The *gpiR* mutation maps at a different location to the *pel* genes on the *Echr* B374 chromosome and the gene product may be a repressor (Reverchon and Robert-Baudouy 1987). Mutants constitutive for expression of all *pel* genes have been isolated by Diolez *et al.* 1986 by marker exchange mutagenesis. These mutants, which may be *gpiR* mutants, produce the same amount of PL in the presence or absence of an inducer.

pecR and negative regulation of PLa

Although the *kdgR* and *gpiR* genes appear to have little affect on the expression of *pelA*, a spontaneous mutant of an *Echr* strain carrying a *pelA-lacZ* fusion has been isolated which leads to a constitutive ß-galactosidase activity (Pupillo *et al.* 1976). In the absence of an inducer *pelA-lacZ* is expressed 100-300 fold higher than the wild type (Reverchon and Robert-Baudouy 1987). This mutation has little effect on the other *pel* genes. As with the *gpiR* gene the *pecR* gene maps away from the affected gene and may therefore produce a repressor of *pelA*.

Yankovsky *et al.* (1989) identified a mutation in a gene from *Echr* ENA49 they designated *ptlR*. Such a mutation led to constitutive expression of *ptlA* (equivalent to *pelA*) in the absence of an inducer. This gene therefore appears to be a negative regulator of *ptlA* and may be functionally equivalent to *pecR*.

pecY and negative regulation of PME

Use of Mu-*lacZ* inserts in *Echr* 3937 have led to the identification of a gene (*pecY*) which appears to negatively regulate the expression of pectin methylesterase

(Boccara and Chatain 1989). The relationship of *pecY* to *kdgR* and *pecI* is unclear.

pecI affects induction of pectinolytic enzymes

In *Echr* 3937 regulation of PLs and PME has been shown to be affected by a further regulatory gene (*pecI*) (Hugouvieux-Cotte-Pattat and Robert-Baudouy 1989). Mu-*lac* insertions in the gene *pecI* lead to constitutive expression of PLs and PME in the absence of an inducer, and a very high induction of PL and PME in the presence of PGA.

1.2.2.1.2.biii Positive regulation

pecA and *pecL* in positive control of PL synthesis

Spontaneous mutants of *Echr* B374 with *pelE-lacZ* or *pelD-lacZ* fusions have been isolated with reduced ß-galactosidase activity in the presence of PGA or galacturonate. The relevant mutations (*pecA*$^-$) lead to reduction in the expression of all *pel* genes in this strain and the corresponding gene may therefore encode a positive regulator. Regulatory mutants with decreased PL synthesis have also been obtained from strain 3937 by screening for Mu-*lac* insertions in the presence of PGA (Hugouvieux-Cotte-Pattat and Robert-Baudouy 1989) These mutants (*pecL*$^-$) showed a phenotype very similar to *pecA* mutants. Whether *pecA* and *pecL* are similar or different genes is presently under investigation (Reverchon *et al.* 1990). Positive regulation of pel genes by *pecA* and *pecL* may work in conjunction with the negative regulatory genes *kdgR* and *pecS*.

pecX and positive regulation of PLa

Reverchon *et al* 1986, using insertion mutagenesis of miniMu*lac*, have defined a

gene (*pecX*) whose product seems to specifically activate *pelA* expression. Insertions in this gene result in a strong decrease in PLa synthesis, suggesting that *pecX* is involved in positive regulation of *pelA*.

In conclusion the isolation of regulatory mutants has shown the existence of three types of regulation affecting PL synthesis (catabolite repression, growth phase dependence and induction by PGA derivatives). In the regulation of PL synthesis there appears to be a complex regulatory circuit with various regulatory genes, the expression of which can vary, and which are probably involved in activating or repressing each other (Reverchon *et al.* 1990).

1.2.2.1.2.biv Pectin lyase regulation

Unlike the induction of PL the synthesis of PNL in *Ecc* is stimulated by agents such as nalidixic acid, U.V. light and mitomycin C (Itoh *et al.* 1980; Kamimiya *et al.* 1974; Tomizawa and Takahashi 1971). In both *Echr* and *Ecc* these agents coordinately induce a bacteriocin called carotovoricin or a temperate phage along with PNL (Itoh *et al.* 1980; Tsuyumu and Chatterjee 1984). By analogy with *E.coli* (Walker 1984) the lack of induction of PNL in *Ecc* in a *recA*$^-$ strain suggest that this enzyme is expressed as part of the SOS response requiring a functional *recA* gene (McEvoy *et al.* 1987, 1990; Zink *et al.* 1985). PNL from *Echr* and *Ecc* is induced *in planta* and DNA damaging agents have been reported in several plant species (Tsuyumu and Funakubo 1985). Therefore PNL induction may play an important role in overcoming plant defences.

1.2.2.1.3 The role of pectinases in the pathogenicity of soft-rot erwinias

The number of PL isozymes produced *in planta* appears to vary between strains (Garibaldi and Bateman 1971; Pupillo *et al.* 1976; Quantick *et al.* 1983). In addition to this, the significance of each in virulence has been shown to vary. The use of antiserum to PLe has shown that this enzyme contributes 50-60% of the total activity of the PL isozymes expressed in plant tissue (Thurn *et al.* 1987; Thurn and Chatterjee 1987). In order to investigate the effect on virulence of individual, or combinations of mutations in the pectinases it is necessary to obtain a mutation within the individual gene(s). This cannot be achieved however by direct mutagenesis of *Erwinia*, since screening would be hindered by the expression of the other pectinases. This problem has been overcome by marker exchange mutagenesis (reverse genetics) (see Section 1.2.bi). Boccara *et al.* (1988) produced a number of *pel*⁻ mutants of *Echr* 3937 in this way and tested their virulence on *Saintpaulia* plants. Similar work was carried out on *pelB*⁻ and *pelC*⁻ mutants of *Echr* CUCPB 1237 (Roeder and Collmer 1985, 1987). From both these studies PLb and PLc were found not to be required for full virulence, whereas PLa, and the alkaline PLs, PLd and PLe, were necessary to elicit the systemic disease. Mutations in PLe led to confined tissue maceration, suggesting the mutant was non-invasive.

Marker exchange mutagenesis has been carried out on the structural *pel* genes of *Echr* EC16 (an *Echr* strain which is naturally PLd⁻) and a number of PL mutants constructed (Ried and Collmer 1988). One mutant deleted in PLa/b/c/e led to a 99.9% decrease in PL activity compared to the wild type, resulting in a reduction in virulence of 79-98%. Since some maceration of plant tissue did occur in the absence of these enzymes workers suggested that PLa/b/c/d/e could not be soley responsible for plant tissue maceration. Using marker exchange mutagenesis Payne *et al.* (1987) deleted the structural gene *pelE*, which led to significantly less maceration than wild type *Echr* EC16, therefore showing the importance of PLe in plant tissue

maceration.

A mutation in the *peh* gene of *Ecc*71 by transposon mutagenesis, resulting in a PG$^-$ strain, behaved like wild type in maceration studies on potato tubers, suggesting a minor role for PG in maceration of potato by *Ecc* (Willis et al 1987). Other enzymes including, pectin methylesterase, pectin lyase, exo-PL and exo-PG are still to be investigated in this way.

The ability of purified PLs from *Echr* to elicit soft rot symptoms on a number of plant tissues has been investigated (Collmer and Keen 1986; Payne *et al.* 1987). All but PLa led to maceration, PLe being more efficient than PLb and PLc (Barras *et al.* 1987; Keen and Tamaki 1986; Payne *et al.* 1987). The ability of the alkaline PLs to macerate plant tissue has been confirmed in both *Ecc* (Hinton *et al.* 1989b; Tanabe *et al.* 1985; Zink and Chatterjee 1985) and *Eca* (Lei *et al.* 1985a; Quantick *et al.* 1983; Tamaki *et al.* 1988).

Another way to investigate the role of pectinases in pathogenicity has been to clone one or more pectinase genes into *E.coli*, then investigate the rotting activity of this strain. *E.coli* is an ideal strain for such studies, since it is closely enough related to *Erwinia* to express the cloned genes but lacks other known pathogenicity determinants. A problem with this system lies in the fact that extracellular enzymes of *Erwinia* are not efficiently secreted through the outer membrane of *E.coli* but remain in the periplasm (Collmer *et al.* 1985; Keen *et al.* 1984; Keen and Tamaki 1986; Lei *et al.* 1985a; Reverchon *et al.* 1985) (see Enzyme secretion). This problem has been overcome to a limited extent however by using expression vectors which over-express these enzymes. Using this technique Keen and Tamaki (1986) were able to show that the *pelE* gene product solely enabled *E.coli* to rot potato tissue under laboratory conditions. In *Ecc* strain EC14 only expression of an endo-PL, an exo-PL and an endo-PG simultaneously was able to cause limited maceration

of potato tuber tissue (Roberts *et al.* 1986a). The expression of *Eca* PG in *E.coli* has led to tuber tissue maceration (Lei *et al.* 1985b). As shown previously however mutants lacking PG remain pathogenic showing that while this enzyme plays a role in pathogenicity, this role is not essential (Willis *et al.* 1987).

From studies using cloned pectinase genes the alkaline PL isozymes appear to be essential for potato tuber tissue maceration and disease development Interpretation of pathogenicity tests using potato tubers have, however, been questioned (Kotoujansky 1987), putting doubt on the validity of this model for pathogenicity in the natural environment. *E.coli* strains carrying *pel* clones producing alkaline or neutral PLs when inoculated into *Saintpaulia* show no symptoms (Boccara *et al.* 1988).

The ability to produce a large number of pectinases enhances plant macerating ability and virulence of the bacterium. In *Echr* strains different pathotypes have been shown to possess different PL profiles (Ried and Collmer 1986). *Echr* strains isolated from dicotyledonous hosts possess an exo-PG, while those from monocots do not (Collmer *et al.* 1982; Ried and Collmer 1986). Together this evidence suggests that *Echr* pectinases, at least, may be adapted to certain plant host pectins which may explain a degree of host specificity. Liao (1989) studied PL profiles in a number of different soft-rot bacterial species including *Erwinia*. *Erwinia* species produced the most complex PL profiles of those bacteria tested, which is probably related to their efficient potato tuber maceration. While some monoclonal antibodies against an extracellular PL of *Ecc* react with most PLs of other *Erwinia* species, they do not with the PL enzymes of *Pseudomonas* or *Xanthomonas* species (Klopmeyer and Kelman 1988). This suggests a diversity in structure of the PLs between different genera.

The production of PLs in *Ecc* has been shown on several occasions to elicit a

phytoalexin response in soybean (Davis *et al.* 1986a; Davis *et al.* 1986b; Davis *et al.* 1984), initiated by oligogalacturonides released from the cell wall. This suggests that while PLs play a major role in virulence they also trigger plant defence mechanisms. The large number and quantity of pectinases produced on infection of a plant would therefore help in the rapid breakdown of substances like oligogalacturonides, to smaller molecules which no longer trigger the host defences. Such an over-production of pectinases can be achieved by suitable regulation (see Section 1.2.2.1.2) and by amplification of *pel* genes as observed in *Echr* (Barras *et al.* 1987; Collmer *et al.* 1985; Kotoujansky *et al.* 1987; Reverchon *et al.* 1986), *Ecc* (Plastow *et al.* 1986; Roberts *et al.* 1986a; Willis *et al.* 1987) and *Eca* (Lei *et al.* 1985a).

1.2.2.1.4 Other extracellular enzymes of *Erwinia*

1.2.2.1.4.a Cellulases

Both *Echr* (Andro *et al.* 1984; Boyer *et al.* 1984; Chambost *et al.* 1987) and *Ecc* (Allen *et al.* 1986) secrete an endo-B-1,4-glucanase with cellulolytic activity, involved in the degradation of cellulose within the primary and secondary cell walls of higher plants (see Section 1.1.4 and Fig. A2). The enzyme has been purified *from Echr* 3665 and 3937, has a pI of 4.3 and is active between pH 6.2 and 7.5 (Boyer *et al.* 1987; Boyer *et al.* 1984) *Eca* also produces a cellulase (Hinton *et al.* 1989a; Weber and Wegener 1986). *cel* genes have been cloned from *Echr* 3665 (Barras *et al.* 1984a; Boyer *et al.* 1987), 3937 (Boyer *et al.* 1987; Kotoujansky *et al.* 1985), B374 (Van Gijsegem *et al.* 1985) *Ecc* EC14 (Allen *et al.* 1986) and *Ecc* SCC3193 (Saarilahti *et al.* 1990b) and SCRI193 (Vick Cooper pers.comm.). *Echr* possess two different endoglucanases encoded by the genes, *celY* and *celZ*. From studies involving *celY-lac*Z and *celZ-lac*Z fusions the regulation of these genes has

been studied (Aymeric *et al.* 1986). The results indicate that the genes are constitutively expressed and subject to catabolite repression (Boyer *et al.* 1984; Chambost 1986). Saarilahti *et al.* (1990b) was the first group to sequence and characterise a Cel enzyme of *Ecc*. This Cel (refered to as CelS) has a pI of 5.5 and a pH optimum of 6.8. *celS* shows little sequence homology to *celY* or *celZ*, but sequence analysis has identified a signal sequence (suggesting a similar export mechanism to that of PL). Genes involved in the catabolism of cellobiose have also been cloned in *Echr* (Barras *et al.* 1984b) and expression of these *clb* genes is positively regulated by the cAMP-CRP complex (Barras *et al.* 1985).

1.2.2.1.4.b Proteases

Echr, *Ecc* and *Eca* secrete proteases (Prt) (Barras *et al.* 1986; Wandersman *et al.* 1986, 1987; Willis and Chatterjee 1987) which are exclusively extracellular and inducible by hydrolysate of casein (Wandersman *et al.* 1986). Two antigenically distinct proteases have been isolated from *Echr* 3937 (Wandersman *et al.* 1986) and a third from *Echr* B374 (Wandersman *et al.* 1987). From a gene library of *Echr* B374 a cosmid was found which confered on *E.coli* the ability both to synthesize and to secrete the three proteases in the absence of cell lysis (Wandersman *et al.* 1987). This cosmid was found to carry genes for two of these enzymes, an intracellular protease inhibitor and the secretion function, the latter of which is described by Delepelaire and Wandersman (1991). PLs are not secreted in an *E.coli* clone simultaneously harbouring *pel* genes and the DNA fragment controlling protease secretion (Barras *et al.* 1986). This therefore suggests the existence of two pathways for protein secretion in *Echr* (Section 1.2.2.1.5). Recently, Dahler *et al.* (1990) have identified three tandem *prt* structural genes, a gene encoding a protease inhibitor, and a region required for extracellular secretion of proteases on a single 14 kb cloned fragment of *Echr* EC16. When individually subcloned downstream

from vector promoters, in the presence of the secretion funtion, all three *prt* genes led to extracellular secretion of proteases by *E.coli*. One *prt* gene (*prtC*) was sequenced and showed high homology to *prtB* of *Echr* B374. Using marker exchange mutagenesis it was shown that *Echr* EC16 strains, defective in one or all of the *prt* structural genes, were not impaired in virulence on plant tissue. Letoffe *et al.* (1991) demonstrated the secretion of a *Serratia marcescens* Prt, expressed from a structural gene cloned in *E.coli*, in the presence of the *Echr* PrtB secretion apparatus. This demonstrates that the mechanism of *S.marcescens* protease secretion is analogous to that of PrtB in *Echr*.

1.2.2.1.4.c Hemicellulases

Hemicellulases have been identified in *Echr* and *Ecc* (Braun and Kelman 1987; Ferraris and Garibaldi 1979; Kaji and Shimokawa 1984) but little work has been carried out on these. Arabinases and xylanases have been found in *Ecc* (Kaji and Shimokawa 1984), while a xylanase from *Echr* has been found in much greater quantities in strains isolated from corn stalks than from those isolated from dicots. Since more xylan is present in grasses, this observation may relate to the host specificity of such strains (Braun and Kelman 1987).

1.2.2.1.5 Secretion of extracellular enzymes

The majority of plant degrading enzymes of *Erwinia* species catabolize polymers in the external environment of the cell (see above). Secretion mutants of *Echr*, termed Out$^-$, have been isolated by insertion mutagenesis of the chromosome using Tn*5* and Mu derivatives (Andro *et al.* 1984; Thurn and Chatterjee 1985). These Out$^-$ mutants retain pectinases and cellulases within the periplasmic space. These

Table 2a. Summary of plant cell wall degrading enzymes of the soft rot erwinias.

	Echr	*Ecc*	*Eca*
Pectate lyase (PG)	PLs a,b,c,d,e	PLs a,b,c,d	7 PLs
PL localisation	Extracel.	PLs a,b Periplasmic	2 PLs Periplasmic
		PLs c,d Extracel.	4 PLs Extracel.
PL regulation +	Inducible by break-	Inducible by break-	ND
	down products	down products	
+	Catabolite repressed	Catabolite repressed	ND
+	Negative regulatory	ND	ND
	genes		
+	Positive regulatory	Positive regulatory	Positive regulatory
	genes	genes	genes
Polygalacturonase	Exo-PG	Endo-PG	Endo-PG
(PG)			
PG localisation	Extracel.	Extracel.	Extracel.
PG regulation	Constitutive	Constitutive	ND
Pectin lyase (PNL)	PNL	PNL	ND
PNL localisation	Extracel.	Extracel.	ND
PNL regulation +	Inducible by DNA	Inducible by DNA	ND
	damaging agents	damaging agents	
Cellulase (Cel)	Cel Y, Z	Cel S, V	Cel
Cel localisation	Extracel.	Extracel.	Extracel.
Cel regulation	Constitutive	Constitutive	Inducible?
Protease (Prt)	Prt	Prt	Prt
Prt localisation	Extracel.	Extracel.	Extracel.
Prt regulation	Induced by peptides	Induced by peptides	Induced by peptides
Hemicellulase	Hemicel.	Hemicel.	ND

enzymes are identical to the extracellular enzymes of the wild type strain (Andro *et al.* 1984; Ji *et al.* 1987; Thurn and Chatterjee 1985; Van Gijsegem 1986), suggesting that *out* mutations effect a mechanism for the secretion of mature proteins out of the cell. Support for the fact that *out* gene products are involved in the transport process through the outer membrane comes from the following observations; a) Out$^-$ mutants are able to export functional enzymes to the periplasmic space but not through the outer membrane; b) Some Out$^-$ mutants lose a periplasmic protein (Ji *et al.* 1987; Thurn and Chatterjee 1985); c) the nucleotide sequence of several *pel* genes from *Echr* and *Ecc* compared with the actual sequence of the mature protein, has shown that PLs are synthesized as precursors with a signal peptide (Keen and Tamaki 1986; Nikaido *et al.* 1985). The secretion of proteases from Out$^-$ mutants is unimpaired suggesting an alternative secretion pathway (Andro *et al.* 1984; Thurn and Chatterjee 1985; Wandersman *et al.* 1986). This pathway is a *sec*-independent pathway, which does not require the presence of a signal sequence (Wandersman 1989) (*sec* is a name given to certain mutants in which secretion of extracellular enzymes is hindered). Information for secretion is located at the carboxy-terminal end of the protease (Delepelaire and Wandersman 1989). *out-lacZ* gene fusion experiments have shown that at least some of the *out* genes are constitutively expressed (Ji *et al.* 1987), although other data suggest they are inducible (Diolez *et al.* 1986). Several *out* genes of *Echr* have been cloned and mapped at three different chromosomal loci (Chatterjee *et al.* 1985; Andro *et al.* 1984). When tested on *Sainpaulia* the Out$^-$ mutants appear avirulent (Andro *et al.* 1984), presumably because the major pathogenicity determinants (PLs) do not get to the plant cell wall efficiently.

He *et al.* (1991a) suggested that all of the *out* genes of *Echr* EC16 were on a cluster of 12kb, because this complemented all known Out$^-$ mutants of this strain. Some of the *out* gene cluster of *Echr* EC16 shows a high degree of homology with both the *pul* secretion gene cluster for pullulanase in *Klebsiella pneumoniae* (Pugsley *et al.*

1991), and the *out* gene cluster of *Ecc* SCRI193 (Reeves *et al.* pers. comm.). However the *K.pneumoniae pul* system is unable to export *pelE* of *Echr* EC16 beyond its periplasm, and the *out* system of *Echr* EC16 is unable to export closely related PLs from *Ecc*. The *out* gene cluster of Echr EC16 did however allow the export of *Echr* EC16 PLs from *E.coli* (He *et al.* 1991a) Py *et al.* (1991) showed that the Cel of *Echr* 3937 was not secreted by *Ecc* SCRI193 and similarly the *Ecc* Cel was not secreted by *Echr*. This suggests that within each bacterial species, both the secretory mechanism and the endogenous secreted enzymes co-evolved to gain a species-dependent specificity. Recently He *et al* (1991b), using pulse chasing, gene fusions to *phoA* and the construction of PelE proteins with a hybrid signal peptide, have presented data which supports the idea of a two-step, *sec*-dependent, secretion pathway for PLe.

1.2.2.1.6 Global regulation of extracellular enzymes

A number of mutants have been isolated which possess reduced production and secretion of a number of extracellular enzymes. Hinton *et al.* (1989a) isolated a number of mutants of *Eca* SCRI1043 which possessed both a reduced production and secretion of PL and PG, while retaining wild type levels of Cel and Prt. These mutants, termed Pep$^-$ (*p*ectolytic *e*nzyme *p*roduction), exhibited reduced virulence when tested on potato plants. Two groups have described the isolation of transposon induced mutants of *Ecc* altered in both production and secretion of a number of extracellular enzymes (Pirhonen *et al.* 1991; Salmond pers. comm.). These mutants are said to be altered in global regulation of these enzymes (Salmond pers. comm.). Pirhonen *et al.* (1991) isolated avirulent mutants of *Ecc* SCC3193 which showed a pleiotropic defect in the production and secretion of pectic enzymes, Cel and Prt. These mutants, termed Exp$^-$ (*ex*oenzyme *p*roduction), synthesized reduced levels of pectic enzymes and negligible amounts of Cel and Prt. Work in this laboratory has

led to the isolation of mutants which synthesize and secrete reduced levels of PL, Cel and Prt in *Ecc* SCRI193. These mutants, termed Sex$^-$ (secretion of extracellular enzymes), show similar global regulatory effects to the Exp$^-$ mutants of Pirhonen *et al.* (1991).

1.2.2.2 Role of cell surface components in pathogenicity

1.2.2.2.a Lipopolysaccharide

Schoonejans and coworkers (1987) isolated LPS mutants of *Echr* 3937 by selecting for spontaneous resistance to phages ϕEC2 and Mu, the receptor of which is LPS (Sandulache and Prehm 1985). All mutants obtained were defective in LPS and they all secreted normal amounts of degradative enzymes. On inoculation into *Saintpaulia* plants mutants lacking only the O-antigen appeared to be as virulent as the wild type. Three mutants lacking the O-antigen and part of the core oligosaccharide, however, were avirulent. This data suggests that the core oligosaccharide of *Echr* is necessary for virulence but the O-antigenic side chains are not. Pirhonen *et al.* (1988) isolated LPS defective mutants of *Eca* and *Ecc* by spontaneous aquisition of T4 resistance. These mutants which were affected in O-side chains and core oligosaccharide were found not to affect virulence. This result is in contrast to the findings of Schoonejans *et al.* (1987). A number of these *Eca* and *Ecc* mutants also exhibited reduced protease production. On reversion of one mutant both the level of protease production and the structure of LPS resembled the wild type strain, suggesting the phenotypic changes in the mutant were due to the same mutation. Reversion of another mutant led to wild type protease production but it remained LPS defective (Pirhonen *et al.* 1988). T4-resistance has also been used in conjunction with transposon mutagenesis to select for transposon mutants altered in their cell surface (Pirhonen *et al.* 1991). From a total of 10,000

transposon induced mutants, 30 were resistant to T4. All 30 strains were defective in their LPS structure when analysed by SDS-PAGE. Only 3, however, were avirulent and these 3 strains also showed a galactose-sensitive phenotype similar to that described for *galU* mutations in *Ecc* by Jayaswal *et al.* 1985 (Pirhonen *et al.* 1991). The LPS sugar composition of *Ecc*, *Eca* (DeBoer *et al.* 1985; Sandulache and Prehm 1985) and *Echr* (Bradshaw-Rouse *et al.* 1988) have been partially characterised, and found to be similar in sugar composition to LPS from both *E.coli* and *Salmonella*.

1.2.2.2.b Outer membrane proteins

Expert and Toussaint (1985) isolated a number of reduced virulent mutants of *Echr* 3937 on whole *Saintpaulia* plants that were resistant to bacteriocins and φEC2 but sensitive to Mu. On cut leaves from the same plant however maceration occurred. No differences were seen in the OM protein profile of these mutants when grown in standard growth media, but under iron-limiting conditions one or more of three polypeptides of 88, 82 and 78 kd were absent. The data suggest that the three polypeptides correspond to the receptor for siderophores. On complementation, restoration of iron transport functions restored the virulence of the mutants (Enard *et al.* 1988). The concentration of ferric ions is very low in the plant environment and there may be competition between the host and the pathogen for iron chelation and acquisition (Leong and Neilands 1982). The loss of the O-antigen side chains on LPS in these mutants was found not to play a role in virulence (Schoonejans *et al.* 1987).

1.2.2.2.c Plant inducible genes

Beaulieu and Van Gijsegem (1990) used random phage Mu dIIPR3 insertion mutagenesis of *Echr* 3937 to isolate mutants altered in plant-inducible genes. This approach, in a single step, allowed both the identification of *Echr* promotors induced by plant extract, and the isolation of mutants which could be directly tested on plants. From 10,000 insertion mutants tested, 30 showed induction by plant extract, of which 10 were characterised. 8 of these mutants were affected in virulence on *Saintpaulia* to varying extents but all retained tuber maceration abilities. 2 mutants failed to grow in iron depleted medium suggesting that these mutants were defective in cation uptake, although the cation involved was not identified. One mutant had reduced PLa synthesis. The *pecX* gene involved in positive regulation of this gene (*pelA*) was identified and found to be as wild type. This suggests that the reduction in PLa synthesis was due to miniMu insertion in an unknown positive regulator of *pelA*, induced by a compound present in the plant extract. Another mutant failed to grow on minimal plates containing galacturonate as a sole carbon source, indicating a mutation in the pectolytic pathway. Beside reduced virulence no other phenotypes were assigned to six of the mutants. Similar approaches to this have been used on *X.campestris* (Kamoun and Kado 1990a; Osbourn *et al.* 1987), *Rhizobium* (Redmond *et al.* 1986) and *A.tumefaciens* (Rong *et al.* 1990; Stachel *et al.* 1985).

In the above section the genetic analysis of pathogenicity in the soft rot erwinias has been the main topic, however, a number of other phytopathogens have been studied in detail and consideration of these may extend our knowledge of pathogenicity in the soft rot erwinias.

1.3 Pathogenicity determinants in other bacterial phytopathogens

Most studies on bacterial plant pathogens have been carried out on *Agrobacterium*, *Xanthomonas*, *Pseudomonas* and *Erwinia* strains, as well as the symbiotic *Rhizobium* (which may help in our understanding of more comlex plant/bacterial interactions). Infection processes vary between these organisms, with *Agrobacterium* and *Rhizobium* species exhibiting a complex relationship with their host. In addition to the above genera the Gram-positive organism *Bacillus*, which is not a pathogen, may contribute to our overall understanding of pathogenicity and virulence in the soft rot erwinias, since enzyme regulation in this genus has been studied in detail.

1.3.1 Enzyme production

Production of extracellular enzymes by phytopathogenic bacteria, other than the soft rot erwinias, has been investigated. A chemically induced mutation in the *Xanthomonas campestris* subsp. *campestris* chromosome abolishing pathogenesis had a "global" effect on the production of its extracellular enzymes and EPS (Daniels *et al.* 1984a). The mutation lay in a cluster of "global" regulatory genes which were involved in the coordinate transcription of the enzyme and EPS genes. This mutant was complemented by a plasmid found to contain at least seven genes (genes A-G) involved in this "global" system which positively regulated enzyme and EPS production (Daniels *et al.* 1984b). Mutations in these genes strictly coordinated the effects on both enzyme and EPS production. The deduced protein product from geneC of this cluster showed a strong homology to well known two-component regulatory elements (Fig. A6) (see Reviews Gross *et al.* 1989; Stock *et al.* 1990). GeneG also showed a regulator domain. The function of the other genes in this region has not yet been elucidated.

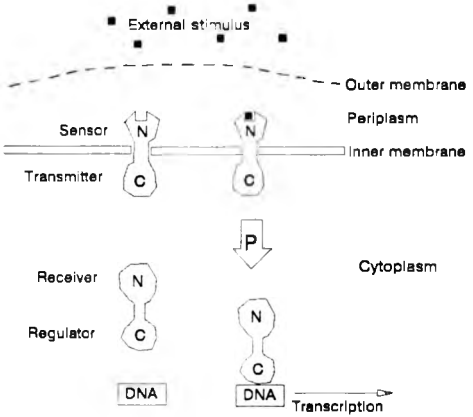


Figure A6. Schematic representation of the interaction between typical sensor and regulator proteins. The cell has a periplasmic sensor with a C-terminal transmitter domain. This transmitter domain is homologous with the N-terminal of a receiver protein with a C-terminal of variable sequence (regulator). The transmitter domain typically contains kinase activity, which phosphorylates the receiver domains thereby modulating the biological process. Adapted from Gross et al. 1989.

The presence of a "balancing" negative regulatory system has also been identified (Tang *et al.* 1990). A mutation in this gene(s) elevated levels of both enzyme and EPS production and wild type genes placed into a wild type strain led to a coordinate depression in levels of the above.

Another two-component regulatory system in *Xanthomonas campestris* subsp. *campestris* has been found which reduces levels of EPS but has no effect on extracellular enzyme production (Daniels *et al.* 1989). It could not however be distinguished from wild type in pathogenicity assays, questioning the role of EPS in pathogenicity.

It can be seen in this Section that a mutation in a single regulatory gene can lead to a "global" regulatory effect on a number of phenotypic properties (Fig. A7). Particularly important is the positive and negative regulatory effects on the whole battery of plant tissue degrading enzymes produced by *Xanthomonas campestris* subsp. *campestris*. The production of EPS is dependent on at least two, two-component systems, suggesting that regulation of this structure could be very complex. Support for the importance of regulation in *X.campestris* comes from the identification of a CAP like protein (CLP) found to have a role in pathogenicity (de Crecy-Lagard *et al.* 1990).

Another regulatory system controlling both secreted and intracellular degradative enzymes in *Bacillus subtilis*, is controlled by a signal transduction pathway defined by at least four regulatory genes: *degS*, *degU*, *degQ* and *degR*. Although this organism is not a plant pathogen its enzyme regulation has been well studied and is therefore worth consideration. The DegS-DegU proteins show amino acid similarities with two-component procaryotic modulator-effector pairs (see Reviews Gross *et al.* 1989; Stock *et al.* 1990). The expression of *degQ* was shown to be subject to both catabolite repression and DegS-DegU-mediated control (Msadek *et*

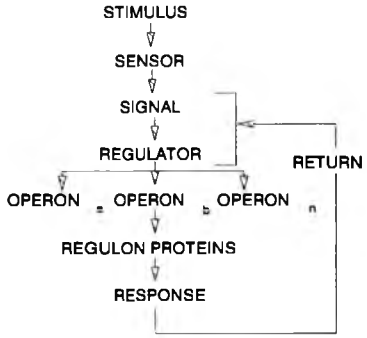


Figure A7. An example of a "global" regulatory process where a single gene can have a regulatory effect on a number of loci. In this example of a regulon the regulator is controlled by both an external stimulus and by feedback from the proteins it produces. From Neidhardt 1989.

al. 1990). Like the pleiotropic phenotypes of the *Xanthomonas campestris* subsp. *campestris* regulatory mutants (above), the Deg mutants were also affected in a number of other determinants including sporulation, transformation and motility. (Ayusawa *et al.* 1975)

1.3.2 Polysaccharide

Gram-negative bacteria produce a range of polysaccharides, polymers of monosaccharide subunits. Lipopolysaccharide (LPS) is a major constituent of the outer membrane and is composed of a lipid portion (Lipid A) which anchors the LPS in the outer membrane, a core oligosaccharide composed of a variety of sugars (including 2-keto-3-deoxyoctanate) and O-antigenic side chain composed of repeating, often branched, sugar units (Fig. A8) (Sutherland 1985). Membrane derived oligosaccharides are low molecular weight polysaccharides composed of glucose units linked by ß1-2 and ß1-6 bonds. They are present in the periplasm of Gram-negative bacteria and are involved in osmoregulation (Miller *et al.* 1986). Finally a number of Gram-negative bacteria produce extracellular polysaccharide (EPS) which may be either acidic or neutral, depending on sugar composition. EPS is often present in pathogenic bacteria and it offers both a method of attachment to host cells and a barrier against host defences (Costerton and Irvin 1981).

1.3.2.a Extracellular polysaccharide

EPS has for many years been implicated in the pathogenicity of phytopathogenic bacteria (Daniel *et al.* 1988). The soft rot erwinias produce very little EPS (Kotourjansky 1987), therefore this substance probably plays little part in the pathogenicity of these species. The effect of EPS in general pathogenicity has,

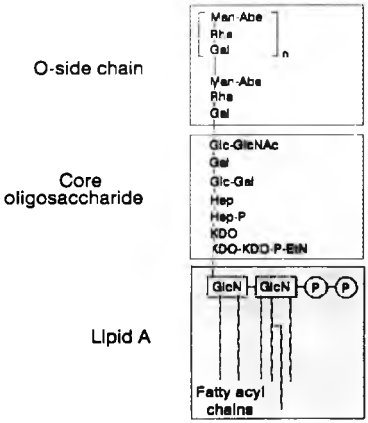


Figure A8. A lipopolysaccharide consists of three regions: Lipid A, core oligosaccharide and O-side chain. The sequence of sugars shown here is from S.typhimurium. Abbreviations used: Abe, abequose; EtN, ethanolamine; Gal, galactose; Glc, glucose; GlcN, glucosamine; GlcNAc, N-acetyl-glucosamine; Hep, heptulose; KDO, 2-keto-3-deoxyoctonate; Man, mannose; Rha, L-rhamnose. Adapted from Freifelder 1983.

however, been studied in detail and may add to our understanding of the biosynthesis and regulation of polysaccharides.

Staskawicz *et al.* (1984), using IS50 mutagenesis of *P.solanacearum*, produced single mutations in the genome which led to a deficiency in EPS (EPS$^-$) production and avirulence. Although this implied that EPS was playing a role in virulence this could have been indirect. Denny *et al.* (1988) described three classes of EPS mutants of *Pseudomonas solanacearum*. One class was reduced in EPS production and had reduced virulence to varying degrees, which correlated with the amount of EPS present. Another class produced by a single mutation was EPS$^-$ and avirulent and was indistinguishable from the "PC" type mutants (see below), and the third class produced nearly wild type amounts of EPS and largely retained virulence. Further analysis has shown that mutants of *P.solanacearum* with reduced EPS production can be complemented to both wild type EPS and virulence and may therefore suggest a role for EPS in virulence (Denny and Baek 1991).

One mutation which reduces EPS production and increases motility in *P.solanacearum* occurs spontaneously and is termed phenotype conversion or "PC" (Boucher *et al.* 1985; Brumbly and Denny 1990). These mutants do not produce wilt symptoms on host plants but the relationship between EPS and virulence is not clear, since these mutants are still able to cause other disease symptoms such as stem necrosis (Denny *et al.* 1988). Recent work by Denny and Baek (1991), studying Tn*5* mutated genes involved in "PC" type mutants, suggests that EPS does play an important role in virulence. Brumbly and Denny (1990) have recently shown by mutagenesis that "PC" is regulated by the *phcA* gene(s), with a functional *phcA* being necessary for maintenance of the wild type phenotype. On conversion to "PC" type, motility is increased. In addition to *P.solanacearum*, phenotype convertion is also seen in *X.campestris*, leading to reduced virulence in host plants (Kamoun and Kado 1990b).

In *E.amylovora* transposon mutagenesis was used to isolate mutants altered in EPS expression (Steinberger and Beer 1988; Bellemann *et al.* 1990). These mutants, which were similar to those isolated by Denny *et al.* (1988), were divided into three classes; those that failed to synthesise EPS and were avirulent on apple seedlings; those with reduced amounts of EPS and showed delayed symptoms; and those that over expressed EPS and were altered in virulence. Cosmid clones able to complement EPS$^-$ mutants and return them to virulence, were able to complement similar mutants in *E.stewartii*, demonstrating common steps in EPS biosynthesis (Coplin and Cook 1990).

The role of EPS in *E.amylovora* pathogenicity is unclear but may mask underlying cell-surface proteins from recognition by plant cell defences and thereby help to avoid a plant defence response (Roberts and Coleman 1990). EPS may also play a role in water retention or even in producing swelling pressure leading to host tissue collapse (Billing 1984; Schouten 1988).

Two other groups of mutants with either reduced or elevated amounts of EPS may define regulatory functions for EPS expression (Roberts and Coleman 1990), involving a protein highly homologous to the RcsA protein in *E.coli* and *K.pneumoniae* (a protein involved in EPS production in these organisms) (Coleman *et al.* 1990; Bernhard *et al.* 1990). Upon infection of plant material EPS expression in *E.amylovora* increases seven fold (Bennet and Billing 1978). Analysis of *rcsA* expression may help explain this phenomenon. The cloned *rcsA* gene is capable of complementing insertion mutations in genes other than *rcsA* and this may indicate the complexity of EPS expression in these bacteria.

Considerable evidence exists for the role of EPS in virulence of *E.stewartii* which causes vascular wilt and leaf blight in corn. Complementation of spontaneous CPS mutants deficient in capsular polysaccharides (cell wall associated EPS) with a

cosmid clone, and transposon mutagenesis of this clone led to the discovery of five linked genes (*cpsA-E*). Mutants defective in these genes lost their ability to cause systemic wilting in corn plants. The CPS$^-$ mutants were still able to multiply and persist in wound inoculated plants however. Apart from *cpsC* and *cpsD* each gene was in a separate operon, all positively regulated by *rcsA* (Coplin and Majerczak 1990). These genes were able to complement *rcs*A mutations in *E.coli* and vice versa (Torres-Cabassa *et al.* 1987).

Another group of mutations involved in EPS production are in the *exo* genes of *R.meliloti*. Mutations in these genes, of which there are 14, reduced or stopped the production of acidic EPS (Leigh and Lee 1988; Reed and Walker 1991; Reuber *et al.* 1991). Strains mutated in *exoB* in addition produced an LPS of altered structure and mutants defective in *exoC* produced an altered LPS, no periplasmic cyclic glucan (Dickstein *et al.* 1988; Leigh and Lee 1988) and were resistant to nine phages (Leigh *et al.* 1985). A mutation in the *exoC* locus is thought to result in a block in the activity of phosphoglucomutase activity, which is involved in the syntheses of UDP-glucose necessary for syntheses of glucan. This block in phosphoglucomutase activity can also account for all the known defects in polysaccharide synthesis in *exoC* mutants (Uttaro *et al.* 1990). *exoB* is thought to be affected in the *galE* gene which could again affect polysaccharide synthesis (Reuber *et al.* 1991). Recent work on the role of the *exoR* gene (a negative regulator of EPS production) in nodulation suggests that an over production, as well as a reduced production of EPS, can lead to a failure of *R.meliloti* to invade alfalfa nodules (Reed *et al.* 1991). Strains mutant in the *exo* genes fail to attach to plant cells of an indeterminant host and induce formation of small white nodules lacking bacteria (Leigh *et al.* 1985, 1987; Leigh and Lee 1988), as opposed to elongated pink nodules containing nitrogen fixing bacteria. On determinant hosts, however, nodulation appears unaffected (Grey and Rolfe 1990; Hotter and Scott 1991). Grey *et al.* (1991) constructed a *Rhizobium* strain deleted in genes involved in EPS

biosynthesis (exo genes) and replaced them with the *exo* genes of another *Rhizobium* species. The results showed that, while the absence of the *exo* genes led to a failure to nodulate the indeterminate host plant, the production of modified acidic EPS was sufficient to nodulate the determinate host.

Mutants affected in the *exo* genes have also been isolated in *A.tumefaciens* (Thomashow *et al.* 1987). In particular mutants defective in the *exo*C gene (also termed *psc*A), homologous to the *exo*C gene of *R.meliloti* (Marks *et al.* 1987), resulted in the inability to synthesize acidic EPS and ß-(1-2)glucan and blocked motility (Cangelosi *et al.* 1987). Since these mutants were non-motile a relationship between motility and ß-(1-2) glucan production has been shown to exist (Uttaro *et al.* 1990).

In contrast to the role of EPS in pathogenicity Xu *et al.* (1990) found mutants of *P.solanacearum* deficient in EPS but highly virulent. Boucher *et al.* (1985) has also reported that EPS$^-$ mutants of this strain remained virulent. Denny and Baek (1991) however suggest that these findings may be because the mutants were poorly characterized.

The role of extracellular polysaccharide (EPS) in virulence may work in association with LPS. Potato tuber agglutinin (which on binding causes invading cells to agglutinate and so hampers their invasion) was shown to bind poorly to virulent strains of *P.solanacearum* but avirulent strains had a much higher binding efficiency (Duvick and Sequeira 1984). EPS produced by the virulent strain inhibited binding of agglutinin to LPS. Evidence for electrostatic interactions was given when anionic polymers such as DNA, dextran sulphate and xanthan, which mimic EPS, also prevented agglutinin binding to LPS whereas an uncharged polymer, dextran, did not (Duvick and Sequeira 1984). Similar findings have been noted elsewhere

(Drigues *et al.* 1985).

1.3.2.b Membrane derived oligosaccharides (ß-(1-2) glucan)

In addition to EPS, bacteria also produce a polysaccharide resident in the periplasm which osmoregulates the cell in varying environments. This function in *E.coli* is performed by the membrane derived oligosaccharides (MDO) (Fiedler and Rotering 1988) and in *R.meliloti* and *A.tumefaciens* by ß-(1-2) glucan (Miller *et al.* 1986).

The *ndvA* and *ndvB* genes of *R.meliloti* were first discovered in 1986 by Dylan *et al.* and are thought to be required for virulence. The *ndvB* mutants are defective in the production of ß-(1-2) glucan due to the loss of a large inner membrane protein (Ielpi *et al.* 1990) involved in the synthesis of the glucan, and the *ndvA* mutants are defective in the export of glucan, both to the periplasm and extracellularly (Stanfield *et al.* 1988). Nodules produced by such mutants appeared similar to those of the *exo* mutants (Leigh *et al.* 1985, 1987) except that in the *ndv* mutants EPS production was unaltered (Geremia *et al.* 1987; Ielpi *et al.* 1990). In these mutants an inability to attach to the plant cells was also observed (Douglas *et al.* 1985; Dylan *et al.* 1986). As well as the above phenotypes these mutants exhibit a number of vegetative alterations including a decrease in motility (flagella$^-$) and phage sensitivity, an increase in sensitivity to hydrophobic antibiotics and low osmolarity. These phenotypes suggest a disturbance of the outer cell surface probably due to a failure to osmoregulate (Dylan *et al.* 1990a).

The *ndv* genes of *R.meliloti* are homologous to a pair of genes in *A.tumefaciens*, *chvA/B* loci respectively. As in the previous example these genes have been implicated in virulence and attachment of this organism to plant cells (Cangelosi *et*

al. 1990; Dylan *et al.* 1986; Inon de Iannino and Ugalde 1989). Like the *ndv* loci they fail to produce ß-(1-2) glucan (Puvanesarajah *et al.* 1985) and are pleiotropic as described above (Douglas *et al.* 1982). As with *ndv* mutants, an increased sensitivity of the cell to hydrophobic antibiotics indicates an alteration in cell surface structure.

The pleiotropic phenotype of the *chvB* mutants suggests that this gene(s) may code for positive regulators, which may in turn control the expression of a set of genes required for infection, amoung which ß-(1-2) glucan may play a role (Zorreguita and Ugalde 1986). In an attempt to locate the *vir* locus of *R.meliloti* revertants for osmoregulation, motility and symbiosis defects have been isolated and studied (Dylan *et al.* 1990b). Pseudorevertants (second site mutations) for symbiosis returned to virulence but not motility or any other vegetative functions, suggesting that these other phenotypes were not involved in virulence. This revertant did not regain its ability to produce or export ß-(1-2) glucan and even on the addition of this polysaccharide, to enhance the *ndv* mutant/plant interaction, no alteration in the phenotype was seen. Pseudorevertants for osmoregulation defects were only possible on the loss of the insertion showing an absolute dependence on the *ndv* genes. Therefore none of the above phenotypes can be correlated directly with the loss of virulence.

1.3.2.c Lipopolysaccharide

In addition to the role of LPS in the virulence of *Echr* (Schoonejans *et al.* 1987) reduced nodulation mutations of *Rhizobium* (Brink *et al.* 1990; Brzoska and Signer 1991; Cava *et al.* 1990; De Maagd *et al.* 1989; Noel *et al.* 1986) and *Bradyrhizobium* species (Puvanesarajah *et al.* 1987), and reduced virulence mutants of *Agrobacterium* (Metts *et al.* 1991) and *Pseudomonas* (Duvick and Sequeira 1984;

Hendrick and Sequeira 1984; Zamza *et al.* 1985) species have been linked to alterations in LPS structure. The loss of O-antigenic side chains lead to a lessening of the virulence of the organism and the extent to which the side chains and core LPS are lacking has been shown to relate to the severity of the mutation (Zamza *et al.* 1985). Three types of mutant are commonly found, those which contain lipid-A, core sugars and O-side chains as normal LPS but with an altered O-side chain structure (LPS III), those which contain lipid-A, core and a reduced amount of side chain (LPS II) and those which contain a lipid-A and core (often incomplete) only (LPS I) (Cava *et al.* 1990; De Maagd *et al.* 1989; Hendrick and Sequeira 1984; Zamza *et al.* 1985). Class I mutants usually have the most severe reduction in virulence which has been related to a loss of important sugars involved in bacteria/plant interaction (De Maagd *et al.* 1989; Hendrick and Sequeira 1984) and to changes in electrokinetic potential of the cell surface (Duvick and Sequeira 1984). The latter is possibly due to the loss of the O-antigen part of the LPS which shields the negative charges of the outer membrane (Carlson 1984). The role of electrokinetic potential (electrostatic charge-charge interactions) between plant and bacterial cells in virulence is supported by several workers (De Maagd *et al.* 1989; Bradley *et al.* 1986). Class III mutants are of interest because, although they contain the normal level of O-side chains, their sugar composition is often different from that of the wild type strain (De Maagd *et al.* 1989; Zamza *et al.* 1985). While DeMaagd *et al.* (1989) found that changes in the sugar composition of smooth LPS in *Rhizobium* did not result in loss of virulence, Zamza *et al.* (1985) found that loss of virulence in *Pseudomonas* occurred on loss or alteration of the side chains. In comparison, work by Schoonejans *et al.* (1987) in *Echr* suggested that loss of virulence only occurred with alterations in the core LPS.

As seen previously (see Sections 1.2.bii, 1.3.2.a, 1.3.2.b), single mutations can lead to pleiotropic phenotypes. This is also the case with mutants affected in LPS. A reduced growth rate, phage resistance, reduced motility, increased sensitivity to

hydrophobic compounds, altered hydrophobicity and surface electrokinetic potential and alterations in outer membrane protein profiles are often seen without alteration in EPS production (De Maagd *et al.* 1989; Hendrick and Sequeira 1984; Zamza *et al.* 1985). LPS mutants often show other phenotypic alterations but, even when another phenotype has not been noted, most workers are reluctant to rule out the possibility that they could be present but undetected. Motility, as previously found, does not, in these mutants, appear to play a direct role in virulence (De Maagd *et al.* 1989). The involvement of alterations in outer membrane protein levels and increased sensitivity to hydrophobic compounds, in bacterial/host interactions has not been ruled out, but more predominant alterations in LPS seem the more likely cause of reduced virulence.

LPS is a major cell surface molecule and is likely to play a role in early bacterial/plant interactions. Goosen-de Roo *et al.* (1991) have shown that after initial infection of roots by *R.leguminisarum*, LPS appears to be actively degraded. The importance of the core oligosaccharide in these interactions is substantiated by the strong correlation between alteration of this structure and loss of virulence (Brink *et al.* 1990; Hendrick and Sequeira 1984; Schoonejans *et al.* 1987), although *Ecc* mutants altered in core LPS unaffected in virulence have been found (Pirhonen *et al.* 1988). It is also clear that substantial alterations in the composition of intact O-side chains can also result in loss of virulence (Cava *et al.* 1990; De Maagd *et al* 1989; Noel *et al.* 1986; Puvanesarajah *et al.* 1987) . Although many mutants defective in LPS possess a pleiotropic phenotype, little evidence has been given to define the exact role of LPS in virulence. Possible roles of LPS in bacterial/plant interactions include [i] a signal molecule, possibly involved in a certain stage of disease development , [ii] a protection barrier against host defences or [iii] a structure involved in suppression of host defence mechanisms (Brink *et al.* 1990; Schoonejans *et al.* 1987).

1.3.3 Motility

Alterations in polysaccharide synthesis are often accompanied by an alteration in motility and/or chemotaxis (see Section 1.3.2), refered to simply as motility in this report. This suggests that coordinate regulatory systems may affect a number of phenotypes. Since one or more of a number of phenotypes could be involved in virulence the role of motility is not easy to assess. Motility in mutants affected in LPS structure, however, is not thought to play a role in pathogenicity (De Maagd *et al.* 1989). Analysis of pseudorevertants of motility mutants of *R.meliloti* affected in the synthesis of ß-(1-2) glucan has indicated that motility, while it may play some part in cell attachment, is not wholly responsible for the loss of nodulation of the mutants, since on plant inoculation of the pseudorevertants only 10% of this population showed symbiotic capabilities (Dylan *et al.* 1990b). The non-motile nature of the *chv* and *ndv* mutants has been shown to be due to a total lack or a reduction in the amount of flagella on the cell surface (Dylan *et al.* 1990a; Geremia *et al.* 1987; O'Connell and Handelsman 1989).

In *P.solanacearum* a regulatory gene, *phc*A, is involved in maintenance of a wild type phenotype (Brumley and Denny 1990). Mutation of this gene leads to an increase in motility and a reduction in EPS production. A similar mutation has also been found in *Xanthomonas campestris* subsp. *campestris* (Kamoun and Kado 1990b). Such mutants did not revert *ex planta*. *In planta* however 10 out of 12 mutants did revert to cause disease in radish plants. These cells had reverted back to EPS production and where non-motile. These results suggest that some plant signals may be involved in the ability to revert to the parental type strain. They also suggest that in the presence of a readily available carbon source the chemotactic ability of the cell may be advantageous and EPS production is not. *In planta* when no carbon source is readily available it may be an advantage for the cell to produce EPS,

which in some way helps in its pathogenic ability. This also suggests that motility is not necessary for this pathogenic process.

The role of motility in virulence of *A.tumefaciens* was investigated by Bradley *et al.* (1984) using mutants resistant to the flagella specific phages GS2 and GS6. From their work they concluded that motility was not required for tumour inducing ability in its host. This work follows a study carried out previously where insertion mutants resistant to these phages were thought to have an altered LPS structure which led to avirulence (Douglas *et al.* 1982). These studies suggest that another phenotype so far unrecognised must be involved in the lack of virulence. Bayot and Ries (1986) working on *E.amylovora* and Panopoulos and Stroth 1974 working on *P.phaseolicola* examined, specifically, the role of motility in pathogenicity of these strains. In both cases non-motile mutants were found to be as virulent as the wild type when tested *in planta*.

From the above information it would appear that motility plays only a small part in the virulence of phytopathogenic bacteria. This role may be on initial infection, eg. entry into wound sites or lenticels (Bayot and Ries 1986; Hattermann and Ries 1989; Panopoulas and Stroth 1974). Insertion mutants of *Eca* SCRI1043 exhibiting reduced virulence have also been isolated. Mutants deficient in enzyme production, growth rate, as well as a number of auxotrophies together with mutants possessing unknown phenotypic changes have been isolated (Hinton *et al.* (1989a). A number of the latter mutants have recently been found to possess reduced motility, although the relationship between motility and virulence has not been proved (Mulholland pers. comm.). Pirhonen *et al.* 1991 isolated a number of transposon induced mutants exhibiting reduced virulence. These mutants were divided into two groups; i) altered enzyme production, ii) defective in motility. To date no other phenotypes have been associated with the reduced virulence of these motility defective mutants, suggesting that they may have a role in bacterial spread within the plant tissue

(Pirhonen et al. 1991).

An example of the complex interaction of genes involved in motility include mutants in *fla* (unable to assemble flagella filamants and with decreased sensitivity to two swarm-specific phages), *mot* (able to assemble flagella filaments but are non-motile and fully sensitive to the phages) and *ple* (non-motile and resistant to the above phages) genes of *Caulobacter crescentus* (Ely *et al.* 1984; Johnson and Ely 1979). Three multigene clusters have been detected and many other sites around the chromosome including 27 *fla* genes, 3 *mot* genes and 3 *ple* genes all affecting motility have been found. The role of the *ple* genes in the regulation of motility includes the activation of flagellum formation and rotation (*ple*A), and the switching off of flagellum rotation (*ple*D) (Sommer and Newton 1989).

1.3.4 Outer membrane proteins

Compared to other cell wall constituents little work has been carried out to study the role of outer membrane proteins on pathogenicity. Outer membrane (OM) proteins may be involved in surface interactions during initial infection of a plant or as a sensor protein in a two-component regulatory system (Gross *et al.* 1989; Stock *et al.* 1990). Another possibility is in the involvement of iron utilization (see Section 1.2.2.2.b). Alterations in outer membrane protein profiles have been found in LPS defective mutants (DeMaagd *et al.* 1989; Metts *et al.* 1991; Noel *et al.* 1986), although no data on their role in pathogenicity is available.

1.3.5 Host specificity and the gene-for-gene hypothesis

All plants are "nonhosts" for the majority of phytopathogens. Basic resistance of a

plant species to a phytopathogenic species is a consequence of general plant defence mechanisms, for example phytoalexin production. Basic compatibility of a pathogen arises by the evolution of pathogenicity factors (encoded by *hrp* genes) necessary to overcome the basic resistance of the plant. Superimposed on the basic resistance of a plant species is cultivar resistance which may be controlled by single plant genes that are matched by single avirulence (*avr*) genes in the bacterial parasite, resulting in a gene-for-gene interaction (Heath 1991). The most likely explanation for this hypothesis is that the avirulence gene within the bacteria controls the production of an elicitor, the recognition of which is controlled by the gene for resistance in the plant. This elicitor may or may not be connected with virulence. If either dominant allele is absent from the genotypes of the interacting organisms, the defence response is not activated, and the plant is therefore susceptible to pathogen attack.

Genetic analysis including physical, chemical and transposon insertion mutagenesis, has been used to identify genes involved in disease development, termed disease specific genes (*dsp*), encompassing factors such as the production of pectolytic enzyme and polysaccharides (see previous Sections). In addition to the *dsp* genes, genetic analysis has begun to identify both hypersensitivity and pathogenicity (*hrp*) genes and avirulence (*avr*) genes. These three classes of genes have been defined as; a) *dsp* genes are involved in disease development in host plants but not with induction of the HR on non-host plants (Boucher *et al.* 1987), b) *hrp* genes are required for both initiation of disease symptoms in host plants and the development of the hypersensitivity response (HR) on non-host plants (Lingren *et al.* 1986), c) *avr* genes are involved in the control of race-cultivar specificity (Staskawicz *et al.* 1984).

Staskawicz *et al.* (1984) first cloned a gene for avirulence from *Pseudomonas syringae* subsp. *glycinea* race 6, the causal agent of bacterial blight in soybean. This gene, termed *avrA* defines the *P.syringae* subsp. *glycinea* race 6 phenotype and

restricts the number of soybean cultivars that the pathogen can attack. Additional *avr* genes have been identified in other *P.syringae* subsp. *glycinea* races (Staskawicz *et al.* 1987; Tamaki *et al.* 1991) in *P.syringae* subsp. *tomato* (Kobayashi *et al.* 1989), *P.solancearum* (Carney and Denny 1990) as well as *X.campestris* (Gabriel *et al.* 1986 ; Minsavage *et al.* 1990; Swanson *et al.* 1988).

hrp genes in phytopathogenic bacteria have been extensively studies over the last 5 years (Willis *et al.* 1991). A cluster of *hrp* genes in *P.syringae* subsp. *phaseolicola* has been identified (Lingren *et al.* 1986; Mukhopadhyay *et al.* 1988). The precise role of most *hrp* gene products in plant-bacterium interactions are unknown, but at least two *hrp* genes have been analysed in detail. *hrpS* encodes a protein that shares sequence homology to NtrC-like regulatory proteins (Grimm and Panopoulos 1989) and *hrpD* is actively expressed at early stages of infection (Lingren *et al.* 1988) and requires the expression of a functional *hrpS* gene (Grimm and Panopoulos 1989). Besides *P.syringae* subsp. *phaseolicola*, *hrp* genes and/or gene clusters have been found in other *P.syringae* subspecies including *P.syringae* subsp.*tomato* (Cuppels 1986), *P.syringae*.subsp. *syringae* (Huang *et al.* 1988; Niepold *et al.* 1985; Salch and Shaw 1988), as well as *P.solanacearum* (Boucher *et al.* 1987), *X.campestris* (Boucher *et al.* 1987; Daniels *et al.* 1988; Stahl and Minsavage 1990) and *E.amylovora* (Steinberger and Beer 1988; Vanneste *et al.* 1990).

To date no *hrp* or *avr* genes have been identified in the soft rot erwinias. This suggests that co-evolution of the host and pathogen leading to the development of specific mutual recognition mechanisms has not occurred in these organisms and plant-*Erwinia* interactions may, therefore, still be relatively primitive (Keen and Holliday 1982).

In the preceeding sections the pathogenesis of erwinias and other phytopathogenic bacteria (including symbionts) has been discussed. In many examples of

pathogenicity in these organisms pleiotropic phenotypes have been observed, many of which include phage resistance. Phage resistance as an initial screen for reduced virulence is therefore, a useful method of identifying such mutants. The use of phages to identify reduced virulence mutants in *Erwinia* is a significant part of this thesis (see Aims - Section 1.6) and as such a number of phages have been isolated and characterised. In the following sections details on phage isolation, characterisation and uses will be given, with particular emphesis on points relating to aspects of this thesis.

1.4 Isolation, characterisation and uses of bacteriophages

1.4.1 What is a bacteriophage?

Bacteriophages, or simply phages, are viruses that infect bacteria. They are not free living organisms but depend on bacterial hosts for multiplication and sometimes for prolonged accommodation during the lysogenic stage of some phage life cycles. The first report of a phage was in 1915 when Twort discovered that lysis could be transferred between bacterial colonies. He termed this "transmissible lysis". A similar event was rediscovered in 1917 by d'Herelle. In the 1920's and 1930's d'Herelle found many examples of this phenomenon, with each phage having a limited range of host bacteria. Phages were visible by light microscopy only as specs of light, and their size was determined initially by the use of filters of graded pore size. It was not until the 1940's with the development of the electron microscope that their structures could be clearly seen and the controversy as to whether they were chemicals or organisms ended. Since the 1940's workers have concentrated on certain phage groups, in particular the T-phages, which has vastly increased our knowledge of these particles. Through the 1960's to the present day phages have been used extensively to study molecular genetic mechanisms (Douglas

1975).

1.4.2 Isolation of bacteriophages and their uses in biological investigation

Phages have been isolated from a variety of sources. Phages of phytopathogenic bacteria (the main topic of this thesis) have been isolated from river water (Seely and Primrose 1980), irrigation water (Zeitoun and Wilson 1969), diseased plant material (Billing 1963; Gross *et al.* 1991; Richie and Klos 1977, 1979; Suzuki and Togashi 1978; Trautwetter and Blanco 1988;; Zeitoun and Wilson 1969), sewage (Bigby and Kropinski 1989; Nordeen *et al.* 1983; Thomas and Leary 1983), soil (Baigent *et al.* 1963; Erskine 1973; Suzuki and Togashi 1978), lysogenic bacteria (Baigent *et al.* 1963; Chatterjee and Brown 1981; Harrison and Gibbons 1975; Okabe and Goto 1963; Resibois *et al.* 1984) and a corn beetle (Woods *et al.* 1981).

Phages, once isolated, can be useful tools in the study of the biology of the host. The study of the biological, structural and physicochemical properties of these phages is of taxonomic importance and in the case of *Erwinia carotovora* subsp. *atroseptica* (*Eca*) this is especially so, since little work on such phages has previously been carried out. Another important use of phages is that of strain identification for taxonomic differentiation of the bacterial host ie. phage typing. This technique has been used for a wide range of bacterial species including phytopathogenic bacteria (Section 1.4.5.1). The use of phages to isolate or identify mutants has been exploited in both the search for virulence determinants of plant pathogens (Section 1.2.bii) and for other genetic studies, eg. use as DNA delivery vectors and cloning vehicles (see later). Their role in the search for virulence determinants will be discussed later in the chapter. Phage enzymes can also be used to our advantage, for example the use of phage lysozyme which may be more

specific to the cell wall of its host than that of a commercial lysozyme, or the use of phage polysaccharide depolymerase to degrade bacterial polysaccharides important in pathogenesis (Hartung *et al.* 1988). The use of phages has for a long time been recognised as a potential biological control agent in an attempt to reduce plant diseases (Okabe and Goto 1963; Vidaver 1976), although to date no significant breakthrough has been achieved. An attempt to control fire-blight caused by *E.amylovora* has shown some degree of success however (Erskine 1973). Another potential use of phages is in estimating the number of bacteria in samples, eg. the estimation of the soft rot bacterium *Erwinia aroideae* in soil (Suzuki and Togashi 1978). Other phage uses include monitoring disease dissemination and epidemiology, including seed infection and overwintering (the survival of bacteria during winter periods) and forecasting disease development (Okabe and Goto 1963). Perhaps their major role as a biological tool is their use in genetic manipulation. The development of phage based DNA transfer systems eg. generalised and specialised transducers (Section 1.5), their use as delivery vectors to allow the *in vitro* penetration of manipulated DNA into cells, eg. the use of Lambda (Salmond *et al.* 1986) and P1 (Quinto and Bender 1984) as vectors for Tn*5* insertion mutagenesis, their use in the study and maintenance of heterologous transformed DNA eg. isolation of restriction-defective mutants (Salmond pers. comm.) and their use in the construction of cloning vectors, eg. the filamentous phages F1 and M13 (Geider 1986).

1.4.3 Bacteriophage taxonomy

In 1981 over 2,000 phages had been isolated (Matthews 1982). Attempts to characterise these phages for taxonomic purposes has been a difficult task due, mainly, to difficulties in selecting, implementing and standardising characterisation methods (Ackermann *et al.* 1978). The ease with which phages have been isolated

meant that in around 1978 about 150 new phages were being described annually, the number in 1982 dropping to about 100 per annum. Although no figures are available for 1991 the number of phages described annually over the past 10 years has probably dropped significantly. There is still an interest in their characterisation however.

1.4.3.1 Methods of phage characterisation

In an attempt to standardise characterisation methods the International Committee on Taxonomy of Viruses (ICTV), established in 1950 (Matthews 1982) proposed a number of criteria for the characterisation of newly described viruses (including phages). Such methods have been described by Ackermann *et al.* (1978) and are based on properties of the virion and its nucleic acid. Possible criteria for the classification of phages include; a) morphology and dimensions of the virion, b) particle weight, sedimentation velocity, and buoyant density, c) type, molecular weight, relative percentage, conformation and composition of the phage nucleic acid, d) serological properties and nucleic acid hybridisation data, and e) physiological properties (including plaque size, latent period, burst size and sensitivity to physical and chemical agents) and host range tests. Recently new methods of characterisation have been employed, including gel electrophoresis of proteins (Jarvis *et al.* 1991) and nucleic acids, the latter involving restriction endonuclease (RE) digestion (Jarvis *et al.* 1991; Nordeen *et al.* 1983; Studier 1979). It has been suggested by Jarvis *et al.* (1991) that RE digestion of DNA is not useful in classifying phages into species, but provides information about relatedness between particular phages. Perhaps the most important of classification methods is analysis of phage morphology, since it is easy to perform by electron microscopy and allows instant categorisation of phages into morphological groups. Extensive

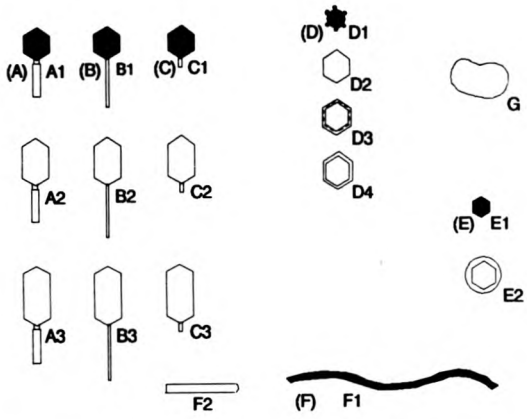


Figure A9 Morphological classification of bacteriophages. Figures in brackets represent Bradleys classification (1967) and figures without brackets represent Ackermann and Eisenstark classification (Reanney and Ackermann 1982).

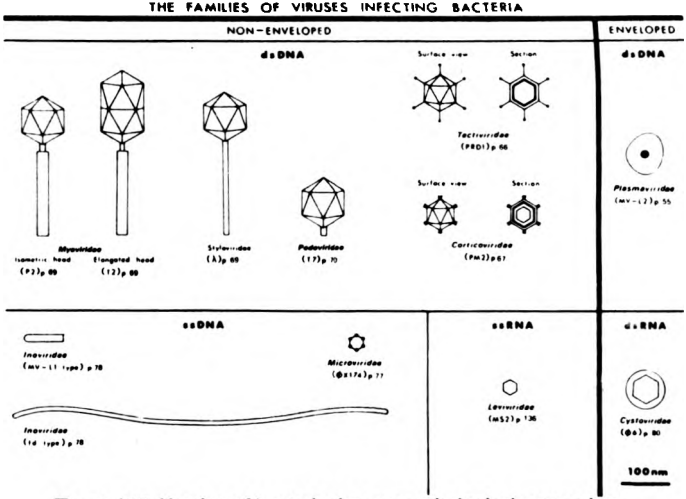


Figure A10. Naming of bacteriophage morphological groups into families (Matthews, International Committee on Taxonomy of Viruses 1982).

work by Bradley (1967) arranged phages into 6 basic morphological types, although this number has now been increased to 18 (Ackermann and Eisenstark 1974; Reanney and Ackermann 1982) (Fig. A9). On the basis of these morphological features the ICTV have placed phages into 10 families (Fig. A10), in some cases containing over 1000 members (Siphoviridae) and in others just a single member (Cystoviridae) (Matthews 1982). Other important criteria for phage characterisation are serology, DNA-DNA hybridisation (both of which correlate well to morphological group), host range and electrophoresis of proteins and DNA, the latter restricted with restriction endonucleases. RE digestion has been used to successfully compare a number of phages, including those of *P.syringe* (Nordeen *et al.* 1983) and *Lactococcus* (Jarvis *et al.* 1991). Physiological properties (such as burst size and latent period) used extensively during the early days of phage characterisation, are now considered to be of little use.

1.4.3.2 Phage morphology

1.4.3.2.a Tailed phages

Tailed phages are the most common type of phages, occurring in over 90 genera of bacteria (Reanney and Ackermann 1982). All known tailed phages contain linear, duplex DNA. Morphological features unique to members of this group include the possession of contractile and noncontractile tails, and various head and tail appendages including collars, base plates, tail fibres and spikes (Fig. A11). Tailed phages are subdivided into 3 basic groups according to tail structure (Bradley 1967): Group A, *Myoviridae*, possessing contractile tails; Group B, *Styloviridae* possessing long noncontractile tails; and Group C, *Podoviridae* possessing short tails. Of the tailed phages described by Reanney and Ackermann (1982) those with isometric heads are most common (A1, B1 and C1) representing over 80% of the tailed

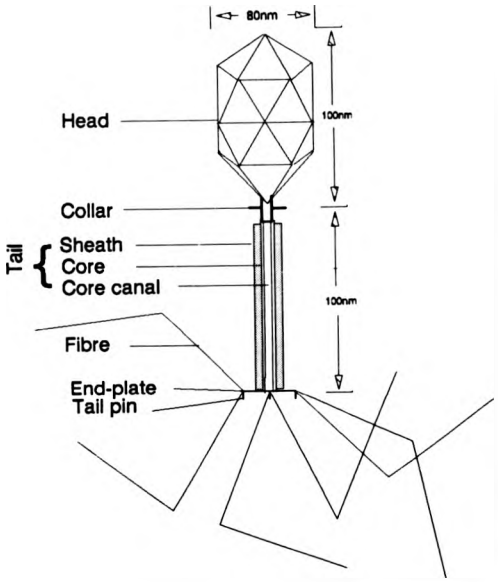


Fig A11
Structure of phage T2 adapted from Hayes (1974).

phages. Phages of B1 morphotype seem especially prevalent and are found in a diverse range of host genera (Reanney and Ackermann 1982). Phages of the family *Myoviridae* possess long contractile tails consisting of a central tube and a contractile sheath separated from the head by a neck. These phages belong to the T-even phage group, the type phage being Coliphage T2. The type phage possesses linear duplex DNA, which is circularly permuted and terminally redundant. T2 is chloroform resistant, adsorbs to the cell wall of enterobacteria and undergoes virulent infection. Phages of the family *Styloviridae* possess long noncontractile tails. They belong to the Lambda phage group, the type phage being Coliphage Lambda. The type phage possesses linear duplex DNA which has cohesive ends. Lambda is chloroform resistant, adsorbs to the cell wall of enterobacteria and undergoes temperate infection. Phages of the family *Podoviridae* possess short noncontractile tails. They belong to the T7 phage group, the type phage being Coliphage T7. The type phage possesses linear duplex DNA and is non-permuted and terminally redundant. T7 is chloroform resistant, adsorbs to the cell wall of female enterobacteria and undergoes virulent infection. In other members of these phage families host ranges include *Agrobacterium*, *Bacillus*, *Clostridium*, *Mycobacterium*, *Pseudomonas*, *Rhizobium*, *Staphylococcus*, *Streptococcus*, *Streptomyces* and *Xanthomonas* (Matthews 1982).

1.4.3.2.b Cubic, filamentous and pleomorphic phages

The remaining phage groups are made up of cubic, filamentous and pleomorphic phages. These taxa are distinctive and are not related to each other in any obvious way. Unlike the tailed phages these groups of viruses are relatively rare and their host ranges are confined to a small group of host genera (Matthews 1982).

Cubic DNA phages: a) Group D1, *Microviridae* (eg. ϕX174) contain circular

ssDNA and adsorb to the cell wall of enterobacteria only; b) Group D2 contains poorly known phages of large size (60 nm); c) Group D3, *Corticoviridae* (eg. PM2) contains circular supercoiled dsDNA, has a 12-14% lipid content making it chloroform sensitive and adsorbs to the cell wall of a single *Pseudomonas* species; d) Group D4, *Tectiviridae* (eg. PRD1) contains linear dsDNA, has a 10-20% lipid content making it chloroform sensitive and adsorbs to the tips of plasmid encoded pili of Gram-negative bacteria harbouring certain drug-resistant plasmids including enterobacteria, *Acinetobacter*, *Pseudomonas*, *Vibrio* and *Bacillus*.

Cubic RNA phages: a) Group E1, *Leviviridae* (eg. MS2) contains linear positive-sense ssRNA, is chloroform resistant and adsorbs to the sides of male bacteria belonging to enterobacteria, *Caulobacter* and *Pseudomonas* species; b) Group E2, *Cystoviridae* (eg. ϕ6, the only member), contains 3 pieces of linear dsRNA, has a lipid content of about 20% making it chloroform sensitive and adsorbs to the sides of pili of *Pseudomonas* species.

Filamentous phages, *Inoviridae*: a) Group F1, genus *Inovirus* (eg. fd), contains circular ssDNA, and adsorbs to the pili or poles of mainly male enterobacteria, *Pseudomonas*, *Vibrio* and *Xanthomonas* species; b) Group F2, genus *Plectrovirus* (eg. MV-L1), contains circular ssDNA, and infects *Acholeplasma*.

Pleomorphic phages: Group G, *Plasmaviridae* (eg. MV-L2), contains circular supercoiled dsDNA, contains lipid in the envelope making it chloroform sensitive, has a rounded pleomorphic appearance and infects *Acholeplasma*.

1.4.3.3 Phage host range

Phages are usually genus specific with important exceptions being a) phages of

enterobacteria where polyvalence is relatively common, for example the temperate coliphage P1 can be accepted by species of *Erwinia*, *Proteus*, *Pseudomonas* and *Serratia* (Murooka and Harada 1979), b) Actinophages which sometimes cross genus boundaries and c) phages specific for bacteria harbouring certain drug-resistant plasmids (Ackermann *et al.* 1978). Polyvalence is so common in phages of enterobacterial species (eg. *E.coli*, *Erwinia*, *Salmonella*, *Shigella*, *Serratia*, *Klebsiella*, *Proteus*) that these phages are placed into a single host group (Reanney and Ackermann 1982). This may also be true for phages which adsorb to appendages such as flagella or sex pili where reversible binding may take place before nonreversible binding to a more specific receptor (Lindberg 1973). The host range of a phage depends on its ability to adsorb to receptors on the bacterial host, usually present in only a limited number of bacterial strains. A receptor may be a protein, a lipopolysaccharide, a teichoic acid, the peptidoglycan or an exopolysaccharide (Lindberg 1973). It is important to note that a phage sensitive strain of bacteria may become resistant by a single mutational step or may become immune by becoming lysogenic.

1.4.4 Phylogeny (Evolutionary history)

Controversy still exists as to the question of phage origins. It has been suggested that bacteriophages may have evolved from the bacterial chromosome as a means of genetic transfer (Bradley 1967), or that a primitive phage was a segment of bacterial chromosome producing a protein that could encapsulate DNA joined to the origin of bacterial chromosomal replication (Zinder 1980). Bradley (1967) has proposed that filamentous phages originated from F-plasmid coded pili and a final example that the genome of phage P1 may have originated from plasmid genes (Abeles *et al.* 1984). Reanney and Ackermann (1982) suggest that bacterial genes could have become components of the genetic information of the tailed phages, a process which

may have arisen before the division of bacteria into Gram-negatives and Gram-positives at least 2 x 10^9 years ago. Genes for cubic, filamentous and pleomorphic phages possibly arose much more recently.

Without the use of molecular techniques studying the evolution of phages was, in the past, almost impossible. More recent techniques in molecular biology, such as heteroduplex analysis and amino acid sequence homologies, are now beginning to uncover molecular relationships between phages (Highton *et al.* 1990), and between phages and their bacterial host(s), eg. the repressor and *cro* genes of Lambda are related to the *lexA* gene of *E.coli* (Sauer *et al.* 1982; Walker 1984). While little attention has been given to this in taxonomy, because of the difficulties in its study, it could potentially expand both the knowledge and interest of this group of organisms.

Phages have mechanisms allowing them to adapt to changing environments. These include single nucleotide changes, frame shift mutations, gene duplication, reassortment of segmented genomes and genetic exchanges with hosts. A popular theory, with strong supporting data, to explain the large number of different morphologies of the tailed phages is the rearrangement of functionally and genetically interchangeable sets of ancestral genes called "modules" (Botstein and Herskowitz 1974). This theory is supported by more recent data using heteroduplex analysis (Highton *et al.* 1990). These modules are flanked on either side by common base sequences, allowing double recombination events to take place within the phage genome. A scheme describing such relationships has been presented (Reanney and Ackermann 1982). Functionally compatible modules which are very different in their nature are flanked by homologous regions, allowing recombination into the phage genome. One method of phage evolution therefore, probably involves the swapping of modules to obtain "favourable" combinations, compatible with the maximum number of combinations of other modules. New modules may arise by

the genetic events listed above, leading to new viable variants or to varying degrees of defective bacteriocin-like particles (a bacteriocin is a nonreplicating, proteinaceous antibiotic generally restricted to closely related bacterial strains or species. High molecular weight bacteriocins appear as phage-like particles under electron microscopy, and are considered to be defective or incomplete phage particles) (Douglas 1975).

1.4.5 Phages of phytopathogenic bacteria

Phages have been isolated on a number of phytopathogenic bacteria (Okabe and Goto 1963; Reanney and Ackermann 1982). A great deal of characterisation has been carried out on *Pseudomonas* phages, and to a lesser extent *Xanthomonas* phages. The tailed phages of these species are summarized by Liss *et al.* (1981) and other phages isolated since 1981 are described by Cuppels (1984), Jindal and Patel (1981), Nordeen *et al.* (1983) and Thomas and Leary (1983). Phages isolated on *Erwinia* species are listed in Table A3. In all cases, where specified, phages are tailed and are members of groups A, B and C of the Bradley classification system (Section 1.4.3.2.a). The phages of *Erwinia* species appear to be very species specific. Only in the work of Richie and Klos (1979) and Woods *et al.* (1981) does a phage appear to infect more than one species, although infection of different strains within a species is common (all examples in Table A3). In the case of a single phage isolated by Gross *et al.* (1991) infection occurred in both *Ecc* and *Eca*. The majority of samples were isolated from bacterial lysogens although some were isolated from plant material (often rotten), soil, irrigation water and a corn beetle (Section 1.4.2). While induction of phages from bacterial cells (eg. using Mitomycin C) enriches for temperate phages, alternative methods of isolation has led to the discovery of both temperate and virulent phages.

Origin of phage	No.	Host bacterium	Reference
E. nigrifluens	-	*E. nigrifluens*	Zeitoun and Wilson 1969
E. amylovora	1	*E. amylovora* *	Erskine 1973
"	3	*E. amylovora*	Richie and Klos 1977
"	14	*E. amylovora* * *E. herbicola*	Richie and Klos 1979
E. herbicola	1	*E. herbicola* *	Harrison and Gibbons 1975
"	9	*E. herbicola*	Tsuyumu pers. comm.
E. stewartii	1	*E. stewartii* *E. herbicola*	Woods *et al.* 1981
E. aroideae	2	*E. aroideae*	Suzuki and Togashi 1978
E. chrysanthemi	32	*E. chrysanthemi*	Paulin and Nassan 1978
"	1	*E. chrysanthemi* strain EC183 and KS605 *	Chatterjee and Brown 1980
"	3	*E. chrysanthemi* strain 3665, 3937, and B374 *	Resibois 1984
E. carotovora	1	*E. carotovora* *	Kishko *et al.* 1983
E. carotovora subsp. *carotovora*	11	*E. carotovora* subsp. *carotovora* .(1 phage subsp. *carotovora* and . subsp *atroseptica*)	Gross *et al.* 1991
E. carotovora subsp. *atroseptica*	2	*E. carotovora* subsp. * *atroseptica*	
E. chrysanthemi	?	*E. chrysanthemi*	"

* = no other strains tested.

Table A3. Phages isolated on *Erwinia* species. Phages infecting *E. carotovora* and *E. amylovora* isolated before 1963 are not included but are referenced (Okabe and Goto 1963).

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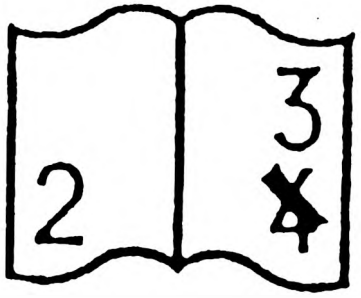


1.4.5.1 Phage typing

In addition to conventional classification methods (eg. serology and biochemical tests) for epidemiological studies, phage typing has proved useful in the identification of bacterial species. Methods for the rapid identification of bacterial phytopathogens include biochemical tests and serology, although these methods alone are often inadequate. This leaves the recognition of a particular phytopathogen to morphological differences in plant lesions (Billing 1963), or more time consuming methods such as the base composition of DNA or nucleic acid hybridization studies (Johnson 1984). An alternative method for the rapid identification of pathogenic bacteria, which has led to variable success, is phage typing. Phage typing relies on the differential susceptibilities of individual strains within a bacterial species to a number of phages, which produce a distinctive pattern of response, allowing segregation of strains into different groups (Lesley 1982). The method of phage typing consists of spotting phage lysates onto a soft agar overlay seeded with a bacterial strain (previously grown in rich liquid broth), followed by incubation to obtain zones of phage action (Billing 1963). Phages are often used at concentrations which are just able to give confluent lysis on the propagating host strain, a concentration called the routine test dilution (RTD) (Cuppels 1984). An alternative method of phage typing was used by Cuppels (1984), who prepared overlays directly from macerated plant tissues. Standardization of media and method is thought to be essential for comparable results (Billing 1963), as is the testing of strains from a number of geographic locations to ensure the reproducibility of a general phage typing system (Liew and Alvarez 1981).

Phage typing has been used in the identification and differentiation of a number of phytopathogenic bacterial species, including *Pseudomonas* (Billing 1963; Cuppels 1984; Vidaver and Buckner 1978), *Xanthomonas* (Liew and Alvarez 1981) and *Erwinia* (Gross *et al.* 1991; Paulin and Nassan 1978). Billing in 1963 isolated a

number of phages which exhibited a high degree of specificity towards either *P.phaseolicola* or *P.syringae* although, due to the nature of genetic variation, these specificities were not absolute. Characteristic patterns of phage sensitivity led to the differentiation of a number of strains, however lack of specificity of some phages meant that their use in diagnosis of disease was limited. Phage typing was useful however, as an additional method of characterisation to the more commonly used biochemical and lesion morphology tests. Phage typing, together with physiological characteristics, has also been used in the identification of a number of *P.syringae* subsp. *tomato* strains (Cuppels 1984). Identical results were obtained with each characterisation method. Since no other *Pseudomonas* species tested was sensitive to these phages and since overlays could be produced directly from infected plant material, phage typing offered a rapid and reliable method of identifying these organisms, without the need for isolation and physiological characterisation. A drawback of this method was that direct testing from leaves required large numbers of bacteria and phage typing was therefore of little use in the screening of symptomless plants. Phage typing systems have also been used to differentiate between cherry and plum isolates of *P.syringae* subsp. *morsprunorum* (Crosse and Garrett 1963) and to distinguish between phytopathogenic and nonphytopathogenic *Pseudomonas* isolates (Thomas and Leary 1983). In addition to phage typing, *P.syringae* strains have been typed successfully using bacteriocins isolated from a range of other *P.syringae* strains (Vidaver and Buckner 1978).

Liew and Alvarez (1981) isolated a number of phages (from soils of cabbage fields and infected seed), specific for *X.campestris* strains (145 *X.campestris* strains from a number of geographic locations were tested for phage sensitivity, as well as 47 other bacterial species, including *Eca* and *Ecc*). Five phages were used to obtain different lysis patterns, which allowed differentiation of the strains into two types, A and B, that were further divided into a number of subtypes (18% of the strains were untypable due to their resistance to all five phages).

Until recently phages for *Ecc*, *Eca* and *Echr* have rarely been isolated (Table A3). Paulin and Nassan (1978) failed to isolate virulent phages on *Echr*, from soil and infected plants, but were able to isolate a number of temperate phages. These temperate phages showed some specificity towards strains originating from specific host plants and were therefore of some use in phage typing. This was the first recorded example of a phage typing system for the erwinias. They concluded from their work that although these phages were specific to *Echr*, due to their temperate nature, they gave only faint turbid plaques and were therefore difficult to monitor. Stock suspensions were also difficult to keep for long periods of time. On the basis of these finding it was concluded that this phage typing system was not ideal for routine use.

Recent work by Gross *et al.* (1991) led to the isolation of a number of phages to *Ecc*, *Eca* and *Echr* in order to type, and therefore trace, strains in epidemiological studies (particularly *Ecc*), since serological methods had failed to differentiate a high proportion of *Ecc* strains. They found that phages for *Ecc* strains were relatively rare compared to *Eca* and *E.herbicola*, but could give no explanation why this should be. *Echr* strains were found to compose less than 1% of the soft rot erwinias isolated from diseased plants in Oregon, yet phages specific to this species were frequently isolated. Gross *et al.* (1991) concluded from this that *Echr* could have been a significant potato pathogen, in this area, under suitable environmental conditions. These workers concluded from their study that phage typing was of only limited use for surveying strain diversity in field populations of *E. carotovora*, particularly since the majority of *Ecc* strains were serologically identified but only 20% were identified into a phage group. They did however, conclude that phage typing was a more useful method of grouping *Eca* strains, since all field strains tested were identified into different phage groups, but fell into a single serological group (serogroup I). Although phage typing was originally proposed as a potentially

better method than serotyping for differentiating between strains of *Ecc*, because specificity would not necessarily be based on LPS receptors, few serologically unreactive strains proved to be sensitive to one or more of the phage isolates. This suggested that the receptor for phage attachment in the majority of cases was LPS, an idea that was further supported by the fact that the lytic action of some phage groups corresponded with specific serogroups.

From the above information it would seem that phage typing of phytopathogenic bacterial strains has been of mixed success. Phage specificity appears to vary between different strains or species, a phenomenon that must be thoroughly investigated before a reliable phage typing system can be implemented. Phage typing of similar species (or subspecies) may have different potentials. For example in the work by Gross *et al.* (1991) serology of *Ecc* was adequate in strain differentiation, but few phages were available for typing, on the other hand *Eca* strains all fell into the same serogroup but were successfully divided into a number of phage groups. Also true is the fact that virulent phages, rather than temperate phages, appear to be more suitable for use in phage typing systems (Billing 1963). In conclusion phage typing does appear to be a useful method for strain differentiation, especially when carried out in conjunction with other methods such as biochemistry and serology.

Generalised transduction is an important method of bacterial gene transfer, along with transformation and conjugation. Because of its importance in gene transfer and its relevance to this work, it will be covered in particular detail.

1.5 Generalised transduction

The generalised transduction process begins when bacterial DNA is encapsulated within a phage head during the lytic cycle of the phage (an accidental consequence of phage replication). This DNA, via a transducing particle, is then carried to a recipient cell where the genomic DNA is injected by the phage. Once inside the recipient the DNA may be lost by degradation or remain in the cytoplasm in a non-replicating state where it will be eventually diluted out of the host population. Occasionally however, it may recombine with the chromosome to yield a stable transductant (Fig. A12) (Masters 1985).

Generalised transduction was first described in 1952 by Zinder and Lederberg who found it was a means of transferring heritable traits between *S.typhimurium* strains by a DNase-resistant filterable agent. It was later found to be due to a sub-class of P22, a phage now well recognised for possessing transducing ability. Lennox in 1955 demonstrated generalised transduction in *E.coli* by P1, a phage 2.4 times the genomic size of P22 and capable, therefore, of transducing fragments of DNA more than twice the length, approximately 2% of the *E.coli* chromosome. Soon after the discovery of these two phages, linked transduction of separate genetic markers was demonstrated, including the cotransduction of up to four *E.coli* genetic markers known to be closely linked in conjugation crosses (Lennox 1955). These two phages are the best characterised generalised transducing phages to date and have continued to be the principle transducing phages used as genetic tools in these organisms (Susskind and Botstein 1978; Walker and Walker 1983).

P1 has an icosehedral head and intricate tail complete with tube, contractile sheath, base plate and tail fibres. P22, in contrast, has a much simpler structure with a simple abbreviated tail. P22 and P1 both contain single linear duplex DNA, of 39-

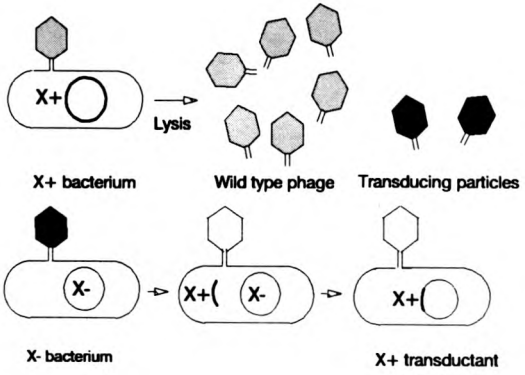


Figure A12

Diagrammatic representation of generalised transduction.

Adapted from Freifelder (1983).

42 kb and 91-100 kb respectively, which is cyclically permuted with terminal redundancies of 2% and 7-12% respectively. Both phages are temperate, although this is a relatively rare event in the P1 life cycle.

1.5.1 DNA Encapsulation

Both P22 and P1 virions are filled by the headful mechanism. This process is initiated at specific sites on the phage genome, in the case of P22 called *pac* sites, which are recognition signals to initiate packaging *in vivo* (Kufer *et al.* 1982; Schmieger 1982, 1984). Restriction analysis of P1 DNA has shown similar sites to be present (Bachi and Arber 1977), although Hanks *et al.* (1988), using Southern hybridizations, failed to find any chromosomal sequences with homology to the phage *pac* site. It is believed that, on entry of P22 or P1 into a donor cell, the incoming phage recognizes regions on the chromosome that resemble such a site and therefore begin packaging chromosomal DNA at these regions, for a limited number of cycles, before phage regulated chromosomal degradation occurs. Different bacteria possess different numbers of such sites for different phages. Chelala and Margolin (1974) suggested for example, that P22 has 10-15 specific start sites around the *S.typhimurium* chromosome and Schmieger (1982) suggested 5-6 such sites. For P1 these regions are thought to represent about 20% of the *E.coli* chromosome (Harrimann 1972). Deletions in the donor chromosome may alter the frequency of transduction of certain markers since they may eliminate or create a *pac*-like initiation points on the chromosome (Chelala and Margolin 1974). The products of specific genes have been assigned to recognition of these sites, eg. gene 3 in P22 (Schmeiger 1972). The DNA contained within P1 transducing phages has been investigated in detail using density labelling of DNA (Ikeda and Tomizawa 1965a, 1965b, 1965c). P1 transducing particles are thought to represent about 0.3% of total phage particles released, with high transducing derivatives producing up to

2%. P22 on the other hand gives normally around 1-5% with high transducing derivatives producing up to 50% (Susskind and Botstein 1978).

1.5.2 Fate of Transduced DNA

On entry into a recipient cell one of three fates await the transduced DNA. Some transduced molecules will recombine with homologous regions of the recipient chromosome, part of the transduced DNA replacing part of the homologous DNA by *rec*-mediated recombination (complete transduction). The use of *rec*A mutants greatly reduces or eliminates the formation of such recombinants (Sandri and Berger 1980a). Non-recombined DNA is either degraded by host nuclease action or more commonly remains in the cytoplasm, without replicating or undergoing recombination with the chromosome (abortive transduction) (Sandri and Berger 1980b).

Two forms of integration may occur; recombination leading to addition or substitution. Addition is characteristic of specialised transduction. In specialised transduction the only genes transduced are those adjacent to the chromosomal site of lysogenisation, which are occasionally incorporated into the phage genome during aberrant excision of the prophage. When a hybrid of phage and bacterial DNA, encapsulated via the initiation site on the phage DNA, is presented to the chromosome in a circular form, it inserts into the chromosome via the Campbell model of integration (Campbell 1962) usually creating a duplication.

Substitution occurs when the linear generalised transducing DNA presents itself in a linear form to the recipient chromosome, where part of the donor DNA is integrated into the chromosome in place of the homologous DNA segment which previously occupied that site. Important evidence of the need for recombination in transduction

comes from work by Hertman and Luria (1967) on P1 and Wing (1968) on P22 who demonstrated a need for the *rec*A system in generalised transduction.

Abortive transductants can be visualized as microcolonies when, for example, wild type genes are transduced into auxotrophic recipients. The donor DNA does not recombine with the chromosome of the recipient but remains in both a supercoiled and relaxed circular form helping to protect it from the *rec*BC system while expressing its genes. This DNA does not replicate but instead, when the cell divides, only one daughter cell receives this fragment. The other daughter cell may undergo a few rounds of replication until the essential gene product remaining in the cytoplasm is depleted. A consequence of this stable, but single, fragment of DNA in a continually dividing population, is a microcolony. Circularisation of the abortive DNA is dependant on a protein component which is often seen in conjunction with a transduced fragment of DNA (Ikeda and Tomizama 1965b).

While most DNA fragments from transducing particles become abortive transductants, only about 2-5% of the DNA from P22 transducing particles integrates into the recipient chromosome as significant segments of covalently bound DNA, these are termed complete transductants. The donor DNA is integrated in double stranded form, as opposed to transformed DNA which integrates in a single stranded form. Ultraviolet irradiation of transducing phage lysates increases the number of complete transductants, indicating a conversion of abortive to complete transductants (Benzinger and Hartman 1962; Newmann and Masters 1980), implying that a repair, or processing, of U.V. irradiated damaged DNA involves recombination.

1.5.3 Transduction frequencies

The efficiency of transduction of chromosomal genes can be affected by a variety of factors. Over 90% of transducing particles give rise to abortive transductants in which the transduced DNA fragment, with the gene(s) of interest, does not integrate into the chromosome but survives in the cytoplasm to produce microcolonies. Complete transductants on the other hand represent both the efficiency of formation of transducing particles containing a particular marker in donor cells, and the effect of stable integration of that marker into the chromosome of the recipient cells.

Even when both abortive and complete transductions are taken into account there can still be as much as 1000 fold difference (in the case of P22), between the frequencies of transduction of particular markers (Schmeiger 1972). This difference may due to a variation in the efficiency of encapsulation. Schmieger (1972) suggested that high-frequency markers represented locations near packaging start signals. An extensive study by Masters (1977) on P1 transduction in *E.coli* demonstrated a preferential transduction of markers around the origin of replication as well as variation in transducing efficiencies for genes located at different positions around the chromosome. In general P1 encapsulates most *E.coli* genes at similar frequencies, with differences ranging from as little as 8-10 fold, without taking into account the high frequencies of transduction around the origin of replication (Masters 1977), whereas P22 encapsulates different *S.typhimurium* genes at very different frequencies (varying over a 1000 fold range) (Schmeiger 1972). The major difference in transduction frequencies therefore appears to be the ability to recognise initiation sites around the chromosome, and since more of these appear to be available for P1 in *E.coli* (Hanks *et al.* 1988) it is not surprising that frequencies are less variable.

It is also believed that there is a discrimination among donor markers by the

recombination process, leading to preferential integration of certain markers and thereby showing large variations in transduction frequencies. Evidence for the latter comes from data obtained after U.V. irradiation of P1 transducing lysates and mutations which affect the recombination pathway. These distinctly reduce the degree of variation in frequencies with which different *E.coli* genes are transduced (Masters *et al.* 1984). Recombination frequency probably plays an important part in frequency variations among P1 transduced *E.coli* genes (Newmann and Masters 1980).

1.5.4 Mutants affected in transduction efficiencies

Mutants of P22 have been produced with decreased or increased transducing frequencies (Schmieger 1972; Schmieger and Backhous 1973). P22 NT, which had a decreased efficiency of transduction, had minor effects on overall transduction frequencies, whereas P22 HT with increased efficiency of transduction showed all genes to be transduced at high frequencies. The lower the frequency of transduction with wild type P22, the greater it was with P22 HT, resulting in a 10 fold variation in frequencies (of specific marker transduction) from what was a 1000 fold variation in wild type P22. Jackson *et al.* in 1982 demonstrated that HT mutants altered the specificity of the initial cut associated with encapsulation. Mutants of P1 with altered transducing frequencies have also been found (Wall and Harriman 1974). A high frequency transducing derivative with 5-10 times higher transduction frequencies was found to possess a suppressible amber mutation defective in a gene involved in the transduction process, indicating that loss of a function increases the ability of P1 to package host DNA preferentially.

1.5.5 Other Generalised transducing phages

1.5.5.a Phage T1

T1 is a highly virulent phage which under special circumstances is able to transduce. Early after infection of an *E.coli* cell the host DNA is degraded to be utilized in the production of progeny phage, and the formation of transducing particles must therefore occur early before the host chromosome is degraded. Amber mutant phage, grown on permissive *E.coli* cells (bearing amber suppressor alleles) allow the production of transducing particles. Like P1 and P22, T1 progeny are filled by the headful process with a 6.5% terminal redundancy on the genome. Unlike the previous phages, low multiplicities of infection (m.o.i.) are used to reduce killing of recipient cells. Ultraviolet irradiation does not increase transduction frequency of most genes which correlates with the apparent absence of abortive transductants. Transducing efficiency is increased by use of phage mutants defective in host chromosome degradation (Drexler 1970).

1.5.5.b Phage T4

T4 requires a series of mutations to act as a transducing phage, eg. phage T4GT7. These mutations effect genes involved in the disruption of the bacterial DNA structure or those which specify an endonuclease which degrades cytosine containing DNA (T4 DNA contains hydroxymethylcytosine) (Wilson *et al.* 1979). This phage is very useful in transduction experiments since it has a genome size of 166 kb and can therefore transduce almost twice as much of the chromosomal DNA as P1. P1 strains are more efficient however, since the T4 transducers appear to be unable to transduce some markers altogether, or do so at undetected frequencies (Young and Edlin 1983). As with P1 and P22, this phage possesses cyclically

permuted, terminally redundant DNA.

1.5.6 Generalised transduction in other bacteria

Many other examples of generalised transducing phages exist both for the above strains and many other bacterial strains. These phages include F126 and F130 (Krishnapillai 1971), E79 (Morgan 1979), F116 (Miller *et al.* 1974), ϕDS1 (Saye *et al.* 1987, 1990) in *Pseudomonas aeruginosa*, $\phi 17\alpha$ and $\phi 56\alpha$ in *Streptococcus thermophilus* (Mercenier *et al.* 1988), ϕCr30 and ϕCr35 in *Caulobacter crescentus* (Ely and Johnson 1977), P78 in *Actinobacter* (Herman and Juni 1973) rp-1 from *Vibrio fisheri* (Levisohn *et al.* 1987), 3M in *Serratia marcescens* (Regue *et al.* 1991), and M1 in *Methanobacterium thermoautotrophicum* (Meile *et al.* 1990). Some transducing phages have special functions, such as KB1, which in *S.typhimurium* is heteroimmune with P22 so each produce plaques on lysogens of the other and transduce with high efficiency (Boro and Brenchly 1971). ES18, another generalised transducing phage of *S.typhimurium* is able to plaque on rough mutants (which lack LPS side chains and often part of the core), whereas most generalised transduction phages of this species are specific to smooth bacterial strains (Kuo and Stocker 1970).

1.5.6.1 Generalised transduction in plant associated bacteria

The first documented evidence of generalised transduction in a plant associated bacterium was in 1967 when Kowalski reported such a phenomenon in the symbiont *Rhizobium meliloti*. After the work by Kowalski further generalised transducing phages were isolated for this species [DF2] (Casadesus and Olivares 1979), and for other *Rhizobium* species, eg. RL38 and RL39 which can transduce between

R.leguminosarum and *R.trifolii* (Buchanan-Wollaston 1979). Within the last 10 years other reports of generalised transducing phages in *R.meliloti* have been described which are able to transduce between various strains used in a number of laboratories, ie. phage 11 (Sik *et al.* 1980), phage N3 (Martin and Long 1984) and M12 (Finan *et al.* 1984) (Table A4).

The first evidence of a generalised transducing phage for a phytopathogenic *Pseudomonas* species was in 1983 with the isolation of Pssy15 in *P.syringae* (Nordeen and Currier 1983). Later CP75 was described in *P.cepacia* (Matsumoto *et al.* 1986).

Chatterjee and Brown in 1981 reported generalised transduction in *Erwinia chrysanthemi* strain EC183. This was the first documented evidence of a generalised transducing phage for any *Erwinia* species. This phage, Erch12, produced from a lysogenic strain of *Echr* has helped in the mapping of a number of genes in *Echr* (Chatterjee *et al.* 1981). However Erch-12 has a restricted host range. Consequently in 1984 workers using *Echr* strain 3937 isolated a temperate generalized transducing phage, ϕEC2 (Resibois *et al.* 1984).

Comparing the phages listed in Table A4 the average frequency of transduction appears to be in the region 10^{-8} to 10^{-6}, although the *Echr* phage Erch-12 transduces at a comparatively poor frequency. All the phages listed transduce at frequencies below those of P1 (10^{-5} to 3×10^{-4}). In several cases it has been shown that ultraviolet light irradiation improves the frequencies of transduction, as seen with P1 and P22 (Benzinger and Hartmann 1962; Wilson 1960).

Table A4

Generalised transducing phages of plant associated bacteria. Phages M12, N3 and RL38 are virulent phages, while 11, Pss15, CP75, Erch-12 and ϕEC2 are temperate. M12 and CP75 fall into group A of the Bradley classification system but other phage groups are not known.

ND = no data.

Phage	Genome Size (kb)	Bacterial Host	Transd. Freq.	U. V. on transd.	Adsorpt. Effic.	M.o.i.	Reference
M12	180	R.meliloti	$1.0x10^{-5}$ - $1.0x10^{-6}$	ND	ND	0.5	Finan et al.
							1984
11	64.5	"	$1.0x10^{-5}$ - $6.0x10^{-7}$	ND	ND	<1	Sik et al. 1980
N3	190	"	$1.1x10^{-5}$ - $8.0x10^{-7}$	ND	90% (3 min)	<0.1	Martin and Long
							1984
DF2	ND	"	$1.2x10^{-5}$ - $9.3x10^{-7}$	ND	99% (2 hours)	<1	Casadisus and
							Olivares 1979
RL38	ND	R.leguminosarum	$2.0x10^{-5}$ - $8.0x10^{-7}$	Inc. freq.	90% (3 hours)	<1	Buchanan-
							Wallaston 1979
Pss15	ND	P.syringae	$1.2x10^{-7}$ - $9.2x10^{-8}$	Inc. freq. x2	ND	10	Nordeen et al.
							1983
CP75	52	P.cepacia	$1.0x10^{-5}$ - $2.0x10^{-6}$	ND	ND	ND	Matsumoto et al
							1986
Erch-12	ND	E.chrysanthemi	$1.0x10^{-7}$ - $7.0x10^{-9}$	Inc. freq. x4-38	99% (30 min)	1 - 10	Chatterjee and
							Brown 1981
ϕEC2	62	"	$1.0x10^{-5}$ - $1.0x10^{-8}$	ND	ND	ND	Resibois et al.
							1984

1.5.7. Expansion of the host range of T4 and P1

Rather than search for a phage capable of generalised transduction in a particular species several attempts have been made to expand the host range of well known transducers, such as T4 and P1. The bacterial host range of P1, using the heat inducible phage P1*clr*100KM, conferring kanamycin resistance, has been extended to a range of Gram-negative bacteria by selection of kanamycin resistant colonies. Goldberg *et al.* (1974) discovered P1 sensitive strains of *Klebsiella aerogenes*, *K.pneumoniae*, *Erwinia amylovora*, *Citrobacter* and *Enterobacter* species, performing transductions between *Klebsiella* species and *E.coli* to create intergeneric hybrid strains. Attempts to isolate kanamycin resistant colonies of *Erwinia carotovora* were successful but no phage release was detected when stabbed into lawns of sensitive bacteria. This was however achieved by Murooka and Harada in 1979, their explanation for previous failures being that different strains were used in each case. Jayaswal *et al.* 1985b examined in more detail the behaviour of P1 in *Ecc* using the phage strain listed above. They concluded that although lysogens of P1 were produced, lytic replication did not occur in P1 lysogens of this species, and P1 DNA was probably integrated into the host chromosome.

The occurrence of T4 receptors (core lipopolysaccharide) on the cell surface of *Ecc* and *Eca* have been observed by phage adsorption studies and phage induced lysis of the bacteria (Pirhonen and Palva 1988). Occasionally lytic growth was also observed. The T4 transducing derivative T4GT7 was employed to transfer plasmids from *E.coli* to both subspecies with success. Plasmid transfer from strains of *Erwinia* carotovora, able to support lytic growth of the phage to *E.coli*, was less successful probably due to restriction within the recipient. It is interesting to note that 7 out of 9 *Eca* strains showed phage sensitivity compared to 1 out of 9 *Ecc* strains, suggesting that *Eca* was more sensitive to T4 than was *Ecc*.

1.5.8. Uses of transduction

Generalised transduction can be used to carry out a range of genetic manipulations;

1.5.8.a Genetic mapping

The mapping of genes by conjugation is well known (Chatterjee *et al.* 1981). While this technique offers rough linkage mapping, transduction allows much finer mapping by the cotransduction of two or more markers. Many examples of this in relation to phytopathogenic bacteria are cited in the literature (Chatterjee *et al.* 1981; Casadesus and Olivares 1979).

1.5.8.b Localized mutagenesis

To obtain a mutation in a particular gene the entire chromosome of a bacterial cell is exposed to mutagenic agent, and so additional mutations may occur in other regions of the genome causing ambiguous physiological effects. Localized mutagenesis allows small segments of the bacterial genome with the gene of interest, contained in a transducing particle, to be exposed to mutagenic agent without effecting the rest of the genome. Although mutations may take place outside the gene of interest, once inside the recipient cell only a very small fragment of the mutagenized DNA will recombine, producing a very clean mutant (Hong and Ames 1971).

1.5.8.c Assessment of intergeneric homology

Some transducing phages have the ability to infect more than one bacterial species or even genera, eg. P1 (Murooka and Harada 1979). Transduction of genes between such bacteria can therefore take place producing intergeneric hybrid strains. This allows the mapping of analogous locations of genes in different bacteria to be made and their relatedness assessed. The use of P1 transduction in genetic transfer of tyramine oxidase (*tynA*) and arylsulphatase (*atsA*) between *K.aerogenes* and *E.coli*

(restrictionless mutant) has allowed the production of intergeneric recombinant strains (Marooka *et al* 1978). Low transduction frequencies of *tynA* and *atsA*, compared to the *leu* marker, and the production of latent arylsulfatase in the *E.coli* recipient, suggested low DNA homologies between the genes from different genera. Similarities in the action of catabolite repression on tyramine oxidase however, suggested a conserved mechanism of gene regulation between the two genera.

1.5.8.d Construction of isogenic strains

Any transduction which introduces a donor allele differing from the original recipient allele it replaces creates a transductant clone isogenic with the recipient strain. This is the fundamental process of transduction and many examples are available. Possibly the simplest example of this is the transduction of a wild type gene into an auxotroph to return the cell to prototrophy. This process is often used simply to study the frequency of transduction by a phage (Buchanan-Wollaston 1979; Chatterjee and Brown 1981).

1.5.8.e Transduction using transposons

Transposon mutagenesis allows the insertion of a transposon into a large number of sites around the bacterial chromosome creating a mutation or inserting next to a gene of interest (Salmond *et al.* 1986). The presence of antibiotic resistance genes on the transposons allows them to be used as selectable markers in the transfer of genes mutated by transposon insertion or tagged by a transposon. In the latter case cotransduction of genes with screenable but non-selectable phenotypes can be useful for strain constructions (Finan *et al.* 1984).

1.5.8.f Transfer of plasmids

Some transducing phages are capable of plasmid transfer between bacterial cells. Plasmids involved in a whole range of cellular processes, including cloning vehicles and chromosome mobilization can be transferred (Buchanan-Wollaston 1979;

Casadesus and Olivares 1979). Transduction of plasmids is much more efficient than transformation, especially with large plasmids and therefore may have a significant advantage in certain circumstances.

1.6 Aims of Thesis

The aims of this thesis were;

a) To obtain phage resistant mutants of *Eca* SCRI1043 altered in their virulence, to characterise these mutants, with particular focus on the role of the cell surface, and to identify pathogenic determinants in these mutants responsible for reduced virulence.

b) To isolate and characterise a number of phages to *Eca* SCRI1043 and *Ecc* SCRI193.

c) To obtain phages capable of generalised transduction in *Eca* SCRI1043 and *Ecc* SCRI193.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2

2 Materials and Methods

2.1 Bacterial strains, bacteriophages and plasmids

The bacterial and bacteriophage strains, and the plasmids used in this study are listed in Tables B1, B2 and B3. Derivatives of *Eca* SCRI1043 produced in this study, and phages isolated during this study will be described in more detail in the text. A number of these phages were isolated by L. Kinersley and G. Pavitt.

2.2 Media

Solutions, growth and assay media are listed in Table B4, 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 autoclaved at 121°C for 20 min. PGA was degraded if autoclaved for longer periods. NB and LB were solidified with 1.5% (w/v) Bacto agar, when necessary. Double Difco medium was used for Lambda work; DDA and soft DDA contained 1% and 0.6% (w/v) Bacto agar respectively. Minimal medium was supplemented with amino acids, bases (both at 20 μg/ml) and sugars (0.2% (w/v), final concentrations) when required. M9 minimal medium was used only for siderophore assay plates. Antibiotics were prepared at 100x final concentrations stock, stored at 4°C and used at 50 μg/ml. TE was used in all DNA manipulations and contained 10 mM Tris-HCl, 1 mM ethylene diamine tetraacetic acid (EDTA), pH 8.0.

Table B1 Bacterial strains

Strain	Characteristics	Plasmid phenotype	Source	Reference
E.coli K12				
DH1	F$^+$,*rec*A1,*end*A1,*gyr*A96, *thi*-1,*hsd*R17($r_k^-m_k^+$), *sup*E44,*rel*A1?,Lambda$^-$	-	J. Hinton	Hanahan, 1983
Erwinia carotovora subsp. *atroseptica*				
SCRI1043	wild-type	-	J. Hinton	Hinton *et al.* 1989a
SCRI1043 derivatives				
HA100	SCRI1043(pHCP2)	Ap^r	J. Hinton	"
HA164	SCRI1043 (pHCP2) *ade*::Tn*5*	Ap^r	J. Hinton	"
HA150	SCRI1043 (pHCP2) *nad*::Tn*5*	Ap^r	J. Hinton	"
TA100	SCRI1043 (pHCP2) *ura*::Tn*5*	Ap^r	This study	
TA200	SCRI1043 (pHCP2) *arg*::Tn*5*	Ap^r	This study	
TA300	SCRI1043 (pHCP2) *cys*B::Tn*5*	Ap^r	This study	
Other *Eca* strains				
SCRI1	wild type	-	Scottish Crop Research Institute	
" 8	"	-	"	
" 9	"	-	"	
" 11	"	-	"	
" 12	"	-	"	
" 13	"	-	"	
" 15	"	-	"	
" 28	"	-	"	
" 31	"	-	"	
" 32	"	-	"	
" 33	"	-	"	
" 34	"	-	"	
" 37	"	-	"	
" 39	"	-	"	
" 41	"	-	"	

Strain	Characteristics	Plasmid phenotype	Source	Reference
" 45	"	-	"	
" 46	"	-	"	
" 48	"	-	"	
" 52	"	-	"	
" 58	"	-	"	
" 68	"	-	"	
" 82	"	-	"	
" 83	"	-	"	
" 84	"	-	"	
" 87	"	-	"	
" 93	"	-	"	
" 94	"	-	"	
" 96	"	-	"	
" 97	"	-	"	
" 98	"	-	"	
" 1034	"	-	"	
" 1035	"	-	"	
" 1036	"	-	"	
" 1037	"	-	"	
" 1038	"	-	"	
" 1039	"	-	"	
" 1040	"	-	"	
" 1041	"	-	"	
" 1044	"	-	"	
" 1045	"	-	"	
" 1046	"	-	"	
" 1047	"	-	"	
" 1049	"	-	"	
" 1050	"	-	"	
" 1051	"	-	"	
" 1052	"	-	"	
" 1053	"	-	"	
BS44	"	-	F. Ellard	
BS131	"	-	"	
BS153	"	-	"	
BS209	"	-	"	
BS227	"	-	"	
C301	"	-	"	
C344	"	-	"	
C361	"	-	"	
C465	"	-	"	
C466	"	-	"	
Erwinia carotovora subsp. *carotovora*				
SCRI193	wild type	-	J. Hinton	Salmond *et al.* 1986
SCRI193 derivatives				
HC131	SCRI193(pHCP2)	Ap^I	J. Hinton	"

Strain	Characteristics	Plasmid phenotype	Source	Reference
HC519	SCRI193(pHCP2) *leu*::Tn*5*	Ap^r	J. Hinton	
HC500	SCRI193(pHCP2) *cys*B::Tn*5*	Ap^r	J. Hinton	Hinton *et al.* 1987
HC512	SCRI193(pHCP2) *lac*::Tn*5*	Ap^r	J. Hinton	"
HC(8.11)	SCRI193(pFE) *trp*::Tn*5*	Ap^r	F. Ellard	Ellard *et al.* 1989
HC(12.19)	SCRI193(pFE) *arg*::Tn*5*	Ap^r	F. Ellard	"
HC(17.5)	SCRI193(pFE) *pur*::Tn*5*	Ap^r	F. Ellard	"

Other *Ecc* strains

SCRI101	"	-	F. Ellard	
" 102	"	-	"	
" 103	"	-	"	
" 105	"	-	"	
" 106	"	-	"	
" 109	"	-	"	
" 110	"	-	"	
" 111	"	-	"	
" 112	"	-	"	
" 113	"	-	"	
" 114	"	-	"	
" 115	"	-	"	
" 116	"	-	"	
" 117	"	-	"	
" 118	"	-	"	
" 119	"	-	"	
" 120	"	-	"	
" 121	"	-	"	
" 122	"	-	"	
" 123	"	-	"	
" 124	"	-	"	
" 125	"	-	"	
" 126	"	-	"	
" 129	"	-	"	
" 130	"	-	"	
" 132	"	-	"	
" 135	"	-	"	

Strain	Characteristics	Plasmid phenotype	Source	Reference
" 139	"	-	"	
" 143	"	-	"	
" 144	"	-	"	
" 149	"	-	"	
" 152	"	-	"	
" 155	"	-	"	
" 166	"	-	"	
" 169	"	-	"	
" 171	"	-	"	
" 172	"	-	"	
" 174	"	-	"	
" 178	"	-	"	
" 182	"	-	"	
" 191	"	-	"	
" 192	"	-	"	
" 198	"	-	"	
" 205	"	-	"	
" 208	"	-	"	
" 211	"	-	"	
" 224	"	-	"	
" 247	"	-	"	
ATCC39048	"	-	"	
Erwinia chrysanthemi				
NCPPB1066	"	-	C. Kell	Gilbert *et al.* 1986
3937	"	-	F. Ellard	
B374	"	-	"	
EC183	"	-	"	
EC16	"	-	"	
Erwinia uredovora				
SCRI431	"	-	"	Ellard *et al.* 1989
" 132	"	-	"	"
" 433	"	-	"	"
Erwinia rubrifaciens				
SCRI445	"	-	"	"
" 446	"	-	"	"
" 469	"	-	"	
" 470	"	-	"	
" 471	"	-	"	
Erwinia rhapontici				
SCRI421	"	-	"	"

Strain	Characteristics	Plasmid phenotype	Source	Reference
" 422	"	-	"	"
" 423	"	-	"	"
" 468	"	-	"	
Erwinia amylovora				
SCRI454	"	-	"	"
" 449	"	-	"	"
" OT1	"	-	"	
Erwinia herbicola				
SCRI463	"	-	"	"
" 424	"	-	"	
" 426	"	-	"	
" 427	"	-	"	"
" 430	"	-	"	"
" 436	"	-	"	"
Erwinia nigrifluens				
SCRI450	"	-	"	
Erwinia quercina				
SCRI452	"	-	"	
" 453	"	-	"	

TableB2 **Bacteriophages**

Phage	Characteristic	Source
Lambda	wild type	G. Salmond
Lambda C1857	ts repressor	G. Salmond
$P1_{vir}$	virulent derivative of P1	G. Salmond
T4	wild type	G. Salmond
A1	isolated on *Eca* SCRI1043	This study
A2	"	"
A3	"	"
A4S	"	"
A4L	"	"
A5	"	"
A6	"	"
M1	"	"
M2	"	"
M3	"	"
M4	"	"
M5	"	"
S21	"	"
S22	"	"
S31	"	"
S32	"	"
S33	"	"
S34	"	"
S41	"	"
S42	"	"
S45	"	"
S62	"	"
S71	"	"
S72	"	"
S65	isolated on *Ecc* SCRI193	This study
S75	"	"
D2	"	"
KP	"	"
AL1	isolated on *Echr* NCPPB1066	This study

A number of the phages listed for this study were isolated by L. Kinnersley and G. Pavitt.

Table B3 **Plasmids**

Plasmid	Characteristic	Phenotype	Source	Reference
pHCP2	pBR322::*lamB*+	Ap^r	J. Hinton	Clement *et al* 1982
pHC79	pBR322::cos	Ap^r, Tet^r	Boehringer Mannheim	Frey *et al.* 1983

Table B4 **Media**

Media	Constituents per Litre
Nutrient Broth (NB)	13 g Oxoid nutrient broth 16 g Bacto agar for plates 7 g Bacto agar for soft agar
Luria Broth (LB)	10 g Bacto tryptone 5 g Bacto yeast extract 5 g NaCl [pH 7.2] 16 g Bacto agar for plates 7 g Bacto agar for soft agar
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)
Phage buffer	7 g Na_2HPO_4 anhydrous 3 g KH_2PO_4 anhydrous 5 g NaCL 2.5 g $MgSO_4.7\ H_2O$ (1 ml 1% (w/v) gelatin)
Pectinase assay medium	16 g Bacto agar (5 ml 20% (w/v) Bacto yeast extract) (10 ml 10% (w/v) $(NH_4)_2SO_4$) (1 ml 1 M $MgSO_4.7\ H2O$ (10 ml 50% (w/v) glycerol) (250 ml 2% (w/v) Sodium polypectate) (200 ml PL phosphate buffer)
Phosphate buffer	15 g Na_2HPO_4 anhydrous 0.7 g $NaH_2PO_4.1\ H_2O$ [pH 8.0]
Cellulase assay medium	10 g Sigma carboxymethyl cellulose 16 g Bacto agar (25 ml 20% (w/v) Bacto yeast extract) (4 ml 50% (w/v) glycerol) (20 ml 50 x phosphate) (10 ml 10% (w/v) $(NH_4)_2SO_4$) (10 ml 1% (w/v) $MgSO_4$)

Media	Constituents per Litre[a]
Protease assay medium	13 g Oxoid nutrient broth 30 g Oxoid gelatin 16 g Bacto agar
Freezing medium	12.6 g K_2HPO_4 anhydrous 0.9 g sodium citrate 0.18 g $MgSO_4.7\ H_2O$ 1.8 g $(NH_4)_2SO_4$ 3.6 g KH_2PO_4 anhydrous 88 g glycerol
Pectate lyase minimal medium	(5 ml 20% (w/v) Bacto yeast extract) (10 ml 10% (w/v) $(NH_4)_2SO_4$) (1 ml 1 M $MgSO_4.7\ H_2O$) (10 ml 50% glycerol) (250 ml 2% (w/v) PGA (Sigma)) (20 ml 50 x phosphate buffer)
Minimal medium	(20 ml 50 x phosphate) (10 ml 10% (w/v)$(NH_4)_2SO_4$) (10 ml 1% (w/v) $MgSO_4.7\ H_2O$)
50 x Phosphate buffer	350 g K_2HPO_4 100 g KH_2PO_4 [pH 6.9 - 7.1]
Double Difco medium (DD)	20 g Bacto tryptone 8 g NaCl (10 ml 20% (w/v) maltose, 10ml 1 M $MgSO_4$ for Lambda work) 9 g Bacto agar for plates 3 g Bacto agar for soft agar
TB	10 g Bacto tryptone 5 g NaCl
MacConkey agar (without carbon source)	40 g Difco MacConkey agar base
Stewarts medium (with pectate overlay)	52 g Oxoid MacConkey agar
Pectate overlay	16 g Sodium polypectate 60 ml ethanol 1 g di sodium EDTA 4 ml NaOH (1 M)

Media	Constituents per Litre[a]
Crystal violet pectate medium:	
a) Basal layer	15 g Bacto agar 5.5 g $CaCl_2.6\ H_2O$ 2 g $NaNO_3$ 1 g Oxoid tryptone 2 ml Crystal violet (0.075% solution) [pH 7.0-7.5]
b) Over lay	25 g Sodium polypectate (H.P. Bulmer, Hereford) (2.5 ml EDTA (5% solution)
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]
Siderophore assay medium	100 ml (10x) M9 salts 30.24 g PIPES 50 ml 50% (w/v) NaOH 15 g Bacto agar 100 ml siderophore assay plate dye solution (see below).
Siderophore assay plate dye solution	
Soln. 1(100 ml)	0.1 ml HCl (38%) 27 mg $FeCl_3.6\ H2O$
Soln. 2(50 ml)	60.5 mg chrome azurol S
Soln. 3(40 ml)	72.9 mg Cetyltrimethyl ammonium bromide (CTAB)
	Solution 2 was mixed in 10 ml of solution 1, followed by addition of solution 3
Tris medium	5.8 g NaCl 3.7 g KCl 1.1 g NH_4Cl 0.15 g $CaCl_2.2\ H_2O$ 0.1 g $MgCl_2.6\ H_2O$ 0.142 g Na_2SO_4 0.272 g KH_2PO_4 12.1 g Trizma base (Sigma) [pH 7.4]

Tryptone swarm medium	10 g Bacto tryptone 5 g NaCl 7 g Bacto agar [pH 7.2]

Antibiotics were added at 50 μg/ml final concentration.
Amino acids and bases were added at 20 μg/ml final concentration.
Sugars and carbon sources were added at 0.2% (w/v) final concentration.

2.3 Chemicals

Media chemicals were generally obtained from Fisons or BDH Chemicals and were of "Analar" grade. Polygalacturonic acid, carboxymethylcellulose and amino acids were obtained from Sigma Chemical Company. All other chemicals, with the exception of those listed below, were obtained from Sigma. Caesium chloride was obtained from BDH, glycerol from Fisons, sodium dodecyl sulphate (SDS) and ammonium persulphate (APS) from Bio Rad. Iso-amyl alcohol was from May and Baker, Formvar was from Agar Aids, silver nitrate was from Johnson Matthey Materials Technol. U.K. and protein molecular weight standards were from Pharmacia. Restriction enzymes were obtained from Amersham International and pHC79 came from Boehringer Mannheim.

2.4 Growth and Maintenance of bacterial cultures

All *Erwinia* strains and *Escherichia coli* strains were grown at 27°C and 37°C respectively unless stated differently in the text. Liquid cultures (10 ml) were grown in 25 ml universals with shaking at 275 rpm in an Aquatherm waterbath.

Strains were maintained on Luria broth agar (LBA) containing appropriate antibiotics and stored at 4°C for one to two months. Cells for long term storage were taken from a fresh overnight and mixed equally with double strength freezing medium and placed at -70°C.

2.5 Isolation of bacteriophages from sewage

500 ml volumes of untreated sewage, activated sludge and effluent were collected

from Finham Sewage Works, Warwickshire, and pooled. The resting mixture was centrifuged in 250 ml pots (9,000 rpm, 15 min, 4°C) in a High Speed 18 centrifuge. The supernatant was collected and the dry constituents of LB added. Chloroform was added to remove bacterial contamination and the centrifugation step repeated. 250 ml supernatant was transferred to a 2 l flask and a 5 ml overnight culture of the bacterial strain added, together with magnesium ions (10 mM), calcium ions (10 mM), tryptophan (0.2%), casamino acid (0.2%) and antibiotics (50 μg/ml) if selection was possible (dependant on enrichment strain).

Originally incubation was carried out at 15°C, 25°C and 30°C to determine the most satisfactory temperature for phage isolation. Subsequent isolations were carried out at 25°C. After 24 to 48 h incubation in a shaking waterbath at 250 rpm, when cells had reached the end of exponential growth, the suspension was recentrifuged and stored over chloroform. After serial dilution the lysate was incubated with 300 μl bacterial culture (25°C, 15 min) and a 0.7% soft agar overlay made on an LBA plate. This was incubated overnight at the temperatures given above.

2.6 Plaque purification and preparation of high titre lysates

Plaques were removed from the bacterial overlay with a Pasteur pipette and resuspended in 200 μl phage buffer containing 10 μl chloroform. Phages were left to elute at 4°C for 4 to 6 hours, before titration. After overnight incubation at 25°C, 2 ml phage buffer was added to each plate and a further 4 to 6 hours allowed for phage elution. The resultant lysate was stored over chloroform at 4°C. The lysates were titred by serial dilution. In some cases titrations were done in duplicate to assess the requirement of magnesium ions (10 mM $MgSO_4$) for phage adsorption.

2.7 Host range determination

A high titre phage lysate (10 μl) was spotted onto a number of soft agar overlays containing the bacterial strains to be tested. After overnight incubation at 25°C the ability of phages to produce plaques on the strains were noted.

2.8 Ultraviolet light inactivation

Phage lysates diluted 1 in 10 in phage buffer, to a final volume of 5 ml, were exposed to short wave ultraviolet light ($8\mu W/cm^2 x100$) for time intervals of approximately 3 sec between 0 and 21 sec. At each time point 100 μl lysate was dispensed into 1.5 ml Eppendorf tubes on ice and titred to obtain isolated plaques after overnight incubation at 25°C.

2.8.1 Ultraviolet light inactivation of generalised transducing phage

Irradiation of the phage lysate was carried out as previously described, but with exposure to U.V. light at time intervals of 5-10 sec between 0 and 40 sec. After each time period 100 μl of lysate was cooled on ice before being added to recipient bacteria and used in transduction assays.

1.9 Heat inactivation

Phage lysates were diluted as above into preheated phage buffer and incubated at 65°C in an Aquatherm waterbath, for time intervals of 5-10 sec between 0 and 40 sec.

2.10 Test for lysogeny

Phage lysate (10 μl) was spotted onto a bacterial lawn and incubated at 25°C overnight. Agar from this region was spread onto LBA media and incubated overnight at 30°C. Individual colonies were streaked once, through a dried phage well on LBA, a sensitive bacterial control being used in each case. Putative lysogens were initially identified by their resistance to phage attack. To confirm the existence of lysogens each was grown up overnight at 30°C and a top agar lawn made of each. Wild type phage (10 μl) was spotted onto the lawn and if no phage action was seen, the culture supernatant of the test strain was spotted onto a wild type bacterial lawn. Phage release from the test host was taken to indicate presumptive lysogeny.

2.11 Electron microscopy

Copper grids (3.09 mm) (Agar Scientific Ltd.) were prepared for electron microscopy by formvar coating. A clean, dry cover slip was dipped into a 1.5% formvar in chloroform solution, allowed to dry in the vapour and, after scoring with a sharp blade, the formvar coat was floated onto a water surface. Grids were placed onto the formvar and the coated grids removed from the water with heavy aluminium foil. A phage suspension (approximately 10^8 p.f.u./ml) was centrifuged (5,000 rpm, 15 min) in an MSE Hi-Spin 21 and the supernatant recentrifuged (13,000 rpm, 90 min). The pellet was resuspended in 2% (w/v) phosphotungstic acid (pH 7.0) and 10 μl placed onto a formvar coated grid, blotted to remove excess liquid and allowed to dry in air. Grids were examined using a Jeol Electron Microscope at 60 kV.

Bacterial samples for examination were grown, without shaking, at 27°C for 18 h and 10 μl placed onto a formvar coated grid for 1 min. The liquid was removed with blotting paper and 10 μl stain added and removed immediately before allowing the grid to air dry.

2.12 Lambda transduction and transposon mutagenesis

Bacterial cells (10 ml) containing pHCP2 were grown at 30°C for 18 hours in DDA containing maltose (0.2%), $MgSO_4$ (10 mM) and ampicillin. The cells were centrifuged in an MSE Chilspin (5,000 rpm, 10 min, room temperature) and resuspended in 1 ml DDA containing 10mM $MgSO_4$. Lambda::Tn*5* was added at a concentration of 10^9 p.f.u./ml and incubated at 30°C for 30 min. The cells were recentrifuged (5,000 rpm, 10 min, room temperature) and washed in 0.1 M sodium citrate before recentrifugation and resuspension in 10 ml LB. The suspension was incubated at 30°C for 30 to 45 min, recentrifuged and resuspended in 1 ml DDA. Samples (100 μl) were spread onto each of three DDA plates containing kanamycin sulphate to select for the presence of the transposon, and the plates incubated for 24 to 48 h at 30°C. Kamamycin resistant colonies were patched onto a number of plates containing minimal media and a range of amino acids. The ability to grow on supplemented plates allowed identification of a number of auxotrophies.

Transduction of plasmids or cosmids into *E.coli* via Lambda was essentially as described above, with the following differences; growth of *E.coli* did not require the addition of Ap, and after incubation to allow expression of antibiotic resistance (if appropriate) cells were plated onto media containing appropriate antibiotics.

2.13 Generalised transduction assays

All phages isolated were tested for the ability to transduce certain markers between strains of *Erwinia*. The assay was carried out essentially as for the production of auxotrophic mutants, except that the auxotrophic mutants were grown in kanamycin initially. The phage to be tested was added at a known multiplicity of infection (m.o.i.) which was varied to offer the greatest chance of success. Adsorption was carried out at 25°C for varying amounts of time and after a final wash in minimal medium the cells were plated onto minimal agar containing glucose as the carbon source. As well as the ability to transduce chromosomal genes between strains the ability to transduce plasmids was also tested, the transduced plasmid (pHCP2) being selected on LB containing ampicillin. The frequencies of generalised transduction were calculated after subtracting the number of spontaneous prototrophic colonies from control plates (as above without the addition of phage).

2.14 Phage adsorption assay

Bacterial cells were grown at 27°C overnight. To 10 ml bacterial cells (approx. 1.0 x 10^8 c.f.u./ml) phage lysate was added to a final titre of approx. 10^9 p.f.u./ml, and the mixture incubated at 27°C. At time intervals of 0, 10, 20, 30, 40 and 60 min, 100 μl of the mixture was removed and diluted 100 fold in ice cold phage buffer, followed by centrifugation in an MSE Chilspin (4,000 rpm, 10 min, 4°C). These supernatants were titred on a permissive host.

2.15 The production of phage resistant mutants

A 10 ml culture of *Eca* SCRI1043 was grown overnight at 27°C. A 0.3 ml sample

of culture was gently mixed with 100 μl of a high titre lysate of phage (approx. 10^{10} p.f.u./ml), a soft agar overlay was made and incubated at 25°C for 48 to 76 hours. After this time colonies that grew were tested for phage resistance by spotting 10 μl of phage onto a lawn of the cells. To confirm that this phage resistance was not due to lysogeny, each colony was grown overnight at 30°C and its supernatant spotted onto a lawn of wild type bacteria. Lack of phage action indicated a phage resistant mutant and not a lysogen.

2.16 Cross testing of the mutants with other phages

A number of phage lysates (10 μl) were spotted onto each of 40 lawns, each seeded with one phage resistant mutant. Phage sensitivity, resistance and decreased efficiency of plating (e.o.p.) was noted in each case.

2.17 Inoculation of miniplants

Growth of potato (*Solanum tuberosum*) plants (cultivar Maris Bard, used because of its particular susceptibility to blackleg infection) was as described by Hinton *et al.* (1989a). When plants were approximately 5 to 10 cm tall and the stems approximately 2 mm thick, virulence tests were performed. Phage resistant mutants were patched onto LB agar and incubated at 27°C for 48 hours. Cells from each plate were inoculated into the top third of a plant stem using a sterile cocktail stick and the wound immediately covered with Vaseline. Inoculated plants were placed inside polythene bags and exposed to a 16 h light regime and 80 to 100% humidity at 20°C for 5 to 7 days. After this time blackleg symptoms were scored using a numerical index; 0, no reaction; 1, slight browning around inoculation site; 2, slight blackening around inoculation site; 3, small black rot spreading from the inoculation

site; 4, medium black rot spreading from inoculation site; 5, large black rot and stem collapse (Fig. B1). Phage resistant mutants which gave symptoms of 1 and 2 were retested on several further occasions on both micropropagated plants and on tuber grown plants (approx. 40 cm tall). Mutants which continued to give such symptoms were termed reduced virulence (Rvi-) mutants.

2.18 Tuber maceration

Bacterial cells were grown in LB (27°C, overnight), washed in diluting buffer and diluted in this buffer to a final density of approx. 1.0×10^7 c.f.u./ml. Potato tubers (Maris Bard) were surface sterilized by immersion in 5% chloros (5 min), rinsed with sterile distilled water and air dried. Using a 200 μl micropipette tip, 10 μl of bacterial cell suspension (1.0×10^7 c.f.u./ml) was injected into an intact tuber, to a depth of 15 mm. Replicates of the same suspension were inoculated up to 5 times into the same tuber. This procedure was repeated for a bacterial suspension of 1.0×10^5 c.f.u./ml. The tubers were placed in a Forma Scientific anaerobic incubator and covered with black polythene bags to reduce exposure to light. The tubers were incubated (20°C, 6 days) and sliced along the axis of injection, and the diameter of the rot measured (mm). Each strain was inoculated on 12 occasions and an average diameter taken.

2.19 Extracellular enzyme plate assays

2.19a Inoculation and incubation

Fresh colonies were picked and patched onto PL, Cel and Prt assay media. Plates were incubated for 24-48 h at 27°C.

Figure B1 Stem rot symptoms caused by Eca SCRI1043 on micropropagated potato plants (Maris Bard).

1 = slight browning around inoculation site
2 = slight blackening around inoculation site
3 = small black rot spreading from inoculation site
5 = large black rot, accompanied by stem collapse

2.19b Protease assay

The medium described in Table B4 was from a recipe of Thurn and Chatterjee *et al.* (1985). After incubation, plates were flooded with 4 M $(NH_4)_2SO_4$. After approximately 30 min protease production was indicated by a clear zone on a white opaque background.

2.19c Pectate lyase assays

PL assays were modified from the method of Andro *et al.* (1984). After incubation on PL media plates were flooded with 7.5% (w/v) copper acetate and left for 1-2 h. PL^+ patches gave a creamy double halo on a translucent light blue background.

2.19d Cellulase assay

Cel assay media was modified from that of Gilkes *et al.* (1984). Cel activity was revealed by flooding plates with 0.2% 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^+ patches gave a red translucent halo on a dark blue background.

2.19e Spectrophotometric enzyme assays

Each strain to be assayed was grown in LB (27°C, overnight). The strains were subcultured 10 μl to 5 ml into PL minimal media containing appropriate antibiotics and incubated at 27°C with shaking in a Aquatherm waterbath (275 rpm) overnight, to similar densities of approximately 4.0 (A_{600}). The cells were pelleted by centrifugation in a MSE Chilspin centrifuge (5,000 rpm, 10 min) and the

supernatant decanted into Eppendorf tubes. Several aliquots of each supernatant were frozen at -20°C until assayed. The remaining cells were washed once in 5 ml PL minimal media, resuspended in 5 ml of this medium and transferred to 25 ml beakers. The suspension was sonicated in a Jencons sonicator using a 3/4 inch probe (3 x 30 sec at amplitude 6, 30 sec cooling intervals). Cell debris was pelleted by centrifugation (5,000 rpm, 10 min) and the sonicate decanted to several Eppendorf tubes and stored at -20 °C until assayed. Samples for analysis were only thawed once.

PL assays were performed as follows; a 22.5 µl sample was added to 876 µl PL reaction mix (Table B4) (preheated to 37°C), in a 1 ml Quartz cuvette and the change in adsorbance at A_{235} (in a preheated cuvette holder at 37°C) monitored over time in a Philips PU 8720 UV/VIS scanning spectophotometer. PL activity was expressed as change in adsorbance (A_{235})/min/ml, against a blank of PL reaction mix alone.

Cel assays were performed as follows; 70 µl sample was added to 287 µl Cel reaction mix (Table B4) and incubated (3 h, 30°C). The reaction was stopped with 3 volumes of ethanol/acetone (2:1) and left for 5-30 min at room temperature. After this time the sample was spun at high speed (13,000 rpm) in a microfuge (10 min) and 0.75 ml supernatant removed and adsorbance measured at A_{550} in an LKB Biochrom tra-spectrophotometer 4050 against water as a blank.

2.19f Isoelectric focusing of PL isozymes

PL isozymes from both supernatants and sonicates (Section 2.19e) were analysed by isoelectric focusing on polyacrylamide gels. The constitution of buffer and solutions is given in Table B5. The gel was poured between glass plates containing a 3 sided

Table B5

Analytical Isolectric Focusing

The Gel:	3.1 ml 29.1% (w/v) acrylamide
	2.7 ml 0.9% (w/v) Bis
	7.6 ml 50% (w/v) glycerol
	0.9 ml 40% ampholytes
or	1.8 ml 20% ampholytes
	2.75 ml or 1.85 ml water
	18 μl TEMED + 1 ml 10 mg/ml APS

PL overlay:

PL overlay	15 ml PL reaction buffer
	15 ml 5.75 mg/ml PGA
	0.3% agarose

PL reaction buffer:

Per 100 ml;

76.8 ml water
23.0 ml 1 M Tris (pH 8.5)
78 μl 1 M CaCl

spacer (1 mm), clamped with 6 bulldog clips and left to polymerize (4 h). One glass plate was removed and the other, carrying the gel, was placed onto a paraffin covered, precooled (6°C) IEF gel rig (LKB Multiphor II electrophoresis unit), avoiding bubbles between the glass plate and IEF rig to allow uniform cooling. Presoaked electrode wicks (1 mm shorter than the gel itself) were placed onto each side of the gel. A 1 M NaOH soaked wick was used as the cathode and a 0.3 M citric acid soaked wick as the anode. Pre-electrophoresis was carried out for 45 min at 12 watts constant power. Samples (15 μl), presoaked into filterpaper wicks, were applied to the centre of the gel and were removed 10 min into electrophoresis. Cytochrome C (5 μl, 10 mg/ml, pI = 10.35) was added as a marker to monitor the progress of electrophoresis. Electrophoresis was carried out at 10 watts constant power until the cytochrome C had formed a tight band (approximately 85 min). The electrode wicks were carefully removed from the gel and a pectate-agarose gel (Table B5) applied for 90 min. After incubation the pectate-agarose gel was stained with 1% cetyltrimethyl ammonium bromide (CTAB) to reveal zones of clearing (PL activity) on an opaque white background.

2.20 Motility assay

Freshly grown (27°C) bacterial colonies were scraped from an agar plate and stabbed into tryptone swarm agar (motility agar), by the method of Wolf and Berg (1989). Incubation was carried out for 24 h and 48 h. After this time the ability of the cells to swarm from the site of inoculation was noted. Cells were also visualised by electron microscopy (Section 2.11) and light microscopy (phase contrast) using an Olympus light microscope.

2.21 Effect of osmolarity on motility

The ability of non-motile cells to swarm on motility agar was investigated at different NaCl concentrations. Cells were inoculated as previously described into motility agar containing zero, 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl, and swarming ability assessed.

2.22 Growth in the presence of surface active agents

Bacterial cells were grown at 27°C overnight, serially diluted and spread onto LBA containing deoxycholate (DOC), sodium dodecyl sulphate (SDS), Triton X-100 or ethylene diamine tetraacetic acid (EDTA) at 0.1%, 0.01% or 0.001% (w/v) for DOC, SDS and EDTA and (v/v) for Triton X-100. A reduction in c.f.u. on these plates indicated an increased sensitivity to the agents.

2.23 Siderophore production and growth in iron depleted medium

Fresh colonies of *Eca* SCRI1043 and mutants A5/22 and A5/8 were streaked onto siderophore medium (Schwyn and Neilands 1987) (along with *Echr* 3937 as a positive control) and incubated at 27°C overnight. The production of siderophores was determined by a colour change from blue to orange. The ability of these strains to grow in iron depleted medium was investigated by the ability to grow in Tris medium for 1-2 days at 27°C. *Echr* 3937 was used as a positive control for this, while AN193-60 was used as a negative control.

2.24 The extraction of outer membrane proteins

Bacterial cells were grown to late exponential phase (10^8 c.f.u/ml) in minimal medium containing glucose at (0.2% w/v) at 27°C. The culture was centrifuged in an MSE multex (4,500 rpm, 15 min) and the supernatant discarded. The pellet was resuspended in 4.5 ml of ice cold culture medium. Further steps were carried out on ice. Immediately before sonication 0.5 ml Triton X-100 (20% v/v), EDTA (1 mM) was added to the suspension and the suspension sonicated (Jencons sonicator) for 5 to 6 one minute pulses at an amplitude of 6 until clear. The sonicated suspension was then centrifuged (8,000 rpm, 4°C) and the pellet discarded. The supernatant was centrifuged in a Beckman L8 ultracentrifuge (25,000 rpm, 90 min) and the supernatant discarded allowing any remainder to drain by inverting the tube. Sample buffer (10 µl) was added (Table B6) and the sample vortexed to resuspend the pellet before overnight storage at 4°C. After this time the sample was warmed at 37°C, vortexed until thoroughly resuspended and pooled in 30 µl fractions. If not used immediately, these were stored at -20°C.

Protein gels were run according to the method of Laemmli (1970). Just before use samples were incubated at 100°C for 5 to 10 min. Samples (30 µl) were load onto an 11% polyacrylamide gel, with a 3% stacking gel (Table B6) and run at 60 volts (using a Bio-Rad Protean gel system) until the tracking dye (bromophenol blue) was through the stacking gel, then at 160 volts until the tracking dye had reached the bottom of the gel. Before staining, the gel was placed in methanol 50% (v/v), acetic acid 10% (v/v) overnight, although to speed up the procedure this step was often omitted without any visible diffusion of protein bands. Protein molecular weight markers (Pharmacia) included Phosphorylase b (94,000), Albumin (67,000), Ovalbumin (43,000), Carbonic Anhydrase (30,000), Trypsin inhibitor (20,100) and α-Lactalbumin (14,400).

Table B6

Electrophoresis of proteins (Silhavy *et al.* 1984)

Acrylamide stock 300 g acrylamide	8 g bisacrylamide water to 1 litre
4 x Lower buffer (1.5 M Tris-HCl, pH 8.8, 0.4% SDS)	181.7 g Tris base 40 ml 10% SDS water up to 1 litre
4 x Upper buffer (0.5 M Tris-HCl, pH 6.8, 0.4% SDS)	60.6 g Tris base 40 ml 10% SDS water up to 1 litre
0.1% Bromophenol blue (BpB)	10 mg bromophenol blue 10 ml water
2 x Sample buffer (SB)	12.5 ml 4 x upper buffer 20.0 ml glycerol water up to 60 ml
2 x Loading buffer (LB)	0.5 ml ß-mercaptoethanol 0.25 ml 0.1% BpB 4.0 ml 10% SDS 5.3 ml 2 x SB
4 x Running buffer (RB)	60 g Tris base 288 g glycine water up to 5 litres
Stain	125 ml isopropanol 50 ml acetic acid 325 ml water 1.25 g Coomassie brilliant blue
Destain	1.0 litre methanol 1.4 litres acetic acid water up to 20 litres
Acrylamide concentration (11% final)	10 ml 4 x Lower buffer 14.6 ml Acrylamide stock 15.2 ml water 0.2 ml 10% Ammonium persulphate
Stacking gel (3%)	2.5 ml 4 x upper buffer 1.0 ml acrylamide stock 6.4 ml water 0.2 ml 10% ammonium persulphate (prep. fresh)

Staining was carried out in 400 ml Coomassie blue (Bio Rad) (25% propan-2-ol, 10% glacial acetic acid, 0.1% Coomassie blue) overnight, followed by 2 changes of destain (25% propan-2-ol, 10% glacial acetic acid) until the background cleared.

2.25 Extraction of lipopolysaccharide

LPS was extracted as described by Westphal *et al.* (1965). Bacteria (2.0 g dry weight) were suspended in 350 ml water at 65-68°C. An equal volume of 90% phenol, preheated to 65-68°C, was added and the mixture stirred vigorously, followed by a further 10-15 min incubation at this temperature. After this time the mixture was cooled to approximately 10°C by placing the vessel in an ice bath, and centrifuged in a Hi-Speed 18 centrifuge (3,000 rpm, 30-45 min). Of the three layers produced (water, phenol and insoluble residue), the water was removed and retained. A further 350 ml of water was added to the phenol and insoluble residue and the process repeated. The combined water extracts were dialysed (3-4 days) against distilled water to remove phenol and small amounts of low molecular weight bacterial substances. The dialysed solution (which appeared slightly opalescent) was concentrated (at 35-40°C) in a rotary evaporator (Buchi Rotovapor-R 382) to a final volume of approximately 100 ml. The concentrate was centrifuged to remove traces of insoluble material and freeze dried (Speedivac model 5PS) to a white fluffy powder.

To remove nucleic acids, the powder was resuspended in double distilled water and centrifuged in a Beckman L8 ultracentrifuge (35,000 rpm, 6-8 h), the sediment resuspended in water and recentrifuged (40,000 rpm, 3h). The final sediment was resuspended in 150 ml water followed by the addition of a 2% aqueous solution of cetavlon. The mixture was stirred (15 min, room temperature), centrifuged (3,000 rpm, 20 min) to remove precipitated RNA, freeze dried, and resuspended in 50-60

ml 0.5 M NaCl. The suspension was poured into a 10 x volume of ethanol to precipitate the LPS, allowed to stand (1-2 h, 4°C), then the precipitate was centrifuged and resuspended in water. After dialysis against deionized water for 2 days to remove the NaCl, the suspension was freeze dried to yield RNA free LPS. The resultant powder was again resuspended in water, and if not used immediately was stored at -20°C.

2.26 Electrophoresis and staining of lipopolysaccharide

Samples of LPS were incubated at 37°C and vortexed until homogeneous, before loading (3-5 μl) onto an SDS polyacrylamide gel (Table B7). Samples were run at 60 V to allow the tracking dye (bromophenol blue) to travel through the stacking gel, followed by 160 V until the dye was 1-2 cm from the bottom of the gel. Staining of the gel was carried out as described by Hitchcock and Brown (1983), in clean glassware, using double distilled water and washed gloves. The gel was placed in 300 ml methanol 50% (v/v), acetic acid 10% (v/v) overnight, followed by 1 h in 300 ml acetic acid 7.5% (v/v) and 10 min in 300 ml periodic acid 0.7% (w/v) containing a 2% (v/v) mixture of methanol 50% (v/v) and acetic acid 10% (v/v). The gel was washed three times in 500 ml water, placed in freshly prepared silver stain for 10 min. Silver stain (300 ml) was prepared by mixing 2.5 ml NH_4OH (25%) into 56 ml NaOH 0.4% (w/v), and during mixing 10 ml of 20% (w/v) $AgNO_3$ was added followed by 230 ml water. Any brown precipitate remaining after the addition of water was removed by adding NH_4OH drop-wise until clear. The silver stained gel was revealed (for 1 l; 50 mg citric acid, 0.5 ml formaldehyde (37%)) until colouration reached its maximum, without background. Colouration was stopped by transferring the gel rapidly to acetic acid 7.5% (v/v) for 10-30 min, followed by washing twice for 15 min in 500 ml water.

Table B7

Lipopolysaccharide electrophoresis buffers and solutions (Hitchcock and Brown 1983)

Lower gel buffer (x4)	1.5 M Tris-HCl, pH8.8
Upper gel buffer (x4)	0.5 M Tris-HCl, pH 6.8
Electrophoresis buffer	0.025 M Tris-HCl 0.18 M glycine 0.18% (w/v) SDS (pH 8.3 -8.7)
Sample buffer	For 100 ml; glycerol 12.5 ml, SDS 1 g, stacking buffer (x4) 25 ml, EDTA (0.5 M) 1 ml; add bromophenol blue as tracking dye.
Loading buffer	as Table 6
Acrylamide stock	as Table 6
Acrylamide concentration (11% final)	10 ml 4 x lower buffer 14.6 ml Acrylamide stock 15.2 ml water 0.2 ml 10% ammonium persulphate
Stacking gel (3%)	2.5 ml 4 x upper buffer 1.0 ml acrylamide stock 6.4 ml water 0.2 ml 10% ammonium persulphate (freshly prepared)

2.27 Chromosomal DNA extraction from *Eca*

DNA extraction was carried out by the method of Marmur (1961) with modifications. Cells were grown to stationary phase in LB (500 ml, 30°C, 150 rpm, 16 h), and the culture 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) and transferred to two 50 ml polycarbonate "Oakridge" tubes, before being mixed with 4 ml lysozyme solution (10 mg/ml, in TES), and kept at 37°C for 30 min. SDS (2ml, 20% (w/v)) was added, the tubes were inverted several times, and were stored at 60°C for 15 min until lysis occurred. Sodium perchlorate (10 ml of 5 M) was then added to help DNA-membrane separation. The solution was transferred to universals and extracted with an equal volume of phenol saturated with TES, followed by gentle mixing of the solution to form an emulsion. The tubes were centrifuged in an MSE Chilspin (5,000 rpm, 5 min, 4°C), and the upper aqueous layer transferred 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 (0.3 M final concentration) followed by an equal volume of 100% ethanol. Solutions were mixed gently and incubated on ice for 15 min. The DNA was collected by centrifugation in an MSE Hi-spin 21 (8,000 rpm, 10 min, 4°C) and the DNA pellet dried under vacuum for 30 min before resuspension in 15 ml TE (overnight, 4°C with gentle agitation). RNAase (2 mg/ml in TE, heat treated, Maniatis *et al.* 1982) was added (100 μg/ml final concentration) and the solution incubated at 37°C for 30 min. After an extraction with phenol/chloroform (section 2.32) and an extraction with chloroform/iso-amyl alcohol, sodium acetate (3 M, pH 4.8) was added to the DNA solution (0.3 M final concentration), followed by an equal volume of 100% ethanol to aid in DNA precipitation (ice, 15 min). After centrifugation (8,000 rpm, 10 min, 4°C) the DNA pellet was washed in 70% ethanol (v/v), followed by recentrifugation and then drying under vacuum (30 min). TE (10-15 ml) was added

to resuspended the DNA (overnight, 4°C).

2.28 Isolation of plasmid DNA

2.28a Large scale preparation

Large scale plasmid DNA isolation was carried out as described by Clewell and Helinski (1970). *E.coli* or *Erwinia* cells were grown to 2-4 x 10^8 c.f.u./ml (A_{600} - 0.5) in 500 ml NB containing appropriate antibiotics for the selection of plasmids. Cells were collected by centrifugation in an MSE Hi-spin 21 Centrifuge using 6 x 300 ml rotor (10,000 rpm, 10 min, 4°C) and resuspended in 16.5 ml Tris-sucrose solution (0.05 M Tris-HCl, pH 8.0, 25% (w/v) sucrose) before being transferred to siliconised 250 ml bottles (Section 2.36). 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. Lysis mix (18 ml, 0.05 M Tris-HCl, 0.0625 M EDTA, 2% Brij 58, 0.4% sodium deoxycholate, pH 8.0) was added to lyse the cells and the mixture was agitated gently until it cleared. This process was aided by incubation at 42°C for a few minutes. The lysed solution was transferred to 50 ml polycarbonate "Oakridge" tubes and the chromosomal DNA, together with unlysed cells and cell debris, was pelleted in an MSE Hi-Spin 21 centrifuge using a 8 x 50 ml rotor (18,000 rpm, 20 min, 4°C). The supernatant was 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 supernatant, 7.38 g CsCl and 0.27 ml ethidium bromide (2.5%, w/v) were gently mixed at room temperature until all the CsCl had dissolved. The resulting solution was transferred to 10 ml Beckman polyallomer tubes and any remaining space was filled with liquid paraffin. Gradients were centrifuged in an L8 ultracentrifuge (36,000 rpm, 60 h, 15°C). The plasmid DNA, viewed under 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 equilibriated 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, and the DNA solution was dialysed against TE (5 l, 4 h), and then overnight in fresh TE (5 l).

2.28b Rapid small scale preparation

The "mini-prep" of plasmid DNA from *E.coli* and *Eca* was based on the method described by Maniatis *et al.* (1982). Cultures were grown overnight in 5 ml NB with antibiotic selection if appropriate. Cells were collected by centrifugation in an MSE Multex centrifuge (5,000 rpm, 10 min), resuspended in phage buffer, and transferred to 1.5 ml Eppendorf tubes. All following centrifugation steps were done in an MSE microcentaur at high speed and at room temperature. After centrifugation (1 min), the cells were resuspended in the drop of liquid remaining in the tube after discarding the supernatant. 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. Following this, 200 µl of solution II (0.2 M NaOH, 1% SDS; stable for 2 weeks at 4°C) was added, and the tubes inverted rapidly before being stored on ice for 5 min. The contents of the tubes were mixed by inversion to obtain an almost clear solution. Ice-cold potassium acetate (150 µl, pH 5.0; made by mixing 60 ml 5 M potassium acetate with 11.5 ml glacial acetic acid and 28.5 ml of water) was added, and the tubes agitated by rapid inversion and brief vortexing. After 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 decanted to a fresh tube. An equal volume (400 µl) of phenol/chloroform (Section 2.32) was added, the tubes

vortexed for 5 sec, and the layers separated by centrifugation (1 min). The upper aqueous layer was transferred to a fresh tube containing 400 μl $CHCl_3$/iso-amyl alcohol (24:1), vortexed for 5 sec and recentrifuged (1 min). The upper layer was again removed to a fresh tube, and two volumes of 100% ethanol (room temperature) were added. After vortexing for 2 sec and incubation at room temperature for 2 min, the DNA precipitate was collected 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 recentrifuged (5 min). The supernatant was carefully removed, remaining drops being 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 incubated at 65°C for 5 min. For restriction digests 3 μl of the DNA solution was used in each case.

2.29 Small scale isolation of phage DNA

Phage DNA was prepared by rapid, small scale isolation as described in Maniatis *et al.* 1982. Phage suspension (approximately 10^6 phage) was mixed with approx. 10^8 bacterial cells in stationary phase and incubated (25°C, 15 min). LB (4 ml) was added and the suspension incubated in a shaking water bath at 25°C for 9 h. In some cases the culture was seen to clear. Chloroform (100 μl) was added to the culture and shaking was continued at 25°C for a further 15 min. The lysate was then transferred to a 5 ml polypropylene centrifuge tube and centrifuged in an MSE Hi-spin 21 (10,000 rpm, 10 min, 4°C). The supernatant was recovered and RNase A and DNase 1 added, each to a final concentration of 1 μg/ml. This was incubated for 30 min at 37°C. An equal volume of a solution containing 20% (w/v) polyethylene glycol and 2 M NaCl in phage buffer was added and the mixture incubated for 1 hour at 0°C (ice water). The precipitated phage particles were recovered by centrifugation (15,000 rpm, 20 min, 4°C). The supernatant was

drained, 0.5 ml phage buffer added, and the phage particles resuspended by vortexing. This was centrifuged (10,000 rpm, 2 min, 4°C) to remove debris. The supernatant was transferred to a fresh 1.0 ml Eppendorf tube, 5 μl of 10% SDS and 5 μl of 0.5 M EDTA (pH 8.0) added and this was incubated at 68°C for 15 min. Extraction with phenol, phenol/chloroform and chloroform was carried out (Section 2.32), the aqueous phase being transferred to a fresh Eppendorf between each extraction. To the final aqueous phase an equal volume of isopropanol was added before storage at -70°C for 20 min. This was then thawed and the DNA pelleted by centrifugation for 15 min at 4°C. The pellet was washed in 70% ethanol, dried and resuspended in 50 μl TE (pH 8.0).

2.30 Restriction endonuclease digestion

The salt buffers (low, medium and high) were made at 10 x concentration and used as described by Maniatis *et al.* (1982). 10 x restriction buffer (1 μl) was added to the DNA (9 μl), restriction enzyme was added and the digestion was carried out at 37°C for at least 90 min. Restriction digests of plasmid DNA prepared by the small scale method were incubated in the presence of RNase A (100 μg/ml).

2.31 Ligation

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

the mixture incubated (15°C for at least 18 h).

2.32 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 mixture was equilibriated by shaking with two changes of 1 M Tris (pH 8.0) at a volume of 0.2, before being stored at 4°C. With gentle shaking, DNA samples were mixed with an equal volume of phenol/chloroform, to form an emulsion. The two layers were separated by centrifugation in an MSE microcentaur (1 min, high speed). The upper aqueous layer was decanted to a fresh tube, and the extraction process was repeated on this layer. To remove any remaining phenol, a further extraction with chloroform/iso-amyl alcohol (24:1) was carried out as described above. Maximum recovery of DNA was achieved by mixing the phenolic phase with an equal volume of TE and centrifuging (1 min, high speed). The aqueous phase was then extracted with chloroform/iso-amyl alcohol and pooled. DNA was recovered by ethanol precipitation.

2.33 Ethanol precipitation

Half a volume of ammonium acetate (7.5 M, pH 7.5) and three volumes of ethanol (-20°C) were added to a DNA solution, mixed by vortexing and chilled at -20°C overnight. The DNA was harvested by centrifugation in an MSE microcentaur (10 min, high speed, room temperature). After discarding the supernatant, ethanol was removed from the tube walls. The DNA pellet was dried under vacuum followed by resuspension in TE buffer.

2.34 Agarose gel electrophoresis

Horizontal agarose slab gels were prepared by boiling agarose in TAE electrophoresis buffer (0.04 M Tris-acetate, 0.001 M EDTA). 0.7% (w/v) gels were used routinely. Before pouring, agarose was cooled to approximately 50°C and 0.5 μg/ml ethidium bromide added. Loading buffer (0.1 volume of a mixture of 0.25% bromophenol blue and 15% Ficoll type 400) was added to the DNA samples before being loaded into the gel slots. During electrophoresis the gel was completely submerged in electrophoresis buffer and run at 80-100 volts, or 25 volts when run overnight. DNA was visualised by transillumination with short-wave UV light (260 nm) (UVP incorporated) and photographed using Polaroid Type 665 film. Restriction fragment sizes of phages and cosmids were determined by comparison with Lambda *Hind*III, after plotting Log10 molecular weight (Y-axis) against migration distance (X-axis).

2.35 Electroelution

*Sau*3A digested chromosomal DNA (20-50) was loaded into a well, spanning the width of a 0.7% acrylamide gel (for gel preparation and equipment see Section 2.34). Initially the DNA was electrophoresed for 2 h at 60 V, after which time a well was cut immediately proceeding the required molecular weight DNA. Electrophoresis buffer (Section 2.34) was lowered to the level of the gel and the well was filled with fresh buffer. The gel was subjected to 100 V pulses for 1 min intervals and the buffer collected and pooled on each occasion. After the required DNA had been removed (approximately 10 cycles), the DNA was phenol extracted (Section 2.32), ethanol precipitated (Section 2.33) and resuspended in 100 μl TE.

2.36 Siliconisation of glassware

Clean glassware was submerged in 2% (v/v) dimethyl dichlorosilane in tetrachloromethane, and left to dry. Following this, glassware was rinsed with double-distilled water.

2.37 Transformation of *E.coli*

Transformation with plasmid DNA was carried out as described by Maniatis *et al.* (1982), with modifications, and using Grade I $CaCl_2$. An overnight culture of DH1 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 treated as described by Maniatis *et al.* (1982). Pre-chilled 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 increased transformation frequencies by approximately 10 times.

2.38 Electroporation

Bacteria were grown in 10 ml of LB (27°C, overnight), pelleted by centrifugation in an MSE Multex (4,500 rpm, 10 min) and washed three times in 10 ml, 5 ml and 1 ml of double distilled water (sterile) respectively. The final pellet was resuspended in 200 μl double distilled water and placed on ice. Cells (40 μl) and DNA (2-5 μl, extracted in phenol and precipitated in ethanol to ensure a salt-free environment) were mixed in a 1 ml Bio Rad Gene pulser cuvette with a 0.2 cm electrode gap, and placed in an electroporator (Bio Rad Gene pulser 4050) preset to 2.5 kV, 25 μFD capacitance and 200 Ohms resistance. After electroporation cells which obtained a time constant below 4.0 ms were discarded. Immediately 1 ml of SOC was added to

the cuvette and the cuvette gently agitated. The contents of the cuvette were decanted into a 1 ml Eppendorf and incubated, with shaking (275 rpm, 27°C, 1 h). Sample (100 µl) was removed and the remaining sample centrifuged, resuspended in 50 µl SOC and plated, together with the 100 µl aliquot, on to LBA containing appropriate antibiotics. This was incubated at 27°C for 2-3 days.

2.39 Construction, maintenance and screening of an *Eca* SCRI1043 gene library

2.39a Construction and maintenance

Chromosomal DNA from *Eca* SCRI1043 (Section 2.27) was digested with *Sau*3a at a concentration of 0.001 units for 1 h and electrophoresed through a 0.7% agarose gel. From the resultant smear, agarose containing DNA in the region of 15-30 kb was cut from the gel and the DNA electroeluted (Section 2.35). Vector pHC79 DNA (Frey *et al.* 1983) was digested to completion with *Bam*HI. The digested DNA was then phenol extracted (Section 2.32), ethanol precipitated (Section 2.33) and resuspended in TE (approximately 30 µl). A 5 µl aliquot of this was removed to use as an unphosphatased control. 1 unit of calf intestinal phosphatase was added to the remainder of the sample and incubated at 37°C for 30 min. 1 µl EDTA (0.5 M) was added to the sample which was again extracted with phenol, ethanol precipitated and resuspended in 20 µl TE (pH 8.0). *Sau*3A digested chromosomal DNA (4-8 µl) was mixed with 1-2 µl of *Bam*HI-digested pHC79, made up to 20 µl and ligated at 15°C for 24 h (Section 2.31), including test ligations containing unphosphatased vector. The ligated mix (2-5 µl) was added to 100 µl aliquots of competent DH1 cells. Following transformation (Section 2.37), cells were incubated in 10 ml SOB (90 min, 37°C) to allow gene expression. A portion was then removed to determine transformation and cloning efficiency, compared to transformation of pHC79 alone. Once a high frequency of transformants, containing

the cosmid library, had been obtained (approx. >200 colonies/100 μl transformant mix), the ligated DNA was *in vitro*-packaged into Lambda (according to the Stratagene Gigapack II protocol). Following *in vitro* packaging of the library, 10 μl or 100 μl was transduced into DH1 (in TB containing 0.2% maltose and 10 mM $MgSO_4$), was incubated with shaking (37°C, 45 min) and after centrifugation in an MSE Multex (4,500 rpm, 10 min) to 1/10 volume, was plated onto LBA containing Ap. After incubation (24-48 h, 37°C) the plates producing >500 colonies were scraped into 1 ml TB and 100 μl subcultured into 5 ml TB Ap and incubated again at 37°C. At an optical density of 0.6 (A_{600}) 250 μl of Lambda CI857 (approx. 10^7 p.f.u./ml) was added to the culture, the culture incubated at 45°C for 2 min followed by incubation at 37°C until lysis ensued. After centrifugation in an MSE Multex (5,000 rpm, 10 min) the lysate was stored over chloroform at 4°C, and titred to ensure a phage concentration of $>1.0 \times 10^9$ p.f.u./ml. From the Lambda CI857 library lysate, cosmid DNA was prepared (Section 2.29) for electroporation into mutants A5/22 and A5/8, and *Eca* auxotrophs (Section 2.38). The gene library was screened for genes encoding PL activity and auxotrophic markers.

2.39b Screening for heterogeneity

The Lambda CI857 lysate containing the cosmid library was transduced into DH1 (Section 2.12) and plated onto PL, Cel and Prt media containing ampicillin (50 μg/ml) to select for cosmid containing transductants. After incubation and development of the plates (Section 2.19), the percentage PL, Cel and Prt producing cells was noted. Any positives were rechecked by patching onto the same media.

Library DNA from the Lambda CI857 lysate was also electroporated (Section 2.38) into a number of *Eca* SCR11043 auxotrophs and the cells plated on minimal glucose Ap media and incubated for 2-3 days. The percentage of cells complemented back to prototrophy was noted (minus spontaneous revertants).

2.39c Screening for complementation of A5/22

Cosmid DNA obtained from a Lambda CI857 lysate (Section 2.29) was electroporated into A5/22 and A5/8 as described in Section 2.38. After electroporation cells were plated onto LB Ap and incubated at 27°C for 2-3 days. Fresh colonies were stab inoculated into motility agar and swarming ability assessed (Section 2.20).

CHAPTER 3

ISOLATION AND CHARACTERISATION OF BACTERIOPHAGES

Chapter 3

3 Isolation and characterisation of bacteriophages

3.1 Introduction

The aim of this work was to isolate phages for a series of laboratory strains of *Erwinia*, to characterise these and use them as tools in the study of *Erwinia* biology. A total of 35 phages were isolated from sewage. The majority of these phages were characterised by plaque morphology, ultraviolet (U.V.) and heat inactivation, structural morphology, restriction pattern and host range. The relationship of the phages to one another and to other phage groups is discussed, together with their use in phage typing. Also discussed are the possible environmental implications arising from this work. In the following two chapters details on the use of the phages will be given.

3.2 Results

3.2.1 Phage isolation

A total of 35 phages were isolated from sewage over a two year period (Table C1). 25 phages were isolated for *Eca* SCRI1043, 5 *for Ecc* SCRI193 and 4 for *Echr* NCPPB1066. The latter will not be discussed in this report.

Phage	Host	Isolation Date	Phage	Host	Isolation Date
A1	*Eca*	6/87	S22	*Eca*	7/88
A2	*Eca*	6/87	S31	*Eca*	11/7/88
A3	*Eca*	6/87	S32	*Eca*	11/7/88
A4S	*Eca*	6/87	S33	*Eca*	11/7/88
A4L	*Eca*	6/87	S34	*Eca*	11/7/88
A5	*Eca*	6/87	S41	*Eca*	25/7/88
A6	*Eca*	6/87	S42	*Eca*	25/7/88
M1	*Eca*	1/88	S45	*Eca*	25/7/88
M2	*Eca*	1/88	S61	*Eca*	16/8/88
M3	*Eca*	1/88	S62	*Eca*	16/8/88
M4	*Eca*	1/88	S65	*Ecc*	16/8/88
D1	*Ecc*	1/88	S71	*Eca*	31/9/88
M5	*Eca*	5/88	S72	*Eca*	31/9/88
D2	*Ecc*	5/88	S75	*Ecc*	31/9/88
S21	*Eca*	7/88	KP	*Ecc*	31/9/88

Table¢1. Dates of isolation of phages on *Eca* SCRI1043 and *Ecc* SCRI193. Phages S21 to KP were isolated by L. Kinersley and G. Pavitt.

3.2.2 Phage characterisation

3.2.2a Plaque morphology

On isolation of the phages a number of different plaque morphologies were observed. Initial characterisation was therefore based on these observations. Plaques could be characterised in terms of size, turbidity and the presence or absence of a halo and these criteria were used in the initial grouping of the phages (Table C2). Clear plaques (CP) ranged from < 1-4mm in diameter, all were circular and those above 1mm possessed clearly visible halos. Turbid plaques (TP) were between < 1mm and 2mm in diameter and had irregular boundaries (Figs C1 and C2).

The majority of *Eca* phages produced clear plaques of varying sizes and were therefore categorised on the basis of these differences. The remaining phages produced turbid plaques, similar in appearance, and were placed in a single group. With the exception of D1 the *Ecc* phages produced turbid plaques, those of ϕKP having a slightly larger diameter than the rest. D1 produced "pin prick" plaques making it difficult to propagate and for this reason little data was obtained on it.

On several occasions during isolation, a phage population of relatively high titre (approximately 10^5-10^7) would emerge. On dilution to single plaques this population appeared homogeneous. To investigate the possibility that this phage population was masking other phage populations of lower titre, a simple screening method was devised. This method involved replating the original phage lysate onto a bacterial mutant resistant to the "high titre" phage. The isolation of mutants resistant to CP phages allowed other phages, both CP and TP, to be exposed. On one occasion this technique uncovered a TP phage (S41) being masked by another TP phage (S45).

Plaque Type	Phages
LCCH (3-4mm)	A1, A4L, A5, M5 S32, S33, S71
MCCH (2-3mm)	S31, S62
SCCH (1-2mm)	M1, M2, M4, S72
SCCH (1mm)	S42, M3
SCCH (< 1mm)	S22
SIT (1-2mm)	A2, A3, A4S, S21,S34 S41, S45, KP, S61
SIT (< 1)	A6, D2, S65, S75
PPP	D1

Table C2 . Plaque morphologies of *Eca* and *Ecc* phages in an LB 0.7% overlay.

LCCH = *L*arge *c*ircular *c*lear plaques with *h*alos
MCCH = *M*edium *c*ircular *c*lear plaques with *h*alos
SCCH = *S*mall *c*ircular *c*lear plaques with *h*alos
SIT = *S*mall *i*rregular *t*urbid plaques
PPP = *P*in *p*rick *p*laques

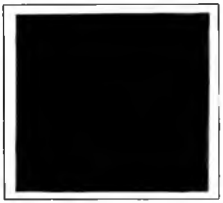
Figure C1

Plaque morphologies of *Eca* phages in a 0.7% soft agar overlayer.

a) Large circular clear plus halo (3-4mm)	phage M5
b) Medium circular clear plus halo (2-3mm)	phage S31
c) Small circular clear plus halo (1-2mm)	phage M2
d) Small circular clear plus halo (1mm)	phage S42
e) Small circular clear plus halo (< 1mm)	phage S22
f) Small irregular turbid (1-2mm)	phage A2
g) Small irregular turbid (< 1mm)	phage A6

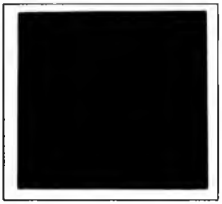
ORIGINAL IN COLOUR

a)

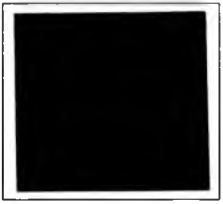


b)

c)



d)



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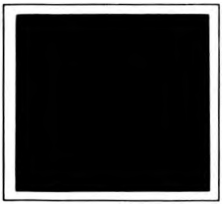
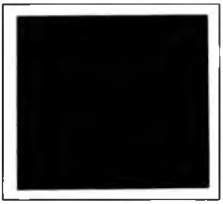


f)



g)

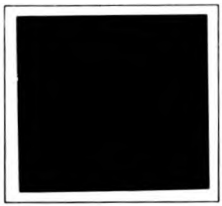
Figure C2

Plaque morphologies of *Ecc* phages in a 0.7% soft agar overlayer.

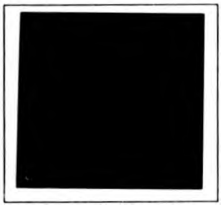
a) Small irregular turbid plaques (1-2mm) phage ϕKP

b) Small irregular turbid plaques (< 1mm) phage D2

a)



b)



Phages isolated in winter time, all produced small, clear plaques. While phages producing similar plaques were isolated at other times of the year, the fact that no TP phages or large CP phages were isolated in winter, may indicate a seasonal variation in phage populations.

3.2.2b Inactivation by ultraviolet irradiation

A number of phages isolated in June 1987 and January 1988 were inactivated by ultraviolet (U.V.) light and their survival over time was compared (Fig C3). The inactivation of all phages produced a linear relationship when survival was plotted against time (inactivation of phages by U.V. irradiation involves mutagenesis of their nucleic acid). Fig. C3a, shows inactivation of TP phages, while Fig. C3b and Fig. C3c show inactivation of CP phages. For comparative purposes the coliphages P1 and T4 were also inactivated and their survival plotted. It should be noted however that the host of P1 and T4 is *E.coli* and as such, a direct comparison with phages propagated on *Erwinia* is open to question. T4 possesses a U.V. DNA repair mechanism and would therefore appear to be inactivated more slowly than would be expected from the size of its genome (Sekiguchi *et al.* 1970; Yasuda and Sekiguchi 1970a, 1970b). P1 DNA (91.5 kb) was inactivated at a slower rate than T4 DNA (166 kb). TP phages were inactivated at similar rates. The CP phages, which, again, had similar rates of inactivation, were inactivated more slowly than the TP phages. This suggests that the genome sizes of the CP phages were smaller than those of the TP phages but larger than that of P1.

3.2.2.c Inactivation by heat

Another method used to compare the phages was inactivation by heat at 65°C. This

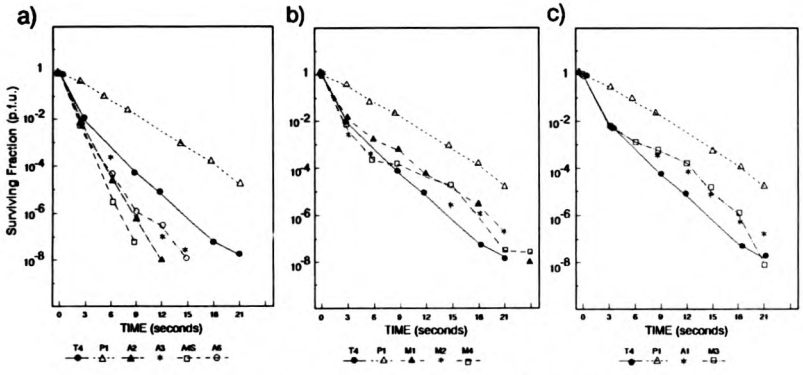


Figure C3. Inactivation of phages by ultraviolet irradiation at 8μW/cm²x100. a) Turbid plaquing phages A2, A3, A4S ans A6, b) clear plaquing phages M1, M2 and M4, and c) clear plaquing phages A1 and M3. Each graph shows the inactivation of T4 and P1 for comparison.

method, like U.V. inactivation, is crude but was included as an alternative method of differentiating between the phages.

As with U.V. inactivation, only a small number of phages were analysed to investigate the value of this technique. The data obtained (Fig. C4) provides little useful information. Two phages, A4S and M1, showed a different inactivation pattern to the rest. In most examples, particularly ϕM1, a biphasic inactivation curve was seen. This indicates the presence of a more heat resistant fraction within each phage population and is a common phenomenon of heat inactivation (Adams 1959).

3.2.2.d Structural morphology

Phage morphology was determined by transmission electron microscopy, allowing differentiation between phages, and classification into Bradley/Ackermann morphological groups (see Section 1.4.3.1). Phages were prepared on formvar coated grids and stained with 2% (w/v) phosphotungstic acid. Three phage groups were observed which were classified according to the Bradley classification system (Fig. A9).

The first group consisted of eight *Eca* phages, possessed isometric heads and contractile tails, and fell into group A (*Myoviridae*) (Fig. C5a). All the phages in group A were of similar size (Table C20). Dimensions were obtained by comparison to Lambda on grids containing a mixture of phages. This group of phages produced turbid plaques and were the only group of *Eca* phages to do so (Fig. C1). Plaques obtained from the group A phages were indistinguishable.

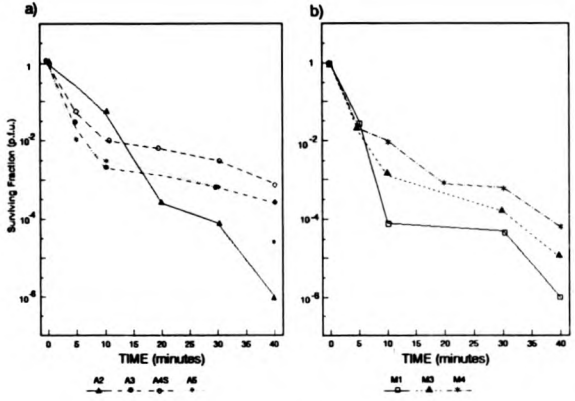


Figure C4. Inactivation of phages by heat at 65°C.
a) Phages A2, A3, A4S and A5. and b) phages M1, M2 and M4.

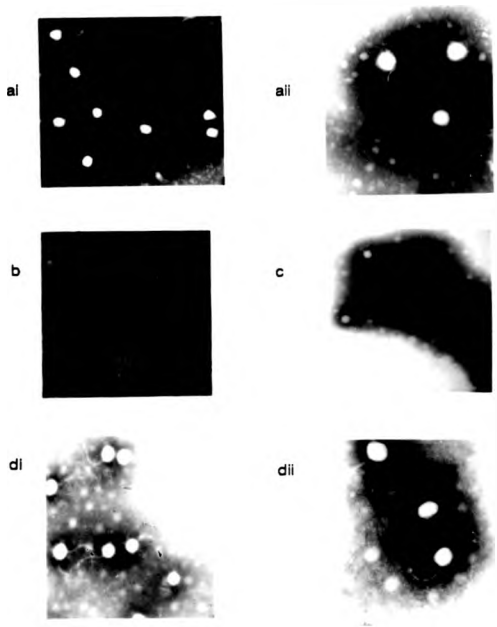


Figure C5. Electron micrographs of Eca and Ecc phages.
a) represents group A phages, b) represents group C phages,
c) represents M2 which possesses a base plate, and d) represents
group B phages. ai, b, c, and di are magnified 50,000x, and aii and dii
are magnified 100,000x.

The remaining sixteen members of the *Eca* phages belonged to group C (*Podoviridae*). Phages in this group were of similar size (Fig. C20) and produced clear plaques, with varying diameters (Fig. C1). The group C phages had isometric heads but possessed only short tails (Fig C5b). In the case of phage M2 a base plate was clearly visible (Fig. C5c).

The remaining phages, all of which were *Ecc*, fell into Bradley group B (*Styloviridae*). The group B phages had isometric heads, long non-contractile tails and were of varying dimensions (Fig. C5d). This group produced turbid plaques which appeared similar in three of the four members (Fig. C2).

3.2.2.e Restriction endonuclease analysis

On the basis of plaque morphology and structural morphology the phages were loosely categorised into three groups (Fig. C16). To compare the phages more closely their nucleic acid was submitted to restriction endonuclease (RE) digestion. Up to six enzymes were used including, *Eco*RI, *Bgl*II, *Bam*HI, *Cla*I, *Hin*dIII and *Hae*III. The nucleic acid of all phages was digested by one or more of the enzymes, indicating that each phage contained double stranded DNA. The migration of restriction bands through agarose gels was compared to a Lambda standard restricted with *Hin*dIII. A plot of the logarithm (log10) of molecular weight (y-axis) against the relative motility (x-axis) was made and band sizes obtained from this. In general it was not possible to obtain values between 1 kb units, due to the accuracy of the technique, so bands which were obviously intermediate could only be estimated.

The DNAs of the group A phages were restricted by *Eco*RI, *Bgl*II, *Bam*HI and *Cla*I. The phages within this group, with the exception of S45 (data not shown),

gave a number of common restriction bands (Table C3, Fig. C6). On digestion with *Eco*RI 7-8 bands were seen in each case. A2 and A3 appeared identical (set 1), as did A4S, A6 and S21 (set 2), with only a single band difference between the two sets. S34 and S41, although different, possessed bands common to both sets. Digestion with *Bgl*II, producing 17-19 bands, also showed similarities within sets 1 and 2 (Table C4, Fig. C7). S41 differed from set 2 by a single band, with S34 showing several differences. The banding pattern of S41 appeared the same as those in set 2 on digestion with *Bam*HI and *Cla*I (5-6 bands and 6-7 bands respectively) (Tables C5, C6, Fig. C8, C9). S34 showed some differences from both sets but common bands were present. The estimated genome sizes of S34 and phages in set 1 were 80-90 kb, compared to 75-90 kb of phages in set 2 (including S41). The group A phage S45 had unrelated banding patterns to the rest of this group when digested with the above enzymes (data not shown). The genome size of S45 was significantly smaller at only 30 kb.

The group C phages were divided into four subgroups on the basis of their restriction patterns (Fig. C16).

Subgroup I contained phages M2, M3, M4, S72 and S42. The phages in subgroup I were digested with *Eco*RI, *Bgl*II and *Bam*HI, producing 3-4 bands, 12-14 bands and 8-15 bands respectively (Tables C7, C8, Figs. C10, C11). The banding patterns of M2 and M4, after digestion with all these enzymes, appeared identical. On digestion with *Bgl*II and *Bam*HI, S72 differed from M2 and M4 by 2-3 bands only. All four bands, produced on digestion of S72 with *Eco*RI, differed from M2 and M4. M3 however, although different from M2, M4 and S72, shared many common bands. The banding patterns of S42 showed some relation to the other phages in this subgroup. Data on the digestion of M3 and S42 by *Eco*RI is not given. The estimated genome sizes of M2, M4 and M3 were 65-75 kb. S72 appeared slightly smaller at 65-70 kb and S42 contained only 60-65 kb.

Table C3

Band sizes (kb) of DNA from group A phages after digestion with *Eco*RI (E). Row 2 indicates the number of bands produced after digestion with *Eco*RI.

Figure C6

Electrophoresis in a 0.8% agarose gel of DNA from group A phages after digestion with *Eco*RI . (a) Direct comparison. (b) and (c) are S21 DNA and S41 DNA respectively showing more clearly the banding patterns produced.

A2	A3	A4s	A6	S21	S34	S41
E 7	E 7	E 7	E 7	E 7	E 6	E 8
25-30	25-30	25-30	25-30	25-30	25-30	25-30
?	?	?	?	?	?	?
23.0	23.0	23.0	23.0	23.0	23.0	23.0
7.8	7.8	7.8	7.8	7.8	8.1	8.5
5.3	5.3	--	--	--	--	--
--	--	4.4	4.4	4.4	--	4.4
--	--	--	--	--	4.3	--
--	--	--	--	--	--	4.1
--	--	2.8	2.8	2.8	--	2.8
--	--	--	--	--	2.3	--
1.5	1.5	1.5	1.5	1.5	1.5	1.5
1.3	1.3	1.2	1.2	1.2	?	1.2

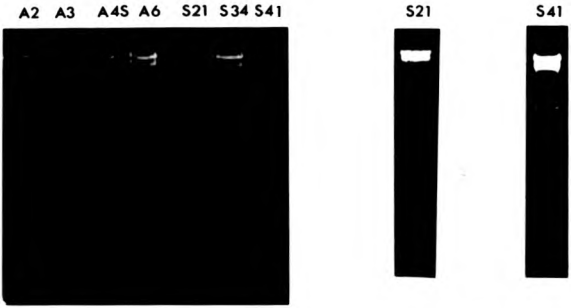


Table C4

Band sizes (kb) of DNA from group A phages after digestion with *Bgl*II (B). Row 2 indicates the number of bands produced after digestion with *Bgl*II.

Figure C7

Electrophoresis in a 0.8% agarose gel of DNA from the group A phages S21, S41 and S34 after digestion with *Bgl*II.

A2	A3	A4s	A6	S21	S34	S41
B 17	B 17	B 19	B 19	B 19	B 19	B 19
15.0	15.0	15.5	15.5	15.5	--	15.5
--	--	--	--	--	14.0	--
11.0	11.0	11.0	11.0	11.0	--	11.0
--	--	--	--	--	--	9.3
--	--	--	--	--	8.1	--
7.9	7.9	7.9	7.9	7.9	--	--
7.4	7.4	7.4	7.4	7.4	7.4	7.4
--	--	--	--	--	6.1	--
--	--	--	--	--	5.8	--
--	--	5.5	5.5	5.5	--	5.5
5.3	5.3	--	--	--	--	--
5.2	5.2	--	--	--	--	--
--	--	4.9	4.9	4.9	4.9	4.9
--	--	4.7	4.7	4.7	4.7	4.7
4.4	4.4	--	--	--	4.4	--
--	--	--	--	--	4.2	--
3.7	3.7	3.7	3.7	3.7	3.7	3.7
3.5	3.5	3.5	3.5	3.5	3.5	3.5
--	--	3.2	3.2	3.2	--	3.2
3.0	3.0	--	--	--	3.0	--
--	--	2.9	2.9	2.9	--	2.9
--	--	2.8	2.8	2.8	--	2.8
2.5	2.5	2.5	2.5	2.5	--	2.5
--	--	--	--	--	2.3	--
2.2	2.2	2.2	2.2	2.2	2.2	2.2
2.0	2.0	2.0	2.0	2.0	2.0	2.0
1.9	1.9	1.9	1.9	1.9	1.9	1.9
1.6	1.6	1.6	1.6	1.6	1.6	1.6
1.5	1.5	1.5	1.5	1.5	1.5	1.5
1.4	1.4	1.4	1.4	1.4	1.4	1.4

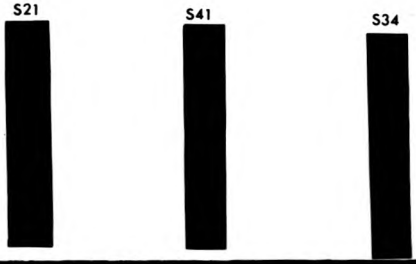


Table C5

Band sizes of DNA from group A phages after digestion with *Bam*HI (M). Row 2 indicates the number of bands produced after digestion with *Bam*HI.

Figure C8

Electrophoresis in a 0.8% agarose gel of DNA from the group A phages after digestion with *Bam*HI.

A2	A3	A4s	A6	S21	S34	S41
M 5	M 5	M 5	M 5	M 5	M 6	M 5
50-55 (X2)	50-55 (X2)	50-55 (X2)	50-55 (X2)	50-55 (X2)	50-55 (X2)	50-55 (X2)
14.0	14.0	--	--	?PART	14.0	--
--	--	9.1	9.1	9.1	--	9.1
--	--	8.9	8.9	8.9	--	8.9
--	--	--	--	--	6.6	--
3.5	3.5	3.5	3.5	3.5	3.5	3.5
2.1	2.1	--	--	--	2.3	--

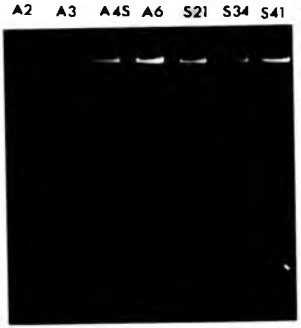


Table C6

Band sizes of DNA from group A phages after digestion with *Cla*I (C). Row 2 indicates the number of bands produced after digestion with *Cla*I.

Figure C9

Electrophoresis in a 0.8% agarose gel of DNA from the group A phages after digestion with *Cla*I.

A2	A3	A4s	A6	S21	S34	S41
C 7	C 7	C 7	C 7	C 7	C 6	C 7
>30	>30	>30	>30	>30	>30	>30
>20*	>20*	--	--	--	>20*	--
--	--	>20	>20	>20	--	>20
14.0	14.0	14.0	14.0	14.0	14.0	14.0
--	--	7.8	7.8	7.8	--	7.8
6.6	6.6	--	--	--	6.6	--
--	--	6.2	6.2	6.2	--	6.2
4.8	4.8	4.8	4.8	4.8	4.8	4.8
--	--	--	--	--	5.5	--
5.3	5.3	5.3	5.3	5.3	--	5.3
4.8	4.8	4.8	4.8	4.8	4.8	4.8
2.8	2.8	--	--	--	--	--

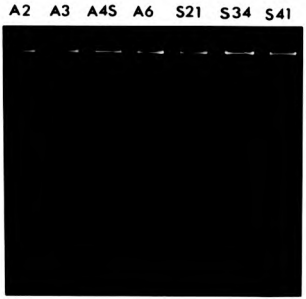


Table C7

Band sizes of DNA from group C phages (subgroup I) after digestion with *Bgl*II (B). Row 2 indicates the number of bands produced after digestion with *Bgl*II.

Figure C10

Electrophoresis in a 0.8% agarose gel of DNA from the subgroup C I phages M2, M4 and S72 after digestion with *Bgl*II and *Eco*R1.

M2	M4	M3	S72	S42
B 14	B 14	B 12	B 14	B 11+
21.5	21.5	21.5	--	--
--	--	--	--	19.0
--	--	--	14.3	--
--	--	--	--	10.2
9.4	9.4	9.4	9.4	--
--	--	--	--	8.7
7.4	7.4	7.4	7.4	--
--	--	--	6.7	6.6?
6.4	6.4	6.4	6.4	--
5.2	5.2	5.2	--	--
5.0	5.0	5.0	5.0	5.0
4.2	4.2	4.2	4.2	4.2
--	--	--	--	3.6
3.2	3.2	--	3.0	2.8?
2.6	2.6	--	2.6	2.6
2.2	2.2	2.2	2.2	--
2.1	2.1	2.1	2.1	2.1
1.8	1.8	1.8	1.8	1.8
1.5	1.5	1.5	1.5	?
1.3	1.3	1.3	1.3	?

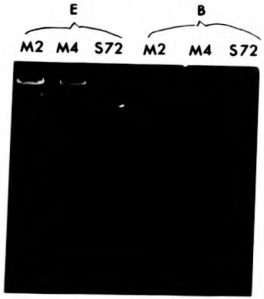


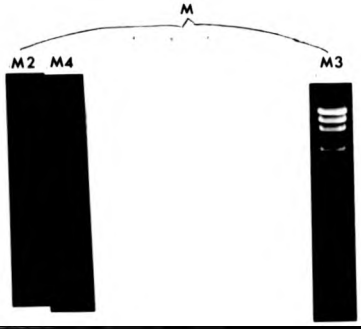
Table C8

Band sizes of DNA from group C phages (subgroup 1) after digestion with *Bam*HI (M). Row 2 indicates the number of bands produced after digestion with *Bam*HI.

Figure C11

Electrophoresis in a 0.8% agarose gel of DNA from the subgroup C 1 phages M2, M4 and M3 after digestion with *Bam*HI.

M2	M4	M3	S72	S42
M 15	M 15	M 12+	M 15	M 8+
--	--	17.5	--	--
--	--	13.5	--	13.8?
12.5	12.5	--	12.5	--
--	--	--	--	11.0 (X2)
10.5	10.5	--	10.5	--
9.4	9.4	9.4	9.4	--
6.9	6.9	--?	6.9	--
--	--	6.6?	--	--
--	--	--	--	6.2
5.0	5.0	5.0	5.0	--
4.1	4.1	4.1	4.1	4.1
--	--	3.5	--	3.6?
3.2	3.2	3.2	3.2	3.2
3.0	3.0	3.0	3.0	--
2.9	2.9	2.9	2.9	2.9
2.7	2.7	2.7	2.7	?
1.7	1.7	1.7	1.7	?
--	--	?	0.8	?
?	0.7	?	0.7	?
?	0.6	?	0.6	?
?	0.5	?	--	?
?	<0.5	?	<0.5	?
?	<0.5	?	<0.5	?



Subgroup II contained phages S31, S32, S33 and S71. On digestion with *Cla*I, which produced only 2 bands, the restriction banding patterns appeared the same in each case. S32, S33 and S71 produced a restriction pattern on *Eco*RI digestion (5-6 bands), which differed to that of S31 by 3 bands (Table C9, Fig. C12). The estimated genome sizes of S32, S33 and S71 were approximately 36 kb, while that of S31 was larger at 42kb.

Subgroup III contained phages A4L, A5, ϕM1 and S62. A4L and A5 gave one banding pattern on digestion with *Eco*RI and another using *Cla*I, producing 9 and 3 bands respectively (Table C10, Fig. C13). On digestion with *Eco*RI ϕM1 shared 7 bands in common with the above, while S62 showed only 4. ϕM1 and S62 appeared similar after digestion with *Cla*I, with 2 bands comigrating with those of S4l and A5. While the estimated genome sizes of A4L, A5 and M1 were approximately 45 kb, that of S62 was smaller at 39 kb.

Of the remaining group C phages only subgroup IV phages A1 and S22 were subjected to RE analysis. The phages differed in both plaque morphology and restriction pattern but were both group C phages (Table C11, Fig. C14).

The *Eca* phages M5 and S61 were placed in a miscellaneous group since no restriction data was obtained for them.

The group B phages contained phages D2, S65, S75 and ϕKP, all of which were isolated on *Ecc* SCRI193. Restriction patterns were obtained for only two of the phages, S65 and ϕKP, which did not appear to be related (Table C12, Fig. C15). The genome size of S65 was approximately 100 kb compared to ϕKP at 46 kb.

Table C9

Band sizes of DNA from group C phages (subgroup II) after digestion with *Eco*RI (E) and *Cla*I (C). Row 2 in each case indicates the number of bands produced after digestion with *Eco*RI and *Cla*I.

Figure C12

Electrophoresis in a 0.8% agarose gel of DNA from the subgroup C II phages S31, S32, S33 and S71 after digestion with (a) *Eco*RI and (b) *Cla*I.

S31	S32	S33	S71
E7	E5-6	E5-6	E5-6
10.0	--	--	--
9.6	9.6 (?X2)	9.6 (?X2)	9.6 (?X2)
7.9	7.9	7.9	7.9
6.0	--	--	--
--	4.3	4.3	4.3
3.6	--	--	--
2.8	2.8	2.8	2.8
2.0	2.0	2.0	2.0

S31	S32	S33	S71
C2	C2	C2	C2
>23	>23	>23	>23
2.0	2.0	2.0	2.0

Table C10

Band sizes of DNA from group C phages (subgroup III) after digestion with *Eco*RI (E) and *Cla*I (C). Row 2 in each case indicates the number of bands produced after digestion with *Eco*RI and *Cla*I.

Figure C13

Electrophoresis in a 0.8% agarose gel of DNA from the subgroup C III phages A4L, A5 and M1 after digestion with (a) *Eco*RI and (b) *Cla*I.

A4L	A5	ϕM1	S62
E9	E9	E8	E7
--	--	--	12.6
9.6	9.6	9.6	--
8.5	8.5	8.5	8.5
--	--	8.3	--
8.1	8.1	8.1	--
6.2	6.2	--	6.2
3.8	3.8	3.8	3.8
3.3	3.3	3.3	3.3
--	--	--	3.2
2.7	2.7	--	--
1.6	1.6	1.6	--
1.4	1.4	1.4	1.4

A4L	A5	ϕM1	S62
C3	C3	C2	C2
>23	>23	>23	>23
?12.9	?12.9	--	--
1.7	1.7	1.7	1.7

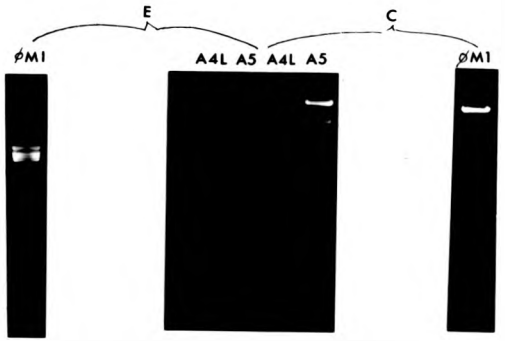


Table C11

Band sizes of DNA from group C phages (subgroup IV) after digestion with *Eco*RI (E), *Bgl*II (B) and *Cla*I (C). Row 2 in each case indicates the number of bands produced after digestion with *Eco*RI, *Bgl*II and *Cla*I.

Figure C14

Electrophoresis in a 0.8% agarose gel of DNA from subgroup IV phages A1 and S22 after digestion with *Eco*RI and *Bgl*II.

A1			S22	
E 6	B 2	C 2	E 6	B 3
8.1	>25	>25	13.6	19.0
7.8	4.0	2.0	8.3	14.1
7.2			6.0	11.0
4.8			3.0	
2.9			2.6	
2.2			2.2	

Table C12

Band sizes of DNA from the group B phages S65 and KP after digestion with *Eco*RI (E), *Bgl*II (B), *Bam*HI (M) and *Cla*I (C). Row 2 in each case indicates the number of bands produced after digestion with *Eco*RI, *Bgl*II, *Bam*HI and *Cla*I.

Figure C15

Electrophoresis in a 0.8% agarose gel of DNA from the group B phage KP after digestion with *Eco*RI, *Bgl*II, *Bam*HI, *Cla*I, *Hae*III and *Hind*III.

S65			φKP		
E 12+	B 15+	C 10	B 8	M 6	C 9
18.0	15.0	18.0	12.0	10.0	8.5
17.0	13.5	19.5	11.0	9.5	8.0
15.0	7.0	15.0	9.5	8.5	6.8
13.5	4.5	11.0	5.0	6.4	6.4
8.3	4.0	10.0	3.3	5.6	5.6
7.2	3.5	8.3	2.7	4.1	5.2
4.6	3.2	7.8	1.3		2.7
3.9	2.9	6.6	0.8		1.7
3.5	2.6	3.9			0.8
3.1	2.5	1.7			
2.8	2.1				
2.3	1.9				
	1.7				
	1.5				
	1.4				

3.2.2.f Host range

Both *Eca* and *Ecc* phages were spotted onto a number of *Eca*, *Ecc* and other *Erwinia* strains, as well as 2 *E.coli* strains. This work was carried out only once and was intended to give an indication of the host ranges of these phages. Lysates were not titred to individual plaques in all cases, so definition of phage sensitivity (in terms of lytic replication) in these preliminary tests has to be loosely interpreted.

The *Eca* strains were divided into 6 divisions on the basis of their sensitivity patterns to the *Eca* phages. From RE data the group A phages appeared to be related and in the case of A4S, A6 and S21, indistinguishable (Tables C3, C4, C5, C6, Fig. C6, C7, C8, C9). The host ranges of these phages on *Eca* strains, however, differed considerably (Table C13). In some cases all strains in a division showed sensitivity to one phage but resistance to another. All but one of the division 2 strains were sensitive to S21, whereas all were resistant to both A4S and A6. Similarly, division 3 strains were sensitive to both S21 and A4S but resistant to A6. S41 showed the largest difference in host range. It was capable of infecting only 5 of the 58 *Eca* strains tested, compared to the most premiscuous phage in the group, S21, able to infect 41 of these 58 strains. Although the variation in host range is presumably related to genetic variation between the phages, no obvious differences in restriction profiles could account for these changes.

The host ranges of subgroup C I phages were very similar, with only 2 *Eca* strains, SCRI41 and SCRI1037, showing variations in sensitivity (Tables C13). Again, these differences could not be related to alterations in restriction profiles.

As with the phages in subgroup C I, those in subgroups C II and C III showed little host range variation (Table C14). For example, the resistance of SCRI1052 to S62, if it is a true phenomenon (ie. repeatable), may relate to any one of a number of

Table C13

Host ranges of *Eca* phage groups A and C (subgroup I) on *Eca* strains (divisions 1-6).

+ = sensitive

- = resistant

NT = not tested

NB. partial sensitivity is also denoted as +.

Strains		Phages										
		A3	A4S	A6	S21	S34	S41	M2	M3	M4	S42	S72
SCRI	1	+	+	+	+	+	−	+	+	+	+	+
"	8	+	+	+	+	+	−	+	+	+	+	+
"	28	+	+	+	+	+	−	+	+	+	+	+
"	52	+	+	+	+	+	+	+	+	+	+	+
"	84	+	+	+	+	+	−	+	+	+	+	+
"	87	+	+	+	+	+	+	+	+	+	+	+
"	**1043**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
D1 "	1044	+	+	+	+	+	−	+	+	+	+	+
"	1049	+	+	+	+	+	−	+	+	+	+	+
"	1050	+	+	+	+	+	−	+	+	+	+	+
"	1052	+	+	+	+	+	−	+	+	+	+	+
"	1053	+	+	+	+	−	−	+	+	+	+	+
"	45	+	+	NT	+	+	+	+	+	+	+	+
"	98	+	+	NT	+	+	−	+	+	+	+	+
"	11	+	−	NT	+	+	−	+	+	+	+	+
"	15	+	−	NT	+	NT	−	+	+	+	+	+
"	31	+	−	−	+	+	−	+	+	+	+	+
"	93	+	−	−	+	+	+	+	+	+	+	+
"	96	+	−	−	+	−	−	+	+	+	+	+
"	1034	+	−	−	+	−	−	+	+	+	+	+
D2 "	1035	+	−	−	+	−	−	+	+	+	+	+
"	1036	+	−	−	+	−	−	+	+	+	+	+
"	1039	+	−	−	+	−	−	+	+	+	+	+
"	1045	+	−	−	+	−	−	+	+	+	+	+
"	34	+	−	−	+	+	−	+	+	+	+	+
"	12	+	−	−	−	−	−	+	+	+	+	+
"	32	+	+	−	+	−	−	+	+	+	+	+
"	37	+	+	−	+	−	−	+	+	+	+	+
D3 "	46	+	+	−	+	NT	−	+	+	+	+	+
"	58	+	+	−	+	−	−	+	+	+	+	+
"	83	+	+	−	+	−	−	+	+	+	+	+
"	1040	+	+	−	+	−	−	+	+	+	+	+
"	33	−	−	−	−	−	−	+	+	+	+	+
D4 "	41	−	+	−	+	−	−	−	−	+	−	+
"	48	−	−	−	+	−	−	+	+	+	+	+
"	97	−	+	−	−	−	−	+	+	+	+	+
"	9	−	−	−	−	−	−	−	−	−	−	−
"	13	−	−	−	−	−	−	−	−	−	−	−
"	39	−	−	−	−	−	−	−	−	−	−	−
"	68	−	−	−	−	−	−	−	−	−	−	−
"	82	−	−	−	−	−	−	−	−	−	−	−
D5 "	94	−	−	−	−	−	−	−	−	−	−	−
"	1038	−	−	−	−	−	−	−	−	−	−	−
"	1041	−	−	−	−	−	−	−	−	−	−	−
"	1046	−	−	−	−	−	−	−	−	−	−	−
"	1047	−	−	−	−	−	−	−	−	−	−	−
"	1051	−	−	−	−	−	−	−	−	−	−	−
"	1037	−	−	−	−	NT	−	−	−	−	−	+
	BS44	+	+	+	+	+	−	+	+	+	+	+
	BS131	+	+	−	+	−	−	+	+	+	+	+
	BS153	+	−	−	+	−	−	+	+	+	+	+
	BS209	NT	−	+	+	+	−	+	+	+	+	+
D6	BS227	−	−	−	+	−	−	+	+	+	+	+
	C301	+	NT	NT	NT	NT	NT	+	+	+	+	+
	C344	+	NT	−	+	+	−	+	+	+	+	+
	C361	−	−	+	+	+	−	+	+	+	+	+
	C465	+	−	+	+	−	−	+	+	+	+	+
	C466	−	−	−	−	−	−	−	−	−	−	−

Table C14

Host ranges of *Eca* phage group C (subgroup II, III and IV) on *Eca* strains (divisions 1-6).

+ = sensitive

- = resistant

NT = not tested

NB. partial sensitivity is also denoted as +.

Strains		Phages										
		S32	S33	S71	A4L	M1	S62	S45	S61	S64	M5	S22
SCRI	1	+	+	+	+	+	+	+	–	–	+	+
"	8	+	+	+	+	+	+	+	–	+	+	+
"	28	+	+	+	+	+	+	+	–	–	+	+
"	52	+	+	+	+	+	+	+	+	–	+	+
"	84	+	+	+	+	+	+	+	+	–	+	+
"	87	+	+	+	+	+	+	+	+	+	+	+
"	1043	+	+	+	+	+	+	+	–	+	+	+
D1 "	1044	+	+	+	+	+	+	+	–	+	+	+
"	1049	+	+	+	+	+	+	+	–	+	+	+
"	1050	+	+	+	+	+	+	+	–	–	+	+
"	1052	+	+	+	+	+	–	+	–	–	+	+
"	1053	+	+	+	+	+	+	+	–	–	+	+
"	45	+	+	+	+	+	+	+	+	–	+	+
"	98	+	+	+	+	+	+	+	–	–	+	+
"	11	+	+	+	+	+	+	+	–	–	+	+
"	15	+	+	+	+	+	+	+	–	–	+	+
"	31	+	+	+	+	+	+	+	–	–	+	+
"	93	+	+	+	+	+	+	+	NT	–	+	+
"	96	NT	+	+	+	+	+	+	–	–	NT	NT
"	1034	+	+	+	+	+	+	+	–	–	+	+
D2 "	1035	+	+	+	+	+	+	+	–	–	+	+
"	1036	+	+	+	+	+	+	+	–	–	+	+
"	1039	+	NT	+	+	+	+	+	–	–	+	+
"	1045	+	+	+	+	+	+	+	–	–	+	+
"	34	+	+	+	+	+	+	–	–	–	+	–
"	12	+	+	+	NT	+	+	+	–	–	+	–
"	32	+	+	+	+	+	+	+	–	–	+	+
"	37	+	+	+	+	NT	+	+	+	–	+	+
D3 "	46	+	+	+	+	+	+	+	+	–	+	+
"	58	+	+	+	+	+	+	+	+	–	+	–
"	81	+	+	+	+	+	+	+	+	–	+	+
"	1040	–	+	+	+	+	+	+	–	–	+	NT
"	33	–	+	–	NT	NT	+	+	–	–	–	+
D4 "	41	+	+	–	–	–	–	+	–	+	–	+
"	48	+	+	+	+	+	+	+	–	–	+	–
"	97	+	+	+	+	NT	+	+	–	–	+	NT
"	9	–	–	–	–	–	–	–	–	–	–	–
"	13	–	–	–	–	–	–	–	–	–	–	–
"	39	–	–	–	–	–	–	–	–	–	–	–
"	68	–	–	–	–	–	–	–	–	–	–	–
"	82	–	–	–	–	–	–	–	–	–	–	–
D5 "	94	–	–	–	–	–	–	–	–	–	–	–
"	1038	–	–	–	–	–	–	–	–	–	–	–
"	1041	–	–	–	–	–	–	–	–	–	–	–
"	1046	–	–	–	–	–	–	–	–	–	–	–
"	1047	–	–	–	–	–	–	–	–	–	–	–
"	1051	–	–	–	–	–	–	–	–	–	–	–
"	1037	–	–	–	–	–	–	+	–	–	–	+
	BS44	+	+	+	+	+	+	+	–	+	+	+
	BS131	–	+	+	–	–	–	+	–	–	+	+
	BS153	+	+	+	+	+	+	+	–	–	+	+
	BS209	+	+	+	+	+	+	+	+	–	+	–
D6	BS277	–	+	+	–	–	–	+	–	–	+	–
	C301	+	NT	+	+	NT	NT	+	NT	NT	NT	NT
	C344	+	+	+	+	+	+	+	–	–	+	–
	C361	+	+	+	+	+	+	+	–	–	+	+
	C465	+	+	+	+	+	+	+	–	–	+	+
	C466	–	–	–	–	–	–	–	–	–	–	–

band changes.

The final subgroup, C IV, contained miscellaneous phages which did not relate to any of the other subgroups (Table C14). These phages, not surprisingly, showed a considerable variation in host range. The most striking feature of this group was the narrow host range of phage S61, infecting only 9 of the 58 strains tested.

Only one strain in division 5, *Eca* SCRI1037, showed sensitivity to the *Eca* phages. All but this strain proved resistant, so to ensure they were in fact *Eca* strains they, together with C466 from subgroup 6, were submitted to biochemical and growth temperature analysis. Only C466 was reclassified as *Ecc*, the rest were confirmed as *Eca* (including SCRI1037).

A number of *Eca* strains showed sensitivity to two of the group B phages (Table C15). D2 infected *Eca* SCRI84 while S65 infected 22 of the 58 *Eca* strains. These latter strains did not include SCRI1043 on which the *Eca* phages were originally isolated.

With few exceptions, namely S62 infecting SCRI126, and S45 and S22 infecting SCRI135, only 2 *Ecc* strains (SCRI105 and SCRI191) were sensitive to the *Eca* phages (Tables C16, C17). These strains were subsequently analysed biochemically and then reclassified as *Eca*.

Unlike the *Eca* phages which, in general, were promiscuous among *Eca* strains, the *Ecc* phages had very narrow host ranges on *Ecc* strains (Table C18). Three strains, *Ecc* SCRI110, SCRI126 and SCRI132, were sensitive to both D2 and S65 but not ϕKP. Two different strains, *Ecc* SCRI105 and SCRI192 were sensitive to S65 only, while *Ecc* SCRI143 was sensitive to D2 only. All group B phages infected *Ecc* SCRI193 (the strain used in the original isolation of the phages). ϕKP had the

Table C15

Host ranges of *Ecc* phage group B on *Eca* strains (divisions 1-6).

+ = sensitive

- = resistant

NT = not tested

NB. partial sensitivity is also denotes as +.

	Strain		Phage		
			D2	S65	KP
D1	SCRI	1	-	NT	-
	"	8	-	+	-
	"	28	-	+	-
	"	52	-	+	-
	"	84	+	+	-
	"	87	-	+	-
	"	1043	-	+	-
	"	1044	-	+	-
	"	1049	-	+	-
	"	1050	-	+	-
	"	1052	-	+	-
	"	1053	-	+	-
	"	45	-	+	-
	"	98	-	+	-
D2	"	11	-	+	-
	"	15	-	+	-
	"	31	-	+	-
	"	93	NT	+	-
	"	96	-	-	-
	"	1034	-	+	-
	"	1035	-	+	-
	"	1036	-	-	-
	"	1039	-	-	-
	"	1045	-	-	-
	"	34	-	-	-
	"	12	-	NT	-
D3	"	32	-	-	-
	"	37	-	-	-
	"	46	-	-	-
	"	58	-	-	-
	"	83	-	-	-
	"	1040	-	-	-
D4	SCRI	33	-	-	-
	"	41	-	-	-
	"	48	-	-	-
	"	97	-	-	-
D5	"	9	-	-	-
	"	13	-	-	-
	"	19	-	-	-
	"	68	-	-	-
	"	82	-	-	-
	"	94	-	-	-
	"	1038	-	-	-
	"	1041	-	-	-
	"	1046	-	-	-
	"	1047	-	-	-
	"	1051	-	-	-
	"	1037	-	-	-
D6	BS44		-	+	-
	BS131		-	-	-
	BS153		-	-	-
	BS209		-	+	-
	BS227		-	-	-
	C344		-	+	-
	C361		-	+	-
	C465		-	-	-
	C466		-	-	-

Table C16

Host ranges of *Eca* phage groups A and C (subgroup I) on *Ecc* strains.

+ = sensitive

- = resistant

NT = not tested

NB. partial sensitivity is also denotes as +.

Strains	Phages										
	A3	A4S	A6	S21	S34	S41	M2	M3	M4	S42	S72
SCRI101	-	-	-	-	-	-	-	-	-	-	-
" 102	-	-	-	-	-	-	-	-	-	-	-
" 103	-	-	-	-	-	-	-	-	-	-	-
" 105	+	-	-	+	-	-	+	+	+	+	+
" 106	-	-	-	-	-	-	-	-	-	-	-
" 109	-	-	-	-	-	-	-	-	-	-	-
" 110	-	-	-	-	-	-	-	-	-	-	-
" 111	-	-	-	-	-	-	-	-	-	-	-
" 112	-	-	-	-	-	-	-	-	-	-	-
" 113	-	-	-	-	-	-	-	-	-	-	-
" 114	-	-	-	-	-	-	-	-	-	-	-
" 115	-	-	-	-	-	-	-	-	-	-	-
" 116	-	-	-	-	-	-	-	-	-	-	-
" 117	-	-	-	-	-	-	-	-	-	-	-
" 118	-	-	-	-	-	-	-	-	-	-	-
" 119	-	-	-	-	-	-	-	-	-	-	-
" 120	-	-	-	-	-	-	-	-	-	-	-
" 121	-	-	-	-	-	-	-	-	-	-	-
" 122	-	-	-	-	-	-	-	-	-	-	-
" 123	-	-	-	-	-	-	-	-	-	-	-
" 124	-	-	-	-	-	-	-	-	-	-	-
" 125	-	-	-	-	-	-	-	-	-	-	-
" 126	-	-	-	-	-	-	-	-	-	-	-
" 129	-	-	-	-	-	-	-	-	-	-	-
" 130	-	-	-	-	-	-	-	-	-	-	-
" 132	-	-	-	-	-	-	-	-	-	-	-
" 135	-	-	-	-	-	-	-	-	-	-	-
" 139	-	-	-	-	-	-	-	-	-	-	-
" 143	-	-	-	-	-	-	-	-	-	-	-
" 144	-	-	-	-	-	-	-	-	-	-	-
" 149	-	-	-	-	-	-	-	-	-	-	-
" 152	-	-	-	-	-	-	-	-	-	-	-
" 155	-	-	-	-	-	-	-	-	-	-	-
" 166	-	-	-	-	-	-	-	-	-	-	-
" 169	-	-	-	-	-	-	-	-	-	-	-
" 171	-	-	-	-	-	-	-	-	-	-	-
" 172	-	-	-	-	-	-	-	-	-	-	-
" 174	-	-	-	-	-	-	-	-	-	-	-
" 178	-	-	-	-	-	-	-	-	-	-	-
" 182	-	-	-	-	-	-	-	-	-	-	-
" 191	+	+	+	+	+	+	+	+	+	+	+
" 192	-	-	-	-	-	-	-	-	-	-	-
" 193	-	-	-	-	-	-	-	-	-	-	-
" 198	-	-	-	-	-	-	-	-	-	-	-
" 205	-	-	-	-	-	-	-	-	-	-	-
" 208	-	-	-	-	-	-	-	-	-	-	-
" 211	-	-	-	-	-	-	-	-	-	-	-
" 224	-	-	-	-	-	-	-	-	-	-	-
" 247	-	-	-	-	-	-	-	-	-	-	-

Table C17

Host ranges of *Eca* phage group C (subgroup II, III and IV) on *Ecc* strains.

+ = sensitive

- = resistant

NT = not tested

NB. partial sensitivity is also denoted as +.

Strains	Phages										
	S32	S33	S71	A4L	M1	S62	S45	S61	S64	M5	S22
SCRI101	-	-	-	-	-	-	-	-	-	-	-
" 102	-	-	-	-	-	-	-	-	-	-	-
" 103	-	-	-	-	-	-	-	-	-	-	-
" 105	+	+	+	+	+	+	+	-	-	+	+
" 106	-	-	-	-	-	-	-	-	-	-	-
" 109	-	-	-	-	-	-	-	-	-	-	-
" 110	-	-	-	-	-	-	-	-	-	-	-
" 111	-	-	-	-	-	-	-	-	-	-	-
" 112	-	-	-	-	-	-	-	-	-	-	-
" 113	-	-	-	-	-	-	-	-	-	-	-
" 114	-	-	-	-	-	-	-	-	-	-	-
" 115	-	-	-	-	-	-	-	-	-	-	-
" 116	-	-	-	-	-	-	-	-	-	-	-
" 117	-	-	-	-	-	-	-	-	-	-	-
" 118	-	-	-	-	-	-	-	-	-	-	-
" 119	-	-	-	-	-	-	-	-	-	-	-
" 120	-	-	-	-	-	-	-	-	-	-	-
" 121	-	-	-	-	-	-	-	-	-	-	-
" 122	-	-	-	-	-	-	-	-	-	-	-
" 123	-	-	-	-	-	-	-	-	-	-	-
" 124	-	-	-	-	-	-	-	-	-	-	-
" 125	-	-	-	-	-	-	-	-	-	-	-
" 126	-	-	-	-	-	+	-	-	-	-	-
" 129	-	-	-	-	-	-	-	-	-	-	-
" 130	-	-	-	-	-	-	-	-	-	-	-
" 132	-	-	-	-	-	-	-	-	-	-	-
" 135	-	-	-	-	-	-	+	-	-	-	+
" 139	-	-	-	-	-	-	-	-	-	-	-
" 143	-	-	-	-	-	-	-	-	-	-	-
" 144	-	-	-	-	-	-	-	-	-	-	-
" 149	-	-	-	-	-	-	-	-	-	-	-
" 152	-	-	-	-	-	-	-	-	-	-	-
" 155	-	-	-	-	-	-	-	-	-	-	-
" 166	-	-	-	-	-	-	-	-	-	-	-
" 169	-	-	-	-	-	-	-	-	-	-	-
" 171	-	-	-	-	-	-	-	-	-	-	-
" 172	-	-	-	-	-	-	-	-	-	-	-
" 174	-	-	-	-	-	-	-	-	-	-	-
" 178	-	-	-	-	-	-	-	-	-	-	-
" 182	-	-	-	-	-	-	-	-	-	-	-
" 191	+	-	+	+	+	+	+	-	-	NT	-
" 192	-	-	-	-	-	-	-	-	-	-	-
" 193	-	-	-	-	-	-	-	-	-	-	-
" 198	-	-	-	-	-	-	-	-	-	-	-
" 205	-	-	-	-	-	-	-	-	-	-	-
" 208	-	-	-	-	-	-	-	-	-	-	-
" 211	-	-	-	-	-	-	-	-	-	-	-
" 224	-	-	-	-	-	-	-	-	-	-	-
" 247	-	-	-	-	-	-	-	-	-	-	-

Table C18

Host ranges of *Ecc* phage group B on *Ecc* strains.

+ = sensitive

- = resistant

NT = not tested

NB. partial sensitivity is also denoted as +.

Strains	Phages			Strains	Phages		
	D2	S65	KP		D2	S65	KP
SCRI101	-	-	-	SCRI132	+	+	-
" 102	-	-	-	" 135	-	-	-
" 103	-	-	-	" 139	-	-	-
" 105	-	+	-	" 143	+	-	-
" 106	-	-	-	" 144	-	-	-
" 109	-	-	-	" 149	-	-	-
" 110	+	+	-	" 152	-	-	-
" 111	-	-	-	" 155	-	-	-
" 112	-	-	-	" 166	-	-	-
" 113	-	-	-	" 169	-	-	-
" 114	-	-	-	" 171	-	-	-
" 115	-	-	-	" 172	-	-	-
" 116	-	-	-	" 174	-	-	-
" 117	-	-	-	" 178	-	-	-
" 118	-	-	-	" 182	-	-	-
" 119	-	-	-	" 191	-	-	-
" 120	-	-	-	" 192	-	+	-
" 121	-	-	-	" 193	+	+	+
" 122	-	-	-	" 198	-	-	-
" 123	-	-	-	" 205	-	-	-
" 124	-	-	-	" 208	-	-	-
" 125	-	-	-	" 211	-	-	-
" 126	+	+	-	" 224	-	-	-
" 129	-	-	-	" 247	-	-	-
" 130	-	-	-	ATCC 39048	NT	NT	+

narrowest host range, plating on *Ecc* SCRI193 and only 1 other strain, *Ecc* ATCC39048, a strain currently under investigation in this laboratory for its antibiotic production.

Spot tests were carried out to study the host ranges of *Eca* and *Ecc* phages on a number of other *Erwinia* species including, *E.chrysanthemi*, *E.uredovora*, *E.rubrifaciens*, *E.rhapontici*, *E.amylovora*, *E.herbicola*, *E.nigrifluens* and *E.quercina*. The sensitivity of these strains to P1 and T4 was also investigated (Table C19). A2, an *Eca* phage, produced a zone of clearing on *E.chrysanthemi* strains NCPPB1066 and B374 as well as *E.herbicola* strains SCRI427 and SCRI430. S75, an *Ecc* phage, produced a zone of clearing on *E.rhapontici* strain SCRI422.

P1 produced a zone of clearing on *E.rhapontici* strain SCRI421, whereas a number of other strains showed clearing on addition of T4. These strains included, *Eca* SCRI1043, HA100 (SCRI1043 containing the *lamB* plasmid pHCP2), *E.rubrifaciens* strains SCRI445, SCRI446, SCRI469 and SCRI470. The most important of these findings was the action of T4 on *Eca* SCRI1043 and its Lambda sensitive derivative, HA100. Since this result showed a zone of clearing and not individual plaques it was important to establish whether individual plaques could be obtained to ensure the phage was replicating inside the host. On dilution and plating of the T4 lysate no plaques were seen on either SCRI1043 or HA100. This result may indicate lysis from without. In this section other zones of phage action may also have been due to this effect, since single plaques were not obtained in all cases.

Table C19

Host ranges of phages A2. S75, AL1 (a phage isolated on *Echr* NCPPB1066), P1 and T4 on a range of *Erwinia* strains and 2 *Escherichia** *coli* strains.

\+ = sensitive

\- = resistant

NT = not tested

NB. Partial sensitivity is also denoted as +. AL1 is an *Echr* NCPPB1066 phage and no other data is given on it in this report. P1 and T4 are both coliphages (see Section 1.5.7).

Strains		Phages				
		A2	S75	AL1	P1	T4
E.carotovora subsp. carotovora	SCRI193	-	+	-	-	-
	MC131	-	+	-	-	-
E.carotovora subsp. atroseptica	SCRI1043	+	-	-	-	-
	HB101	+	-	-	-	-
	NCPPB1066	+	-	+	-	-
	3937	-	-	-	-	-
E.chrysanthemi	B374	+	-	-	-	-
	EC183	-	-	-	-	-
	EC16	-	-	-	-	-
* *E.coli*	*E.coli*B	-	-	-	+	+
	W3110	-	-	-	+	+
	SCRI431	-	-	-	-	-
E.uredovora	" 432	-	-	+	-	-
	" 433	-	-	-	-	-
	" 445	-	-	-	-	+
	" 446	-	-	-	-	+
E.rubrifaciens	" 469	-	-	-	-	+
	" 470	-	-	-	-	+
	" 471	-	-	-	-	-
	" 421	-	-	-	+	-
E.rhapontici	" 422	-	+	-	-	-
	" 423	-	-	-	-	-
	" 468	-	-	-	-	-
	" 449	-	-	-	-	-
E.amylovora	" 454	-	-	-	-	-
	OT1	-	-	-	-	-
	" 424	-	-	-	-	-
	" 426	-	-	-	-	-
E.herbicola	" 427	+	-	-	-	-
	" 430	+	-	-	-	-
	" 436	-	-	+	-	-
	" 463	-	-	+	-	-
E.nigrifluens	" 450	-	-	-	-	-
	" 452	-	-	-	-	-
E.quercina	" 453	-	-	-	-	-
	" 456	-	-	-	-	-

3.2.2.g Generalised transduction

Generalised transduction is an important tool in the study of bacterial genetics (see introduction). Since it is characteristic of only certain phages, it is useful as a further means of differentiating between them. All phages were assayed for transducing ability but only two, the *Eca* phage ϕM1 and the *Ecc* phage ϕKP, were found to be capable of this. This screen was carried out on a single occasion and the inability of the other phages to transduce is, therefore, not absolutely certain. Details on generalised transduction in these phages will be given in detail in the next chapter.

All characterisation results are listed in Table C20.

3.3 Discussion

3.3.1 Phage isolation and characterisation

Sewage was chosen as a medium for phage isolation since it was rich in bacteria and had been used successfully in the past for the isolation of phages to other phytopathogenic bacteria (Ayers *et al.* 1979; Bigby and Kropinski 1989; Nordeen *et al.* 1983). Chatterjee and Starr (1980) have suggested that although phages against *E.amylovora* and *E.herbicola* are readily isolated from soil, sewage and plant material, those for *Ecc* and *Echr* are rarely isolated from such sources. Since 35 phages were isolated from only 10 visits over the two year period of this study, the search for additional phage source was unnecessary.

The technique of uncovering low titre phages by the use of phage resistant mutants, proved very successful on a number of occasions. The use of mutants resistant to

Group	Phage	Plaque Type	Morph. Class	Genome Size (kb)	Virion Size (nm) Head	Tail	Collar
	A2	SIT (1-2mm)	A	80-90	70-85	90-100	10-20
	A3	SIT (1-2mm)	A	80-90	70-85	90-100	10-20
	A4s	SIT (1-2mm)	A	75-90	70-85	90-100	10-20
A	A6	SIT (<1mm)	A	75-90	70-85	90-100	10-20
	S21	SIT (1-2mm)	A	75-90	70-85	90-100	10-20
	S34	SIT (1-2mm)	A	80-90	70-85	90-100	10-20
	S41	SIT (1-2mm)	A	75-90	70-85	90-100	10-20
	S45	SIT (1-2mm)	A	30	70-85	90-100	10-20
	M2	SCCH (1-2mm)	C	65-75	60	10-20	--
	M3	SCCH (1mm)	C	65-75	60	10-20	--
CI	M4	SCCH (1-2mm)	C	65-75	60	10-20	--
	S42	SCCH (1mm)	C	60-65	60	10-20	--
	S72	SCCH (1-2mm)	C	65-70	ND	ND	--
	S31	MCCH (2-3mm)	C	42	45-60	10-20	--
CII	S32	LCCH (3-4mm)	C	36	60	10-20	--
	S33	LCCH (3-4mm)	C	36	60	10-20	--
	S71	LCCH (3-4mm)	C	36	45-60	10-20	--
	A4L	LCCH (3-4mm)	C	45	60	10-20	--
CIII	A5	LCCH (3-4mm)	C	45	60	10-20	--
	øM1	SCCH (1-2mm)	C	45	60	10-20	--
	S62	MCCH (2-3mm)	C	39	60	10-20	--
	M5	LCCH (3-4mm)	C	ND	60	10-20	--
CIV	S22	SCCH (<1mm)	C	36	60	10-20	--
	A1	LCCH (3-4mm)	C	33	45-60	10-20	--
	øKP	SIT (1-2mm)	B	46	60	120-135	--
B	D2	SIT (<1mm)	B	ND	85-95	225	--
	S65	SIT (<1mm)	B	100	70-85	225	--
	S75	SIT (<1mm)	B	ND	60-70	200	--

Table C20. Summary of phage characterisation studies. Genome and virion sizes are approximate only.

LCCH = Large circular clear plus halo
MCCH = Medium circular clear plus halo
SCCH = Small circular clear plus halo
SIT = Small irregular turbid

CP phages as indicators allowed TP phages of lower titre to be uncovered. On one occasion the use of a resistant mutant to a CP phage, S42, led to the unmasking of a TP phage, S45. When a mutant resistant to S45 was used, another TP phage, S41, was uncovered. Besides allowing more phages to be isolated, the success of this technique points to obviou s differences in the biology of phages isolated from the same samples. The ability of one phage to form plaques on a bacterial host resistant to another phage, indicates the phages have either different adsorption sites or different replication systems. While this differentiation is important in distinguishing between CP and TP phages, it is even more interesting to observe this between two TP phages. The fact that S41 was able to form plaques on a host resistant to S45 indicated a basic difference between these phages. This was further supported by restriction analysis, which suggested that S45 was unrelated to the rest of the *Eca* TP phages.

Plaque morphology served as a good initial screen to study variation between the phages (Table C2). Differences were observed in plaque turbidity, size, shape and the presence or absence of halos. The results, however, were interpreted with caution. Identical plaques did not necessarily indicate identical or even related phages, and similarly, different plaque types did not necessarily indicate unrelated phages. A tentative categorisation based on clear or turbid plaques could, however, be made.

Inactivation by U.V. irradiation is a crude method of phage characterisation but a number of tentative conclusions were made from this study (Fig. C3). The inactivation of all the phages followed first order kinetics indicating that a single lesion in any phage genome was sufficient to prevent plaque formation. A clear difference was observed between the inactivation rates of the TP and CP phages. The TP phages were inactivated at a faster rate than the CP phages, suggesting that their genome sizes were considerably larger than those of the CP phages, since a

larger genome increases the likelihood of U.V. mediated mutation (this was subsequently shown to be correct by restriction endonuclease digestion of the phage genomes). It was not possible to differentiate between genome sizes within these groups even though, from restriction analysis, the genome size of A1 was half that of M2. Using restriction analysis the TP phages were shown to have genome sizes of up to 90kb, but they were inactivated more rapidly than T4. Since the size of the T4 genome is 166 kb, the slower rate of T4 inactivation was probably a consequence of either subsequent plating on a different host (*E.coli*), or the operation of the T4 DNA repair mechanism (Sekiguchi *et al.* 1970). In general U.V. inactivation did allow a distinction to be made between TP and CP phages, although relating these difference to the size of individual genomes was not possible.

Heat inactivation, which differentiates phages on the grounds of capsid stability, offered little data towards the characterisation of the phages (Fig. C4). The results suggested that capsid stability in ϕM1 differed from that of M3 and M4. Differences in DNA RE patterns did suggest a fundamental difference between these phages, although these differences are not necessarily related to capsid stability. The inactivation of A4S also appeared to differ from A2, A3 and A5. Although restriction analysis placed A4S in the same group as A2 and A3, undetected variation in the genome of A4S may have led to apparent alteration in capsid stability.

Structural morphology was the first method of characterisation to show clear differences between the phages. All phages possessed isometric heads but three tail types were observed; contractile tails, long and short non-contractile tails (Fig. C5). On this basis the phages were placed into groups A, B and C of the Bradley classification system (Figs. A9 and C16) (Bradley 1967). All group A and C phages were isolated on *Eca* SCR11043, while the group B phages were isolated on *Ecc*

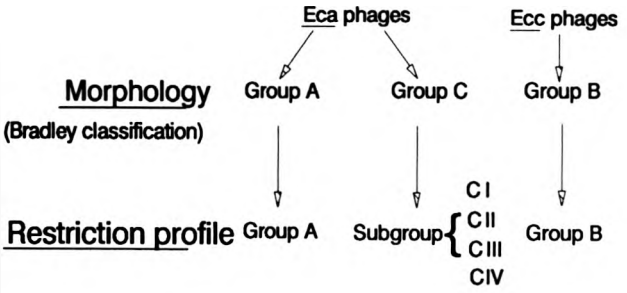


Figure C16. Diagrammatic representation of the Eca and Ecc phages based on structural morphology and RE profiles.

SCR1193. The relevance of this finding is unclear.

RE analysis was the most informative method of phage characterisation. Identical or similar restriction patterns were often seen, clearly showing a relationship between some of the phages. This method also clearly differentiated between phage groups. While some phages were obviously related, eg. A2 and A3, it must be stressed that bands of similar size were not necessarily the same fragment of DNA. While caution should be exercised when studying the less related phages, eg. S42 of subgroup CI (Tables C7, C8), there is a high likelihood that phages possessing a significant number of common bands were related. If more time was available, DNA homology using hybridisation could be used to confirm the identity of co-migrating bands.

Using RE analysis it was clear that all but S45 of the *Eca* TP phages (group A) were closely related (Tables C3, C4, C5, C6). Their banding patterns were similar, and in some cases appeared identical. Of the CP phages, at least five different phage DNA types were present (Tables C7 to C11). Restriction analysis was carried out on only two of the group B phages and it was therefore difficult to speculate on any relationship between them (Table C12). Their morphological dimensions, however, did differ (Table C20).

RE analysis in this study clearly indicated that some phages were closely related. Alterations however, in plaque morphology and host range, between phages showing only minor alterations in RE banding patterns, could clearly be seen. S41 showed only a small number of band alterations, compared to other members of the group A phages, yet its host range was significantly reduced on *Eca* strains. The subgroup C I phages, M3 and S75, produced different plaque morphologies to the closely related M2 and M4 (Fig. C1). As in the previous example, only a small number of band alterations seem to correlate with this.

The phages isolated in this study fell into distinct groups based on their RE banding patterns. Within these groups some banding patterns appeared identical, eg. A2 and A3, but in others a number of differences were apparent, although the close genetic relationship between them was apparent (Fig. C6 to C9). This suggests that genetic change within a phage population may occur continuously. To examine this in more detail, especially exchange of modules, RE analysis would need to be supported by DNA homology studies. RE analysis did however, allow the genetic relatedness of phages to be assessed, and along with structural morphologies was the most productive method of phage characterisation to be used.

Host range studies, although originally carried out as a simple indication of phage sensitivity on a number of strains, proved very interesting (Tables C13 to C19). The *Eca* phages were, on the whole, very promiscuous on *Eca* strains but infected only a small number of *Ecc* strains (Tables C16, C17). The *Ecc* phages were very host specific, infecting only a handful of *Ecc* strains (Table C18). ϕKP was particularly limited in host range infecting only two *Ecc* strains, both of which are important laboratory strains.

The reason for differences in promiscuity between *Eca* and *Ecc* phages is not clear. It may be related to differences in phage receptors or restriction systems between the phages. Differences in the host ranges of *Eca* phages on *Eca* strains suggest that variations in phage receptors and not replication may be involved, since a number of strains are sensitive to one group of phages but show either sensitivity or resistance to another group (eg. the division II *Eca* strains show sensitivity to group C I phages but either sensitivity or resistance to group A phages. These differences suggest variations in receptor site of the phages. Further support for variations in receptors and not replication systems comes from the observation that *Ecc* phage S65 was capable of infecting both *Ecc* and *Eca* strains. The serogroups of *Ecc* and

Eca strains relate to variations in their LPS O-antigen structures. *Eca* strains commonly fall into a single serogroup (Group I), whereas *Ecc* strains belong to a number of different serogroups (De Boer *et al.* 1979, 1985). The promiscuity of *Eca* phages on *Eca* strains may relate to their ability to adsorb to these similar LPS structures. Differences between group A and group C phages is unclear but could relate to differences in adsorption site on the LPS structure. If the *Ecc* strains did possess a number of different serogroups, it would suggest that the host ranges of phages adsorbing to specific serogroups would be limited.

Two phages (A2 and S75) had hosts outside *Eca* and *Ecc* strains (A2 plating on *Echr* and *E.herbicola* strains and S75 plating on an *E.rhapontici* strain). This is of interest since these phages have crossed species barriers. It is important to note that these experiments were not repeated to obtain individual plaques and the zone of clearing obtained could have been due to lysis from without. The same is true for the phage action of P1 on the *E.rhapontici* strain SCRI421, and T4 action on *E.rubrifaciens* SCRI445, SCRI446, SCRI469 and SCRI470. Since four out of five *E.rubrifaciens* strains appeared sensitive to T4 and all other *Erwinia* strains resistant, replication in these strains seems likely. The action of T4 on *Eca* SCRI1043 and its Lambda sensitive derivative HA100 was studied in more detail, since at that time a phage capable of generalized transduction was actively being sought in this strain. An attempt to obtain single plaques failed, indicating that T4 action on *Eca* SCRI1043 was due to lysis from without. A similar result has been shown previously (Pirhonen and Palva 1988).

3.3.2 Genome comparison of *Erwinia* phages with other phage groups

An intensive study into the similarities and differences between phages from different bacterial groups would not be possible, especially since phages have been

isolated on over 90 different bacterial genera (Reanney and Ackermann 1982). It may be of value, however, to briefly compare the phages with those of other phytopathogens and the better known coliphages. It is important, firstly, to point out that 95% of all known phages possess tails and that 90% of these phages have isometric heads. Also all tailed phages contain double stranded DNA (Ackerman *et al.* 1978). The phages from this study therefore, fall into the most common group of phages.

Coliphages have been isolated that belong to most morphological classes. The T-phages represent the three classes of tailed phages. The T-even phages T2, T4 and T6 fall into Bradley group A, but due to their elongated heads, fall into group A2 of the Ackermann system (Ackermann and Eisenstark 1974) (Fig. A9). T1 and T5 fall into group B and T7 into group C. The phages isolated in this study belong to these three groups.

Vidaver and Schuster (1969) isolated 10 phages for *Xanthomonas phaseoli*. These phages also infected a small number of *Pseudomonas* strains. The phages had either elongated or isometric heads and had long non-contractile tails. 8 out of 10 phages were serologically related.

While a large number of phages have been isolated on *Pseudomonas* species, only two studies will be discussed here. The *Pseudomonas* phages represent all morphological groups except group D (Bradley 1967). Thomas and Leary (1983) isolated 56 phages for *P.syringae* pv. *glycinea* from sewage. All the phages possessed tails and contained double stranded DNA. Their host range was wide, infecting a number of phytopathogenic Pseudomonads. As in this study, phages were readily isolated from sewage in an area where the host was not known to exist but no explanation for this fact was given. Noreen *et al.* (1983) isolated a number of phages from both culture supernatants and sewage. While phages from culture

supernatants produced only turbid plaques, as expected, those from sewage produced both turbid and clear plaques. All the phages were tailed, with the majority falling into group B. The CP phages ranged in genome size from 24-49 kb, while the genome sizes of TP phages were 39-52 kb. Phage genomes in this study were, on the whole, larger than the genomes of these *Pseudomonas* phages, in particular those of group A which were up to 90 kb.

There are several reports on the isolation and characterisation of *Erwinia* phages. The largest study prior to this report by Gross *et al.* (1991) has been discussed previously (Section 1.4.5.1). In the report by Gross *et al.* the use of these phages in phage typing was extensively discussed, although their virulent nature was their only mentioned characteristic. Examples of phages isolated for *E.amylovora* and *E.herbicola* are given below. Erskine (1973) isolated a clear plaquing phage, S1, from soil, on *E.amylovora* strain PR1. This phage had an isometric head and short tail (group C) and contained double stranded DNA. Harrison and Gibbins (1975) isolated a temperate phage, Y46/(E2). This was a group A phage with a very limited host range, infecting only two *E.herbicola* strains out of 30 *E.herbicola*, 6 *E.stewartii*, 4 *E.amylovora* and 1 *E.coli* strain tested.

The above information is only a fraction of that available on phages (including those of plant pathogens). It is clear that tailed phages are relatively easy to isolate from a variety of sources. This observation is supported by the ease with which tailed phages were isolated in this report.

While a number of reports describe the isolation and characterisation of a small number of *Erwinia* phages, this is the first report that characterises and compares an interactive phage population from a single source over a period of time. While little ecological data can be obtained from this, it does seem that while several groups of phages were isolated in summer, only a single group (group C) was isolated in

winter. This may relate to the ability of the phages to replicate at low temperatures, a characteristic which could be easily investigated.

3.3.3 Phage typing

Phage typing is a common technique used to identify bacteria by their pattern of sensitivity to test phages. Many examples of phage typing systems are available, including those used to differentiate between strains of phytopathogenic bacteria (Section 1.4.5.1). On two occasions phage typing systems have been developed for strains of *Erwinia* (Paulin and Nassan 1978; Gross *et al.* 1991).

Paulin and Nassan (1978) developed a phage typing system using temperate phages for *Echr* strains, which were obtained from lysogenic bacteria since they failed to isolate *Echr* phages from the environment. This was the first reported phage typing system in *Erwinia*. However, the faint turbid plaques that were produced and the inability to maintain high stock suspensions, meant that this system was not ideal for routine use.

A very recent example of phage typing in *Ecc*, *Eca* and *Echr* strains has been discussed (Gross *et al.* 1991). As in this study, Gross *et al.* isolated a high proportion of *Eca* phages but relatively few *Ecc* phages. In this study only a small number of *Echr* phages were isolated but Gross *et al.* were able to isolate them quite readily, although this species was not common to that area (Oregon, U.S.A.). Gross *et al.* concluded that phage typing, to differentiate between *Erwinia* strains, was varied in its application. More *Ecc* strains were differentiated by serology than phage typing (only 20% of strains could be placed into phage groups). This was not the case with *Eca* strains, however, since many fell into a single serological group (serogroup I). Unlike serogrouping of the *Ecc* strains, phage typing was able to

place all the *Eca* strains into phage groups. In this study the majority of *Eca* strains were placed into phage groups, while those of *Ecc* were not. This finding is in agreement with Gross *et al.*. Since serology has been carried out on only a handful of strains involved in this study (Perombelon pers. comm.), similar comparisons to the work of Gross *et al.* cannot be made. It would seem highly likely, however, due to the nature of *Eca* and *Ecc* serotypes that similar results on serological classification would be obtained (De Boer *et al.* 1979, 1985).

Gross *et al.* (1991) originally developed a phage typing system to differentiate strains on criteria other than LPS, to complement the serological data. They found that few serologically unrelated strains were sensitive to one or more of the phages. A relationship, therefore, existed between phage adsorption and serology, and it was suggested that the receptor for phage attachment in the majority of cases was LPS. The lack of serological data for this study makes any similar conclusions impossible, although the theoretical basis to this has been discussed (Section 1.4.5.1).

The success of the phage typing system in this study can be clearly seen on the recatagorisation of two *Ecc* strains, SCRI105 and SCRI191. These strains, originally thought to be *Ecc* were sensitive to a large number of *Eca* phages (Table C15). Repeating the biochemical analysis of these strains led to their reclassification as *Eca*. Since a high proportion of *Eca* strains (division V) were resistant to the *Eca* phages, they were also tested biochemically but only 1 strain out of 13 analysed was recatagorised to *Ecc* (C466). Although phage typing is not an infallible method of identifying *Eca* and *Ecc* strains, the results do prove its usefulness, especially in association with biochemical tests.

3.3.4 Environmental implications

The findings of this study suggest that phages for *Eca* strains are readily isolated from sewage, while those of *Ecc* are not. This finding is curious since *Ecc* is widely distributed ecologically (being found readily in plants, soil and water) but *Eca* is rarely isolated from the environment, although it is the major cause of blackleg in the U.K. (Perombelon pers. comm.). Although *Echr* is not indigenous to the U.K., four phages were also isolated for this species. The isolation of phages on *Echr* strains by Gross *et al.* (1991), led to the conclusion that, while only 1% were isolated from diseased plants in the Oregon area, this species may still have been a significant potato pathogen under suitable environmental conditions. A second theory however, seems equally likely. Since both *Eca* and *Echr* are not readily isolated from the environment (particularly in the case of this study, ie. in a temperate climate), it is likely that these species/subspecies are not the natural hosts of the isolated phages. These phages therefore, must have alternative hosts in the natural environment. If this is the case, this begs the question as to whether horizontal gene transfer by these phages takes place in such an environment. To date horizontal gene transfer has been found for phages in only a small number of genera (Kokjohn 1989).

There is also the possibility that more phages are isolated on *Eca* strains because, as already mentioned, if they adsorbed to the LPS, they would have only a single serotype to adapt to (a serotype which may be associated with a number of other Enterobacteriaceae). Phages isolated on *Ecc* strains, on the other hand, would have to alter their host range in accordance with the serotype. Too little serological data on the strains from this study has been obtained, and the relevant phage receptors are not known. The theory of horizontal gene transfer could be investigated by using a number of other bacterial species, in particular members of the Enterobacteriaceae, to identify strains sensitive to the isolated phages. On

identification of such strains their serotypes could be determined and compared to those of strains in this study. The possibility of other adsorption sites could also be investigated.

In conclusion a number of phages were isolated from sewage which propagated on *Ecc*, *Eca* and *Echr* strains. Five times more phages were isolated on *Eca* than on the other 2 strains. While phages to *Echr* were not investigated, those of *Eca* and *Ecc* were. These phages fell into different groups based on structural morphology, RE analysis, plaque morphology and host range. The *Eca* TP phages were members of Bradleys group A, based on their structural morphology but based on their RE analysis were placed into 2 different groups. Similarly some *Eca* phages which fell into Bradleys group C based on structural morphology, were placed into 5 different groups based on RE analysis. The *Ecc* phages gave turbid plaques and belonged to Bradleys group B, but little other information was obtained to allow further groupings. Even between genetically related phages host ranges varied considerably. This suggests that genetic variation between the phages may play some part in their adaption to different hosts.

CHAPTER 4

GENERALISED TRANSDUCTION

Chapter 4

4 Generalised transduction

4.1 Introduction

Within the last 5 years *Ecc* SCRI193 and *Eca* SCRI1043 have become amenable to genetic manipulation by techniques such as conjugation, transposon mutagenesis and transformation (Hinton 1985). A very important technique, initially unavailable for the study of these organisms, is generalised transduction. The use of generalised transduction in molecular biology has been introduced in detail in Section 1.5.

Attempts have previously been made to obtain T4 or P1 sensitive strains of *Erwinia* (Pirhonen and Palva 1988; Murooka and Harada 1979). In this study however, *Eca* SCRI1043 and *Ecc* SCRI193 were found not to be susceptible to T4 or P1 infection (see Section 1.5.7). Because of the great genetic utility of generalised transduction part of this study was directed at isolating and characterising transducing phages.

In this study 25 phages were isolated for *Eca* SCRI1043 and 5 for *Ecc* SCRI193. These phages were used in a primary assay to screen for phages capable of generalised transduction. In this chapter the characterisation of 2 phages, ϕKP and ϕM1, both capable of generalised transduction in *Eca* SCRI1043 and *Ecc* SCRI193 respectively, is described. This is the first report of a generalised transducing phage for *Eca* and only the second for *Ecc* (the first being reported by Mukvick *et al.* 1987).

4.2 Results

4.2.1 Phage ϕKP

4.2.1.1 Screening for lysogeny

Bacteria from the centre of several turbid plaques, created by ϕKP, were streaked twice to single colonies. These colonies were tested for the production of phage and for immunity to ϕKP by spot tests. From over 100 colonies taken, none showed signs of lysogeny indicating that ϕKP was probably a virulent phage.

4.2.1.2 Test for transduction

ϕKP was assayed for generalised transduction by the method described in Section 2.13. Initial screens were carried out using wild type ϕKP and a *cysB* auxotroph (containing *cysB*::Tn*5*). Phage adsorption to the auxotroph was carried out at 25°C for 30 min at an m.o.i. of 1. Plating of the transduction mix onto minimal medium produced approximately twice the number of colonies produced on the control plates (auxotroph plus phage buffer). This prompted further investigation in an attempt to improve the frequency of transduction by ϕKP.

4.2.1.3 Optimization of transduction

To increase the frequency of transduction various approaches were tried. These included the use of adsorption studies, the effect of m.o.i., variation in temperature and the addition of divalant cations.

4.2.1.3.a Adsorption studies

Adsorption of ϕKP to *Ecc* SCRI193 was carried out at an m.o.i. of 1 and the phage population remaining unadsorbed at set time intervals was titrated, indicating the degree of adsorption over this time period (Fig. D1). Rapid adsorption took place over the first 45 minutes resulting in adsorption of up to 90% of the phage population. After this time the process slowed dramatically. At 30 minutes up to 85% of the population had adsorbed and this time period was therefore chosen as standard, since it would minimize the time of the transduction assay but allow a high proportion of the phage population to adsorb.

The possibility of transducing markers between similar bacterial strains or species is highly desirable since similarities in gene expression, for example, can be studied (Murooka *et al.* 1978). For this reason an attempt was made to study the adsorption of ϕKP to *Eca* SCRI1043 (Fig. D1). It was clear from this investigation that ϕKP was unable to adsorb to *Eca* SCRI1043 and transduction between them, via ϕKP, would therefore not be possible.

Some phages require the presence of divalent cations for adsorption, possibly the best known example being Lambda which requires magnesium ions (Arber *et al.* 1983). The plaquing efficiency of ϕKP was tested with and without the presence of magnesium ions (10mM magnesium sulphate). The values obtained (Table D1) show similar plaquing efficiencies in both cases and magnesium was not therefore, used routinely.

4.2.1.3.b Multiplicity of infection

The efficiencies of transduction at different m.o.i.s were studied. At 30°C an m.o.i.

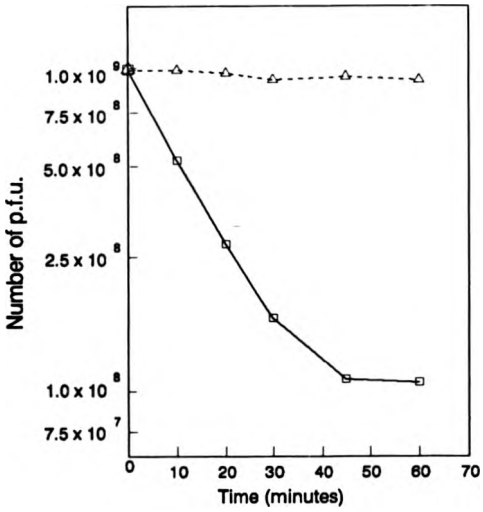


Figure D1 Adsorption of ϕKP to Ecc SCRI193 and Eca SCRI1043 at 25°C and an m.o.i. of 1.

——— = Eca SCRI1043
..... = Ecc SCRI193

Temperature	ϕKP Titre	Relative e.o.p.	Host killing	Magnesium Requirement Mg+	Mg-
18°C	6.7×10^9	0.56	----	----	----
25°C	1.2×10^{10}	1.00	1.5×10^8	5.6×10^9	1.5×10^{10}
30°C	4.3×10^9	0.34	2.0×10^8	----	----
37°C	no plaque	–	----	----	----

Table D1. Efficiency of plating of ϕKP on *Ecc* SCRI193 and host killing at various temperature, together with e.o.p. with and without magnesium ions (25°C).

Temperature	m.o.i.. 10	1	0.1
25°C	1.4×10^{-7}	7.0×10^{-7}	$< 1.0 \times 10^{-7}$
30°C	1.8×10^{-7}	6.8×10^{-6}	2.0×10^{-7}
37°C	$< 1.0 \times 10^{-9}$	2.0×10^{-7}	$< 1.0 \times 10^{-7}$

Table D2. Transduction frequencies of ϕKP at various temperatures and m.o.i.. Frequencies are given per p.f.u..

of 1 gave frequencies over three times those produced at an m.o.i. of 10 and 0.1 (Table D2). The low values obtained at 10 were probably due to lysis from without. From this study an m.o.i. of 1 was chosen for routine use.

4.2.1.3.c Effect of temperature

In order to obtain maximum transduction of a marker, the killing of host cells by wild type phage particles must be minimized (a problem particularly associated with virulent phages) (see Sections 4.3.2.1). Such a reduction can be achieved by controlling the m.o.i. (Section 4.2.1.3.b), but also by controlling the temperature at which the phage replicates or adsorbs. A reduction in host killing would suggest an adverse temperature effect on phage replication, while a reduction in the efficiency of plating (e.o.p.) could result from either a reduction in replication or adsorption. The e.o.p. of φKP on *Ecc* SCRI193 at 30°C was reduced by 65% compared to the e.o.p. at 25°C (Table D1). This reduction in e.o.p. was supported by an increase in transduction frequency at 30°C (Table D2), but little difference in host killing was seen at 30°C and 25°C (Table D1). This latter finding suggests that the reduction in e.o.p. was mainly due to a reduced ability to adsorb and not a reduced ability to replicate. At 37°C no plaques were obtained suggesting an inability of φKP to adsorb or to replicate at this temperature. These findings suggested that, after phage adsorption, further incubations could be carried out at 37°C, allowing expression of markers but preventing host killing by φKP. In practice however, fewer transductant colonies were produced at 37°C than at 30°C (data not shown). This may have been due to a less healthy growth of the cells at the higher temperature. From the three temperatures used in this study, 30°C was chosen as the most suitable for transduction.

4.2.1.4 Generalised nature of transduction

The ability of ϕKP to transduce the *cysB* marker was insufficient evidence to suggest generalised transduction. In order to test this phenomenon, a number of other markers were selected and frequencies for their transduction obtained (Table D3). All markers tested (including the plasmid pHCP2) were transduced at similar frequencies to the *cysB*$^+$ marker, with the exeption of the *lac* marker which transduced at a frequency 13 times lower. These findings suggest that ϕKP is a generalised transducing phage.

4.2.1.5 Secondary transposition

During transduction, transposons may excise from their original site and insert elsewhere on the chromosome or replicative copies may also arise in the chromosome. This phenomenon is called secondary transposition (or transposon hopping) and is dependent on the site of the insertion. Transduction of a number of Tn*5* insertion mutations, eg. *cysB*::Tn*5*, into wild type *Ecc* SCRI193 allowed the percentage of secondary transposition to be calculated for each marker. This was done by comparing the number of kanamycin resistant prototrophic colonies with the number of kanamycin resistant *cysB* auxotrophs (kan^r prototrophs / kan^r auxotrophs x 100). From these studies percentages of secondary trasposition ranging from 6.5% to >90% were obtained (Table D4).

4.2.1.6 Ultraviolet irradiation

The effect of U.V. irradiation on the transducing efficiency of generalised transducing phages has been well documented (see Sections 152, 153 and 1561).

Recipient	Selected Marker	Reversion/Spontaneous Resistance Frequency	Transduction Frequency
HC519 (*leu*::Tn*5*)	*leu*+	2.0×10^{-8}	2.8×10^{-6}
HC500 (*cysB*::Tn*5*)	*cysB*+	2.9×10^{-7}	1.0×10^{-6}
HC8.11 (*trp*::Tn*5*)	*trp*+	4.7×10^{-8}	1.4×10^{-6}
HC12.19 (*arg*::Tn*5*)	*arg*+	1.0×10^{-8}	1.4×10^{-6}
HC512 (*lac*::Tn*5*)	*lac*+	$< 10^{-8}$	2.1×10^{-7}
HC17.5 (*pur*::Tn*5*)	*pur*+	5.3×10^{-7}	2.0×10^{-6}
SCRI 193	Ap^r	$< 10^{-8}$*	2.4×10^{-6}

Table D3. φKP-mediated transduction of auxotrophy markers, and plasmid pHCP2, in *Ecc* SCRI193. Frequencies of transduction are shown (per p.f.u.) together with reversion / spontaneous resistance frequencies.

φKP Lysate	Selected Marker	Kan^r Colonies	Prototrophic Colonies	% Secondary Transposition
φKP.*leu*::Tn*5*	Kan^r	639	58	9
φKP.*cysB*::Tn*5*	Kan^r	1148	0	undetectable
φKP.*trp*::Tn*5*	Kan^r	809	655	80
φKP.*arg*::Tn*5*	Kan^r	552	46	8
φKP.*lac*::Tn*5*	Kan^r	712	334	47
φKP.*pur*::Tn*5*	Kan^r	891	29	3

Table D4. Percentage secondary transposition of Tn*5* from various auxotrophy markers transduced into prototrophic *Ecc* SCRI193.

Transductions were carried out at an m.o.i. of 1.

The effect of U.V. irradiation on transducing efficiencies of ϕKP was investigated. Two markers, *leu*$^+$ and *cysB*$^+$, were chosen for U.V. studies because of the contrast in their transduction frequencies, transducing at relatively high and low frequencies respectively. At set time intervals during U.V. irradiation 100μl of the lysate was used to transduce the markers into recipient cells, which were plated onto minimal medium and prototrophic colonies counted (as previously described) (Fig. D2). As phage inactivation occurred the number of transductant colonies increased to a maximum (equivalent to a three log drop in phage titre). Further inactivation led to a decrease in the number of transductant colonies. Although both the *leu*$^+$ and *cysB*$^+$ markers showed a maximum transduction frequency after a three log fall in phage titre, the frequency of *cysB*$^+$ transduction increased by a factor of 3.7 compared to only 1.7 for the *leu*$^+$ marker.

4.2.1.7 Host range

From 49 Ecc strains tested ϕKP was able to plaque on only 2 (SCRI193 and ATCC39048). The inability of ϕKP to transduce in SCRI1043 has been described above. Recent data shows that ϕKP is also able to transduce in ATCC39048 (G. Salmond pers. comm.). From a range of other bacterial strains tested (see Section 322f) no susceptible strains were found.

4.2.2 Phage ϕM1

4.2.2.1 Screening for lysogeny

Bacteria from the centre of several ϕM1 plaques on *Eca* SCRI1043 were tested in a similar way to those of ϕKP. From the 100 colonies taken, none showed signs of

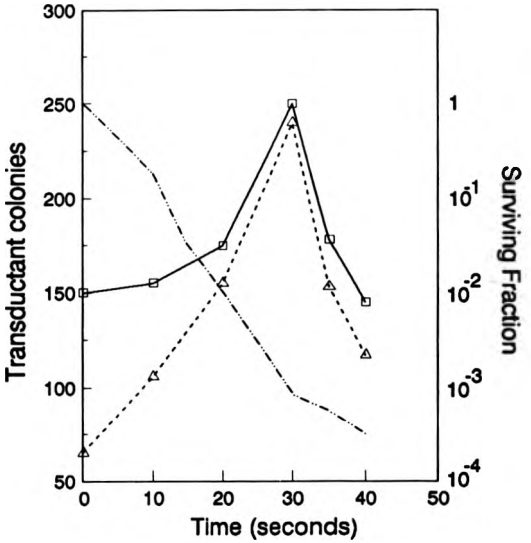


Figure D2 Inactivation of ϕKP by U. V. irradiation , and its effect on the transduction of 2 auxotrophic markers (leu+ and cysB+) from a ϕKP.wild type lysate, into 2 auxotrophs of Ecc SCRI193 (below).

——— = Transduction of leu+ marker into HC519.
- - - - - - = Transduction of cysB+ marker into HC500.
-·-·-·- = ϕKP titre.

lysogeny indicating that ϕM1 was probably a virulent phage.

4.2.2.2 Test for transduction

ϕM1 was assayed for generalised transduction by the method described in Section 2.13. As with ϕKP, initial screens were carried out using the wild type phage and a *cysB* auxotroph of SCRI1043. After an initial transduction assay, the number of transductant colonies was greater than the number of revertants. A further investigation was carried out to optimize the frequency of transduction. In the case of ϕM1 no secondary transposition experiments were carried out.

4.2.2.3 Optimization of transduction

Attempts to optimize the frequency of transduction were carried as described with ϕKP.

4.2.2.3.a Adsorption studies

Adsorption of ϕM1 to *Eca* SCRI1043 was carried out at an m.o.i. of 1 and an adsorption curve obtained as previously described (Fig. D3). By comparison with ϕKP adsorption was very slow, with 34% adsorbing within the first 30 minutes and only 43% after 2 hours. 30 min was chosen as a standard adsorption time, since 35% of the total phage population had adsorbed after this time, compared to only 9% more after 2 hours. Adsorption of ϕM1 to *Ecc* SCRI193 did not take place (Fig. D3). The plaquing efficiency of ϕM1 was tested with and without the presence of magnesium sulphate (10 mM). The values obtained (Table D5) show

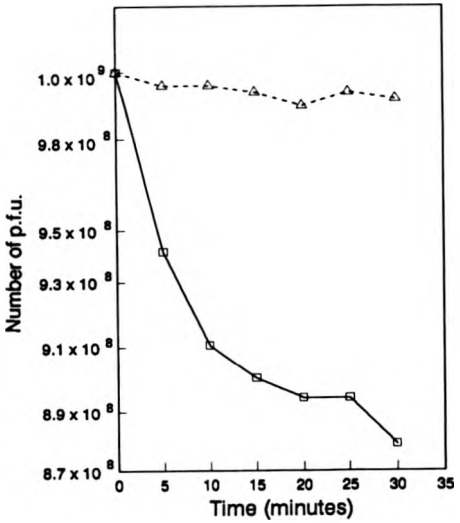


Figure 53 Adsorption of ϕM1 to Eca SCRI1043 and Ecc SCRI193 at 25°C and an m.o.i. of 1.

——— = Eca SCRI1043

..... = Ecc SCRI193

Temperature	ϕM1 Titre	Relative e.o.p.	Magnesium requirement Mg+	Mg-
18°C	1.7×10^{12}	1.90	---	---
25°C	9.0×10^{11}	1.00	8.0×10^{11}	1.0×10^{12}
30°C	no plaques	---	---	---

Table D5. Efficiency of plating of ϕM1 on *Eca* SCR11043 at various temperatures, together with e.o.p. in the presence and absence of magnesium ions (25°C).

Temperature	m.o.i. 10	1	0.1
25°C	1.8×10^{-8}	2.0×10^{-6}	4.2×10^{-6}

Table D6. Transduction frequencies (of the *cysB*$^+$ marker into *cysB*$^-$ auxotrophs of *Eca* SCR11043) of ϕM1 at 25°C and various m.o.i.s. Frequencies are given per p.f.u..

plaquing efficiencies similar in both cases, suggesting that magnesium ions did not improve phage adsorption.

4.2.2.3.b Multiplicity of infection

The efficiencies of transduction of the *cysB*$^+$ marker at m.o.i.s of 0.1, 1 and 10 were studied at 25°C (Table D6). An m.o.i. of 10 gave a transduction frequency of 1.8×10^{-8} (18 colonies per 100 μl of transductant cells). An m.o.i. of 1 gave an average of over 200 transductant colonies per plate compared to 42 at 0.1, their transduction frequencies being 2.0×10^{-6} and 4.2×10^{-6} respectively. Although an m.o.i. of 0.1 gave the greatest frequencies of transduction per p.f.u., an m.o.i. of 1 gave the greatest number of colonies per plate. An m.o.i. of 1 was therefore used in further assays. Higher numbers of transductants may have been possible however, by increasing the number of bacteria in the transduction assay and adding phage at an m.o.i. of 0.1.

4.2.2.3.c Effect of temperature

The e.o.p. of ϕM1 was tested at 18 °C, 25°C and 30°C (Table D5). The number of plaques produced at 18°C was slightly greater than at 25°C. At 30°C however, no plaques were seen, even though adsorption was done at 25°C. The failure of ϕM1 to plaque at 30°C, even at high titre, appears to be due to an inability of the phage to replicate. At a high m.o.i. a large percentage of host cells would contain phage DNA, which on plating would lead to lysis of the bacterial lawn, if replication was occurring. This effect however, was not seen. From the temperatures used in the study, 30°C was chosen as the optimum for transduction.

4.2.2.4 Generalised nature of transduction

The ability of ϕM1 to transduce a number of markers was tested, and transduction frequencies for each marker obtained (Table D7). All markers were transduced at similar frequencies, with less than a 6 fold difference between the lowest (*nad*+) and highest (*ade*+) frequencies. These findings suggest that ϕM1 is a generalised transducing phage.

4.2.2.5 Ultraviolet irradiation

U.V. irradiation of ϕM1 was undertaken in an attempt to increase the frequencies of transduction (Fig. D4), in this case the transduction of the *ura* allele into an *ura*$^-$ auxotroph. This experiment was performed as previously described (Section 2.8.1) but with two exceptions. Firstly the lysate was irradiated in LB (the storage medium of the stock ϕM1 lysate), since the titre of the lysate was too low for dilution in phage buffer. Secondly the lysate was irradiated in a 2.5 cm petri dish, not a 5 cm petri dish as previously used. This was to reduce the volume of lysate needed for the study, but may also have increased the depth of the lysate, making U. V. penetration more difficult. The result of this may have been to significantly reduce the rate of ϕM1 inactivation, taking 28 seconds to fall by 2 logs (Fig. D4), compared to less than 10 seconds in previous studies (Fig. C3). Although the ϕM1 population was inactivated by little more than 2 logs, a sharp rise in the number of transductant colonies was seen. Since no fall in the number of transductants was seen after 30 sec, it is not clear whether this was the optimum time of irradiation. At 30 sec however, the frequency of transduction had increased by a factor of 2.5. Futher inactivation of the lysate may have led to an additional increase in the number of transductant colonies obtained.

Recipient	Selected Marker	Reversion/Spontaneous Resistance Frequency	Transduction Frequency
TA 100 (*ura*::Tn*5*)	*ura*+	6.8×10^{-7}	8.4×10^{-6}
HA (7.40) (*ade*::Tn*5*)	*ade*+	3.0×10^{-8}	9.7×10^{-6}
TA 200 (*arg*::Tn*5*)	*arg*+	1.0×10^{-8}	5.9×10^{-6}
TA300 (*cysB*::Tn*5*)	*cysB*+	1.0×10^{-8}	2.6×10^{-6}
HA150 (*nad*::Tn*5*)	*nad*+	3.0×10^{-8}	1.7×10^{-6}
SCRI 1043	Ap^r	$< 1.0 \times 10^{-8}$	5.3×10^{-6}

Table D7. ϕM1-mediated transduction of a number of auxotrophy markers, and plasmid pHCP2, in *Eca* SCRI1043. Frequencies of transduction are shown (per p.f.u.) together with reversion / spontaneous mutation frequencies.

Transductions were carried out at an m.o.i of 1.

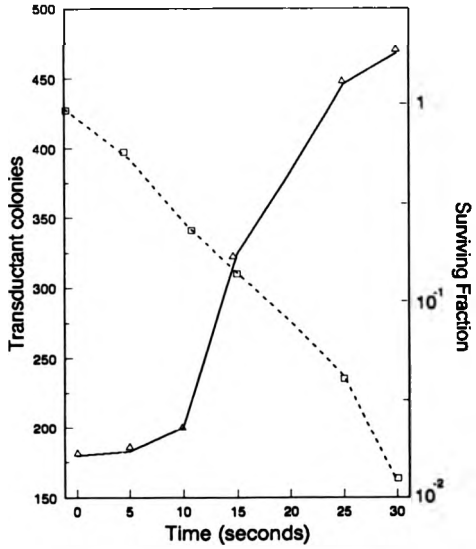


Figure D4 Inactivation of ϕM1 by U. V. irradiation and its effect on the transduction of an auxotrophic marker (ura+) from a ϕM1.wild type lysate into an auxotroph (below) of Eca SCRI1043.

—— = Transduction of ura+ marker into TA100

- - - = ϕM1 lysate

4.2.2.6 Host range

The host range of ϕM1 has been described previously in Chapter 3. ϕM1 is able to produce zones of clearing on 38 *Eca* strains (from a total of 58 strains tested), although the ability to produce plaques on all of these was not examined. From a range of other bacterial strains tested (see Section 3.2.2.f), non were susceptible to ϕM1 infection. Of the *Eca* strains which appeared susceptible to ϕM1, only in SCRI1043 was generalised transduction attempted.

4.3 Discussion

4.3.1 Generalised transduction in ϕKP and ϕM1

Two phages, ϕKP and ϕM1, were found to be capable of generalised transduction in *Erwinia* strains *Eca* SCRI1043 and *Ecc* SCRI193 respectively. The phages were characterised (Chapter 1), the optimum conditions for transduction determined and a number of markers transduced.

ϕKP, one of four *Ecc* phages tested for generalised transduction, was a virulent phage and belonged to group B of the Bradley classification system. It contained double stranded DNA of approximately 46 kb which, due to the transducing nature of the phage, was probably packaged by the headful mechanism. 137 *Erwinia* strains, of which 49 were *Ecc*, were tested for their sensitivity to ϕKP. The host range of ϕKP was very narrow infecting only two *Ecc* strains, SCRI193 on which it was isolated and ATCC39048. ATCC39048 produces carbapenem antibiotic and has recently been shown to be sensitive to ϕKP mediated generalised transduction (G. Salmond pers. comm.).

Of the 25 *Eca* phages tested only ϕM1 appeared capable of generalised transduction. ϕM1 was a virulent phage and belonged to group A of the Bradley classification system. It contained double stranded DNA of approximately 45 kb and due to its transducing nature was probably packaged by the headful mechanism. The host range of ϕM1 was wide amoung *Eca* strains infecting 38 of 58 strains tested, but no other species/subspecies were sensitive to it.

Once the transducing ability of both phages had been established, attempts were made to improve transduction frequencies. Adsorption rates were found for each phage on its respective host and on an alternative host. The adsorption rate of ϕKP to its host *Ecc* SCRI193 was greater than the adsorption rate of ϕM1 to its host *Eca* SCRI1043. At 25°C, incubation for 30 min led to 85% adsorption of the ϕKP population compared to only 35% adsorption of ϕM1. Although incubation of ϕM1 for longer periods may have increased the number of transductants, it may also have led to an increase in host killing by the phage. A phage able to transduce markers between different organisms, whether genera, species or subspecies, would be of enormous value in comparing their genetic systems. For this reason attempts were made to adsorb each phage to an alternative host (ie. ϕKP to *Eca* SCRI1043 and ϕM1 to *Ecc* SCRI193), in the hope of obtaining a transducing phage capable of exchanging markers between these subspecies (a tool of enormous potential for the genetic analysis of these two organisms). In both cases the lack of phage adsorption suggested that transduction between the 2 subspecies was not possible. In neither ϕKP nor ϕM1 were magnesium ions able to increase e.o.p. suggesting that these divalent cations did not improve phage adsorption. Whether other cofactors were capable of this was not investigated.

The virulent nature of each phage made it important to investigate the effects of their m.o.i. on transduction. In the case of both phages an m.o.i. of 1 resulted in the greatest number of transductant colonies. An m.o.i. of 10 led to reduced

numbers of transductants, probably due to multiple infection of transductant cells resulting in their lysis (an effect that would be less significant in the case of a temperate phage). An m.o.i. of 0.1 was probably too low to allow a significant number of transducing particles to adsorb to the relevant host. The number of transductant colonies may have been increased at an m.o.i. of 0.1 however, by increasing host cell numbers.

During transduction assays, once phage adsorption had taken place, host cells were washed to remove excess phage. At this stage the cells could have been plated directly onto selective media or, in the case of selection for antibiotic resistance, incubated to allow expression of this resistance. It was decided to include an incubation step in all transduction assays in an attempt to standardize the method. During this incubation period phage replication, lysis and reinfection could have occurred. To minimize these effects incubation was carried out for only a short period of time (30-45 min) and incubation temperatures were adjusted to obtain the least host killing and to reduce or prevent reinfection. At 30°C the e.o.p. of ϕKP was significantly reduced compared to 25°C. This may have been due to a decrease in ability to adsorb to or replicate within the host. At 37°C no plaques were produced on the host indicating an inability of the phage to adsorb to or replicate within the host. Incubation at 37°C produced low numbers of poorly growing transductants, probably relating to the fact that this temperature is higher than the optimum growth temperature for *Ecc* strains (Perombelon 1985). 37°C was therefore unsuitable for post-adsorption incubation. *Eca* is known to grow at cooler temperatures than *Ecc* (see Section 1.1.3). This being the case, phages infecting this subspecies may have a lower replication/adsorption temperature than phages infecting *Ecc* strains. The e.o.p. of ϕM1 on SCRI1043 supports this idea, since unlike ϕKP on SCRI193, ϕM1 is unable to plaque on SCRI1043 at 30°C.

To investigate the generalised nature of ϕKP and ϕM1 transduction, the ability of

both to transduce a range of markers was tested. All markers tested, including the plasmid pHCP2, were transduced suggesting the phages were generalised transducers. Frequencies of transduction by ϕKP were similar in each case, with the exception of the *lac*$^+$ marker which transduced at a frequency 13 times lower than the most highly transduced marker (*leu*$^+$). In general the frequencies of markers transduced by ϕM1 were greater than those of ϕKP. The difference between the highest and lowest transduction frequencies was less than 6 times. Secondary transposition of Tn*5*, within the host SCRI193, showed wide variation between markers. These results clearly demonstrate the use of generalised transducing phages in the study of transposon insertion stability.

The effect of U.V. irradiation on increasing transducing efficiencies of a number of phages has been shown and is described in Sections 152 and 153. An increase in the number of transductants has been shown to be a consequence of increased recombination (between transduced DNA and the recipient chromosome), leading to the conversion of abortive to complete transductants (Benzinger and Hartman 1962; Newman and Masters 1980; Wall and Harriman 1974). Differences in the recombinational ability of markers means that frequencies can vary by as much as 25 times (in the case of P1, Newman and Masters 1980). On exposure to U.V. irradiation recombination in P1 is thought to reach a maximum level, with differences relating to gene dosage and not differences in packaging ability (Newman and Masters 1980). In the case of both ϕKP and ϕM1 U.V. irradiation had the effect of increasing transduction frequencies of the markers tested. The transduction frequency of the marker *leu*$^+$ by ϕKP was 2.8 times greater than that of *cysB* before U.V. irradiation, but only 1.3 times greater afterwards (Fig. D2). The increase in transduction frequency of the *cysB* marker suggests an increased recombination leading to the conversion of abortive to complete transductants, a phenomenon which was also observed by counting abortive and complete

transductant colonies (data not shown). An increase in transduction frequency of the *ura*$^+$ marker by ϕM1 after U.V. irradiation (Fig. D4) suggested a similar fate for abortive transductants. In both the case of ϕKP and ϕM1, the possibility of preferential packaging and gene dosage would need further study.

4.3.2 Comparison of ϕKP and ϕM1 with other generalised transducing phages.

Many generalised transducing phages have been isolated but it would be impossible to compare ϕKP and ϕM1 with all of these. In this discussion ϕKP and ϕM1 will be compared briefly to the coliphage P1, T1, T4 and P22 (P22, for convenience, will be refered to as a coliphage in this discussion) and to generalised transducing phages of phytopathogens.

ϕKP and ϕM1 belong to the Bradley morphological groups B and C respectively. From this observation the structural morphology of ϕKP resembles T1 and ϕM1 resembles P22. The genome sizes of ϕKP (46 kb) and ϕM1 (45 kb) resemble that of T1 (47 kb) and P22 (42 kb) but are much smaller than those of P1 (99 kb) and T4 (166 kb). P1, P22, T4 and T1 all contain linear dsDNA which is cyclically permuted and terminally redundant to varying extents. In all these phages DNA is packaged from long concatemers by the headful mechanism (Masters 1985). It is clear from restriction data that both ϕKP and ϕM1 contain linear dsDNA (Chapter 3). The ability of the coliphages to transduce appears to be a consequence of their DNA packaging system. If this is the case it seems likely that ϕKP and ϕM1 also package DNA in this manner. Some evidence for this comes from the ability of both ϕKP and ϕM1 to transduce the plasmid pHCP2. Packaging of plasmids by T1 has been shown to occur by the formation of plasmid DNA into concatemers which replicate by the rolling circle method and thereby package by "headfuls" (Drexler and Kylberg 1975). This mechanism of packaging has also been shown to occur in

plasmid transduction by T4 (Takahashi and Saito 1982), and by the *Pseudomonas aeruginosa* transducing phage D3 (Sharp *et al.* 1990). If this is a general method of plasmid transduction it seems possible that φKP and φM1 also transduce plasmid DNA in this fashion. The virulent phages T1 and T4 require one or more mutations within their genome to successfully transduce (Masters 1985) (Section 1.5.5). These mutations either reduce the efficiency of host DNA degradation (T4) or prevent phage replication within the host (T1). φKP and φM1 both appear to be virulent although they are able to transduce at relatively high frequencies without the need for mutation. Nevertheless, mutations similar to those used in T1 and T4 may improve transduction frequencies.

Transduction frequencies vary considerably between the coliphages (Masters 1985). P1 transduces various markers at relatively high frequencies (10^{-5} to 3×10^{-4}), while P22 transduces at low frequencies (10^{-9} to 10^{-6}). T4 has the greatest range of frequencies (0 to 10^{-4}), while T1 has values intermediate of the other coliphages (5×10^{-7} to 2×10^{-5}). φKP and φM1 transduce markers at frequencies of 2.1×10^{-7} to 2.8×10^{-6} and 1.7×10^{-6} to 9.7×10^{-6} respectively, values intermediate of those of the coliphages.

P1 is able to infect a number of Enterobacteriaceae and other Gram-negative bacteria including, *E.coli*, *Klebsiella*, *Enterbacter*, *Citrobacter*, *Salmonella*, *Serratia*, *Proteus*, *Pseudomonas*, *Agrobacterium* and *Erwinia*. This makes possible the construction and study of inter-generic hybrids of these organisms as well as allowing generalised transduction within each genus (Murooka and Harada 1979). Pirhonen and Palva (1988) attempted to obtain T4 sensitive strains of *Ecc* and *Eca*. On the whole *Ecc* strains showed resistance to the phage whereas most *Eca* strains showed phage action. On further analysis it was shown that T4 was adsorbing to, and causing lysis of, these strains without replicating within them (1 *Ecc* strain and 1 *Eca* strain did however, show lytic growth of T4). Although transduction into

these strains was demonstrated, no transducing lysates were produced on them.. In this study attempts were made to increase the host range of T4 and P1 *on Eca* SCRI1043 and *Ecc* SCRI193. While no phage action was seen on addition of P1 to the strains, T4 spotted onto a lawn of SCRI1043 produced zones of clearing. On dilution no single plaques were obtained suggesting T4 was adsorbing but not replicating within the strain, as with many *Eca* strains tested by Pirhonen and Palva (1988).

1.3.2.1 Comparison of φKP and φM1 with generalised transducing phages of plant associated bacteria

The characteristics of generalised transducing phages of phytopathogens are summarised in Table A4. These phages, all of which possess tails, fall into three morphological classes (groups A, B and C). Genome sizes (where noted) vary widely, CP75 of *P.cepacia* possesses a genome of 52 kb (Matsumoto *et al.* 1986) and N3 of *R.meliloti* possessing a genome of 190 kb (Martin and Long 1984). In comparison φKP and φM1 possess small genomes of only 46 kb and 45 kb respectively. Phages RL38 of *R.leguminosarum* (Buchanan-Wollaston 1979), N3 of *R.meliloti* (Martin and Long 1984) and M12 of *R.meliloti* (Turlough *et al.* 1984) appear virulent. These phages do not require mutations to allow transduction as in the case of T1 and T4, but do require very low m.o.i.s. The requirement for low m.o.i.s, eg. 0.5, 0.1 and 1 for infection by the phages M12, N3 and RL38 respectively, suggests a low m.o.i. is needed to prevent host killing by these phages. A similar situation has been shown in transduction by φKP and φM1, which both show large reductions in transducing frequency at m.o.i.s above 1. As in the case of φKP and φM1, adsorption frequencies vary widely. While 90% of RL38 particles adsorb to their host in 3 hours, a similar percentage of Pssy15 adsorbs in 3 mins. The slow adsorption rate of the virulent phage RL38, as in the case of φM1,

may lead to a low level of host killing and thereby help to improve transduction frequencies. Frequencies of transduction vary from as high as 10^{-6} to 10^{-5} in the case of M12 to as low as 8.2×10^{-8} to 1.3×10^{-7} in the case of Pssy15. The majority of phages transduce at frequencies in the range 10^{-6} to 10^{-8}. This being the case, ϕKP and ϕM1 have transduction frequencies greater than average at 2.1×10^{-7} to 2.8×10^{-6} and 1.7×10^{-6} to 9.7×10^{-6} respectively. From information available, host ranges of the generalised transducing phages listed appear, on the whole, very narrow. In the majority of these phages however, only a single wild type bacterial strain, in each case, was tested making further host range analysis impossible. Only in the case of RL38 is a phage reported to infect more than a single species. This phage is able to transduce from *R.leguminosarum* to *R.trifolii* but not *vice versa*. From available data, the ability of ϕM1 to infect 38 *Eca* strains (out of 58 tested) suggests this phage has a larger host range than the majority of the generalised transducing phages listed in Table A4. The effect of U.V. irradiation on the tranduction frequencies of ϕKP and ϕM1 has been demonstrated (Sections 4.2.1.6 and 4.2.2.5), showing improved frequencies of 3.7 and 2.5 times respectively. U.V. irradiation has also been reported to improve transduction frequencies in several other of the listed phages (phage 11, RL38 and Pssy15), improving transduction frequencies of Pssy15 two-fold.

4.3.2.2 Generalised transducing phages of *Erwinia* species

The *Echr* generalised transducing phages, Erch-12 and ϕEC2 are both temperate phages (Table A4). The genome size of ϕEC2, at 62 kb, is slightly larger than ϕKP and ϕM1. Transducing frequencies of Erch-12 are generally lower than those of ϕKP and ϕM1 (7.0×10^{-9} to 1.0×10^{-7}). U.V. irradiation does however, have the effect of increasing frequencies by 4-38 times in Erch-12. This phage adsorbs quickly to its host (*Echr* EC183), with 99% adsorbing in 30 minutes, and is used

for transduction at a m.o.i. of between 1 and 10, probably because of its temperate nature. The host ranges of both Erch-12 and ϕEC2 are narrow. Erch-12 infects only two strains of *Echr*, including EC183, a strain used in the study of enzyme production (see Section 1.2.2.1). ϕEC2, similarly, infects only two *Echr* strains, B374 and 3937. Both these strains have been used extensively in the elusidation of *Erwinia* pathogenicity, including enzyme secretion (see Sections 1.2.2.1 and 1.2.2.2).

Two temperate phages ϕ49 and ϕ59 which infect *Ecc* strain 268 and *E.horticola* strain 450 are capable of transduction (Mukvich *et al.* 1987). Markers transduced include *his*, *ile*, *ser*, *thr*, *thy*, *trp* and *ura* with frequencies varying between 1.1 x 10^{-8} to 1.8 x 10^{-6}. These frequencies are, on the whole, lower than those of ϕKP and ϕM1. Cotransduction of markers has also been carried out using these phages. ϕ59 has an isometric head, a long non-contractile tail and a base plate, therefore belonging to group B of the Bradley classification system and similar to ϕKP in this respect. Its genome size is smaller than those of ϕKP and ϕM1 at approximately 39 kb. (Kishko *et al.* 1983).

In chapter 3 the isolation of a number of phages to *Eca* SCRI1043 and *Ecc* SCRI193 was described, together with details on their characterisation and use in phage typing. In this chapter the transducing abilities of 2 of these phages have been described. Such phages are now being used to help in the genetic analysis of many biological processes in *Erwinia*. The *Erwinia* strains commonly used in this laboratory (*Eca* SCRI1043, *Ecc* SCRI193 and *Ecc* ATCC39048) are now amenable to generalised transduction by ϕKP and ϕM1. The use of ϕKP and ϕM1 have allowed, a) mutated genes to be transduced into "clean" backgrounds, b) cotransductional mapping of transposon linked mutations, and of the *out* gene cluster, and c) localized mutagenesis (G. Salmond pers. comm.).

One of the major reasons for isolating phages against *Erwinia* was for use as "probes" for the identification of cell surface mutants, which could be affected in pathogenicity. This is the topic of the next chapter.

CHAPTER 5

AVIRULENT, PHAGE RESISTANT MUTANTS OF *Eca* SCRI1043

Chapter 5

5 Avirulent, phage resistant mutants of *Eca* SCRI1043

5.1 Introduction

The mechanisms of both pathogenicity and virulence in *Erwinia* species, as for many other pathogenic bacteria, has been studied intensely for many years. The use of transposon mutagenesis, which creates gene disruptions randomly around the chromosome, has been very successful in these studies (see Section 1.2.1). A less commonly adopted approach is the use of bacteriophages to isolate mutants altered in their cell surface, eg. lipopolysaccharide (LPS) mutants (Schoonejans *et al.* 1987) or outer membrane protein (OMP) mutants (Expert and Toussaint 1985) (see Sections 1.2.bii and 1.2.2.2). In *Erwinia carotovora* subsp. *atroseptica* strain SCRI1043, the transposon mutagenesis approach has been used with great success (Hinton *et al.* 1989a). T4 resistance has previously been used to isolate LPS defective mutants of *Eca*, although these LPS defective mutants were found not to affect virulence (Pirhonen *et al.* 1988). T4 resistant mutants have also been shown to possess both LPS defects (thought not to be associated with virulence) and a galactose-sensitive phenotype (Pirhonen *et al.* 1991). We have previously reported the first successful isolation of phages *for Eca* SCRI1043 and report here their use, as a follow up experiment to that of Hinton *et al.* (1989a), in the search for virulence determinants of this strain.

5.2 Results

5.2.1 Phage resistant mutant isolation and cross-resistance

10 phage resistant mutants were obtained for each of the phages A1, A4L, M1, M2 , M3 and M4. 40 mutants were obtained which were resistant to phage A5. All mutants were cross tested to a number of other phages (Table E1). The majority of mutants gave identical patterns of resistance, showing resistance to all CP phages and sensitivity to all TP phages. These patterns appeared to vary in only four of the mutants, originally selected for their resistance to phage A5. Three of the four TP phages (A2, A3, A4S) showed reduced e.o.p.s on mutants A5/12, A5/33, A5/8 and A5/22, while the forth (A6) showed a reduced e.o.p. on A5/8 and was unable to plaque A5/22 (Fig. E1).

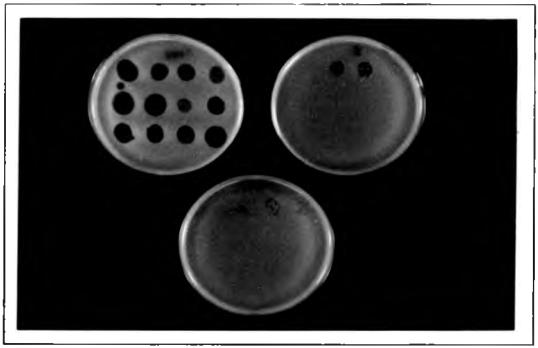
5.2.2 Reduced virulence *in planta*

All mutants were stab inoculated into the stems of potato plants, sealed with petroleum jelly and incubated at 20°C and 100% humidity for 5-7 days. After this time blackleg symptoms were scored on a scale from 1 to 5, 1 showing the least symptoms and 5 showing a full stem rot (Fig. B1). Any mutants giving a score of 1-3 were retested. Table E2 indicates the number of mutants which consistently exhibited reduced symptoms. Of the Rvi- mutants obtained two were chosen for further study. These mutants differed from the other mutants in their patterns of cross resistance to other phages and were termed A5/8 and A5/22 (Table E1). Mutant A5/22 gave an Rvi- phenotype of value 1-2, while A5/8 gave an intermediate value of 3 on at least six retests (Fig. E2). Since A5/22 exhibited a significant reduction in virulence this mutant was characterised in particular detail.

Phage	A5/8	A5/12	A5/22	A5/33	Other mutants
A1	-	-	-	-	-
A2	(+)	(+)	(+)	(+)	+
A3	(+)	(+)	(+)	(+)	+
A4S	(+)	(+)	(+)	(+)	+
A4L	-	-	-	-	-
A5	-	-	-	-	-
A6	(+)	+	-	+	+
M1	-	-	-	-	-
M2	-	-	-	-	-
M3	-	-	-	-	-
M4	-	-	-	-	-
M5	-	-	-	-	-

Table E1. Plaquing ability of phages on a number of phage resistant mutants. Headings beginning A5/ denote mutants of *Eca* SCRI1043 resistant to phage A5. - = phage resistance, + = phage sensitivity and (+) = reduced e.o.p. The e.o.p.s of phages A2, A3 and A4S on *Eca* SCRI1043 were 5.7×10^{10}, 6.1×10^{10} and 6.5×10^{10} respectively, compared to 6.0×10^{3}, 3.2×10^{3} and 7.6×10^{3} on mutant A5/22.

SCRI1043 A5/8



A5/22

Figure E1. Phage action on Eca SCRI1043, A5/8 and A5/22 after incubation at 27°C overnight.

For each plate; Row 1 = Phages A1, A2, A3, A4 S
Row 2 = Phages A4L, A5, A6, M1
Row 3 = Phages M2, M3, M4, M5

Phage No.	Mutants	No. Reduced Virulence
A1	10	2
A4L	10	0
A5	40	5
M1	10	6
M2	10	4
M3	10	4
M4	10	5
S22	20	0
S31	20	4
S32	20	0
S33	20	0
S42	20	0
S62	20	0
S71	20	0
S72	22	0

Table E2. Number of reduced virulence (Rvi-) mutants of *Eca* SCR11043 obtained for different groups of phage resistant mutants.

Figure E2

Stem rotting symptoms of *Eca* SCRI1043, A5/22 and R1 on potato plants, as described in Section 2.17.

Plate 1

Eca SCRI1043 A5/22

Plate 2

Eca SCRI1043 A5/22 R1

5.2.3 Tuber Maceration

Eca SCRI1043, A5/22 and A5/8 were stab-inoculated into potato tubers, wrapped in plastic film and moist tissue paper and incubated in plastic bags, to obtain an anaerobic environment, at 25°C for 6 days. After this time the diameter of each rot was measured (Fig. E3). Each inoculation was repeated twelve times and an average taken. While this was a very crude method of testing virulence it did show that SCRI1043 was able to macerate tuber tissue more rapidly than the mutants over the time period, from an initial concentration of 1×10^5 cells per micro site. Differences in rotting ability from this initial inoculum were supported by statistical analysis, ie. the use of a t-test at 95% significance. SCRI1043 was shown to be statistically different from A5/8 and A5/22 when a t-test was performed. The rotting abilities were found not to be significantly different by the same method analysis. Although a rot did occur over this time period, little difference was seen in rotting ability from an initial inoculum of 1×10^3 cells. In all cases t-tests showed the values obtained were not significantly different.

5.2.4 Mutant identification

To ensure mutants A5/22 and A5/8 were *Eca* they were tested using a number of criteria. These tests, which included biochemical tests (Table A1), the ability to grow at 30°C and 37°C on LBA, to produce the enzymes pectate lyase, cellulase and protease, and to exhibit cross resistance to *Eca* phages, were carried out both on bacteria used to inoculate plants and on bacteria obtained from infected plants.

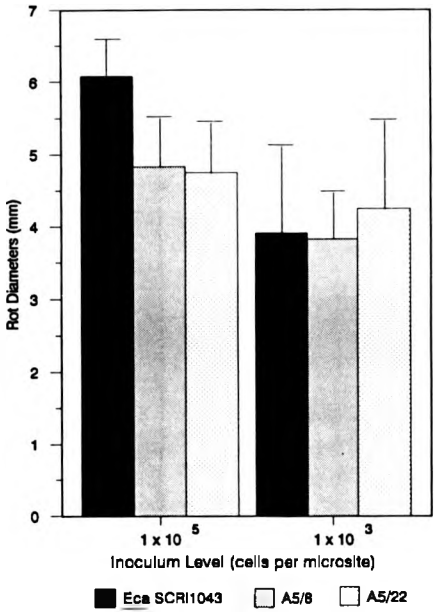


Figure E3 Rot diameters (mm) of tubers (Maris Bard) inoculated with Eca SCRI1043, A5/8 and A5/22 after 6 days incubation at 27°C and under anaerobic conditions.

T = Error bars at 95% confidence limit

5.2.5 Mutant characterisation

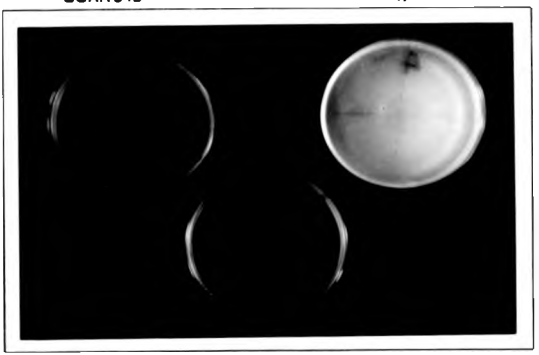
On confirmation that the strains were *Eca*, the mutants were characterised in an attempt to identify phenotypic alterations responsible for reduced virulence.

5.2.5.1 Reversion analysis

During the course of the experiment attempts were made to obtain revertants of A5/22. Growth of SCRI1043 on Stewarts medium led to the formation of large red colonies, which left deep pits in the pectate overlay, due to the utilization of PGA. A5/22 however, produced smaller pink colonies with smaller pits. Revertants appeared similar to the wild type and were clearly visible among the mutant colonies. Of the two revertants obtained only one was characterised (R1). Reversion was confirmed by increased sensitivity to phages A1, A5, A2 and A3, although CP phages (A1 and A5) produced turbid zones, suggesting a degree of resistance remained (Fig. E4). R1 was characterised at a later date to SCRI1043 and the mutants, and not all relevant aspects of its phenotype were investigated. Where carried out, however, the phenotype of R1 was compared directly with SCRI1043, A5/22 and A5/8.

5.2.5.2 Nutrient requirements

A5/22 and A5/8 were grown on both minimal medium (MM) agar containing glucose and on McConkey agar containing a range of sugars including glucose, sucrose, galactose, mannose, raffinose, arabinose, sorbitol and lactose (each at 0.2% (w/v)). The mutants grew on MM containing glucose, which indicated they were prototrophic. The mutant and wild type strains were able to ferment all sugars



FigureE4 The action of phages A1, A2, A3 and A5 (10 μl ≈ 10 p.f.u./ml) on strains Eca SCRI1043 A5/22 and R1 at 27°C.

A = Phage A1
B = phage A5
C = phage A2
D = phage A3

except sorbitol. The ability to ferment galactose, however, appeared to be reduced in both mutant strains compared to the wild type strain (data not shown).

5.2.5.3 *In vitro* growth rate

The growth rates of SCRI1043, A5/22, A5/8 and R1 were compared, over a 3 day period, in MM containing glucose, xylose, lactose, glycerol, galactose, PGA or PGA plus glycerol (each at 0.2% (w/v)) (Figs. E5 and E6). This was carried out in triplicate and an average growth rate taken. In all media with the exception of galactose and possibly PGA plus glycerol, growth rates, monitored by changes in optical densities (A_{600}), were similar. Growth of A5/8 in galactose was significantly reduced compared to both SCRI1043 and R1, while A5/22 appeared to grow at a very reduced rate on this medium (Fig. E6). Using t-tests both A5/8 and A5/22 were found to be significantly different from each other and from *Eca* SCRI1043. This result suggested a defect in galactose utilisation in both mutants, being more severe in A5/22. When both mutants were grown in galactose plus glucose (Fig. E6), glucose was utilized in preference to galactose, suggesting that catabolite repression was taking place. In MM galactose plus glycerol all strains grew at similar rates for the first 25 hours (Fig E7). After this time the growth rate of A5/22 plateaued, exhibiting very slow growth during this time. Statistical analysis (t-tests) suggested that from 25 to 67 hours A5/22 grew at a significantly reduced rate to SCRI1043, A5/8 and R1. A5/8 on the other hand was statistically different from SCRI1043 between 55 and 67 hours. This result is unclear, but may suggest an inability of A5/8 to utilize galactose but not to produce galactose intermediates which cause bacteriostasis, eg. as with GalU mutants (see Section 5.3). On addition of glucose rapid growth of all strains was seen. These results suggest that galactose was having a bacteriostatic effect on A5/22 which was suppressed by the addition of glucose. Growth rates on PGA plus glycerol (Fig. E6)

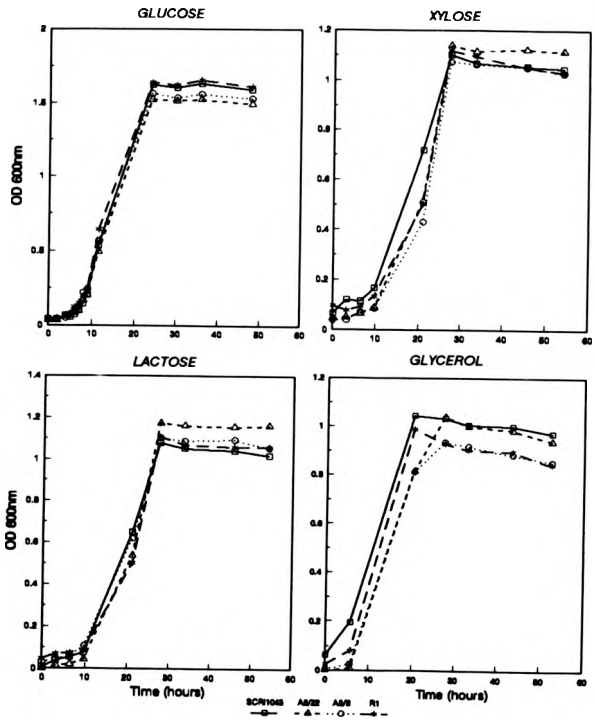


Figure E5. Growth rate (27°C) of Eca SCRI1043, A5/22, A5/8 and R1 in MM containing the carbon sources glucose, xylose, lactose and glycerol (all sugars at 0.2%(w/v)).

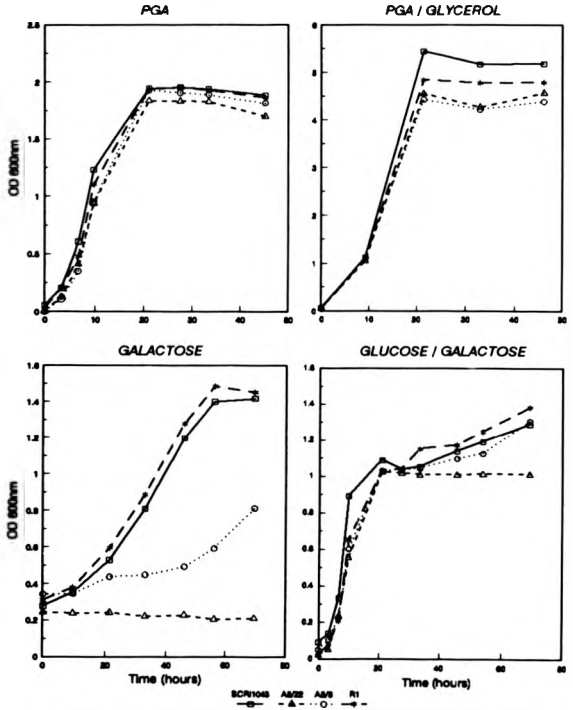
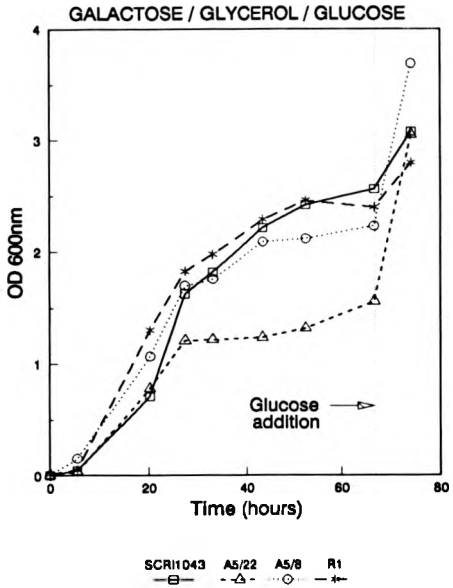


Figure E6. Growth rate (27°C) of Eca SCRI1043, A5/22, A5/8 and R1 in MM containing the carbon sources PGA, PGA plus glycerol, galactose and glucose plus galactose (all sugars at 0.2% (w/v)).



FigureE7 Growth rate (27°C) of Eca SCRI1043, A5/22 and A5/8 A5/8 in MM containing the carbon sources glycerol and galactose, followed at 67 h by the addition of glucose (all sugars at 0.2% (w/v)).

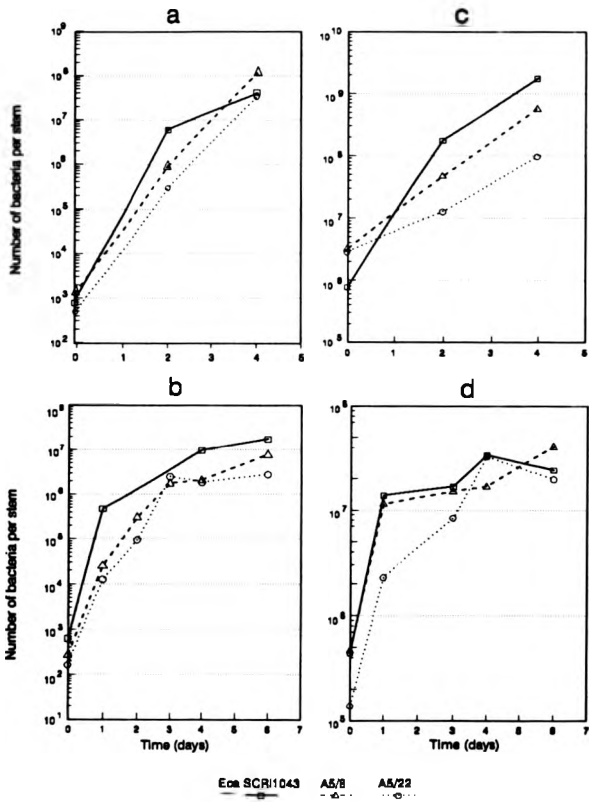
appeared similar in all cases, but final optical densities appeared progressively lower in R1, A5/8 and A5/22 respectively. Although the reason for this is not clear, using a t-test optical densities of A5/8 and A5/22 were found to be significantly different from SCRI1043 between 20 and 40 hours.

5.2.5.4 *In planta* growth rate

In planta growth rate experiments were carried out as described by Hinton *et al.* (1989a), on two occasions and with up to three replicates for each time point. Fresh overnight cultures of mutants A5/22, A5/8 and wild type SCRI1043 were adjusted to similar optical densities (OD) at A_{600}, serially diluted, and plated onto crystal violet pectate (CVP) medium to obtain cell counts. Two suspensions of approximately 1.0×10^5 colony forming units (c.f.u.) and 1.0×10^3 c.f.u. were inoculated into the upper third of tuber grown plants in 10 μl volumes using a small Gilson tip. These two different inoculum levels were used to study the ability of each strain to grow from both high and low initial bacterial numbers. Stem cuttings were taken and bacteria counted in one case on days 0, 2 and 4 (Fig. E8a/c) and in another case on days 0, 1, 2, 3, 4 and 6 (Fig. E8b/d). As well as the number of bacteria per stem being measured at each time point, the degree of rot was also noted (Table E3). Figure E8a represents the growth of SCRI1043, A5/22 and A5/8 from an initial inoculum of approximately 1×10^3 c.f.u.. The growth rate of the wild type strain appeared slightly greater than A5/8, which in turn appeared slightly greater than A5/22. After four days the number of wild type bacteria, which had increased to 5×10^7, was less than that of A5/8 and approximately equal to A5/22 suggesting, therefore, that both mutants were capable of replicating from an original inoculum of 1×10^3 to at least 1×10^7 *in planta* during the first few days of infection. On analysis of cell numbers on day 4 using a t-test, no significant differences were seen between the three strains. From Table E3a/c it can be

Figure E8

In planta growth of *Eca* SCRI1043, A5/8 and A5/22 from initial inocula of approximately 10^3 (a and b) and 10^5 (c and d) bacteria per micro site, carried out over a 4 day (a and c) and a 6 day (b and d) period.


a
c
b
d
Number of bacteria per stem
Number of bacteria per stem
Time (days)
Time (days)
Ecs SCRi1043
A5/8
A5/22

A

Days	Strains		
	1043	A5/22	A5/8
0	-/-/-	-/-/-	-/-/-
2	1/1/1	-/-/-	-/-/-
4	3/3/1	-/-/-	-/-/-

C

Days	Strains		
	1043	A5/22	A5/8
0	-/-/-	-/-/-	-/-/-
2	3/3/1	-/-/1	1/3/3
4	3/3/3	1/1/2	2/3/3

B

Days	Strains		
	1043	A5/22	A5/8
0	-/-/-	-/-/-	-/-/-
1	-/-/-	-/-/-	-/-/-
2	-/-/-	-/-/-	-/-/-
3	-/-/1	-/-/1	-/-/1
4	1/1/1	-/1/1	-/1/1
6	1/3/1	1/1/1	1/1/1

D

Days	Strains		
	1043	A5/22	A5/8
0	-/-/-	-/-/-	-/-/-
1	-/-/-	-/-/-	-/-/-
2	1/1/1	-/-/1	-/-/1
3	1/1/1	-/1/1	1/1/1
4	2/1/1	1/1/1	2/1/1
6	3/1/1	1/1/1	2/1/1

Table E3. Degree of stem rot (in triplicate samples) over a 4 day period (A and C) and a 6 day period (B and D) from initial inocula of approximately 1 x 10^3 (A and B) and 1 x 10^5 (C and D) cells per plant micro site. - = no reaction, 1 = slight browning around inoculation site, 2 = slight blackening around inoculation site, 3 = small black rot spreading from inoculation site.

seen that a wild type population of 5 x 10^7 was capable of causing a significant rot. The experiment was repeated over a six day period to study the effect of rotting ability on bacterial numbers (Fig. E8b, Table E3b). When repeated over this period similar growth curves were obtained, although after four days the number of bacteria began to plateau, suggesting on optimum level had been achieved. After six days insignificant rotting had taken place to compare with bacterial numbers. Statistical infomation obtained from t-tests suggested that cell numbers of SCRI1043 were significantly different to A5/22 but not A5/8 on day 6.

Figure E8c represents growth from an initial inoculum of approximately 1 x 10^6 to 5 x 10^6 over a four day period. SCRI1043 had a faster growth rate than A5/8, which in turn was faster than A5/22. Again statistical analysis on day 6 suggested cell numbers of SCRI1043 were significantly different to A5/22 and A5/8. All reached levels of 1 x 10^8 c.f.u. per stem or higher and all showed a degree of rotting. In the case of SCRI1043 and A5/8 a moderate rot had occurred with numbers reaching 2 x 10^9 and 6 x 10^8 respectively. When repeated over a six day period bacterial numbers reached around 2 x 10^7 to 5 x 10^7 in each case, with only the early stages of a rot taking place in SCRI1043 and A5/8 (Table E3b/d). In this case no significant difference was seen between cell numbers of SCRI1043 and A5/22 on day 6 using a t-test.

5.2.5.5 Enzyme production

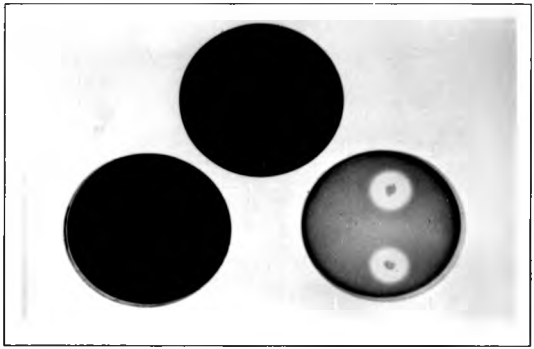
Previous attempts to isolate Rvi- mutants of *Eca* SCRI1043 by Tn*5* mutagenesis resulted in mutants with a reduction in extracellular enzyme production (*pep* mutants reduced in both synthesis and secretion of PL and PG, Hinton *et al.* 1989a). To investigate this possibility in our mutants, and the possibility of other enzyme reductions, the mutants and wild type were patched onto PL, Cel and Prt

plates and incubated at 27°C. This growth temperature was chosen for its optimum enzyme production (Lanham *et al.* 1991; Salmond pers.comm.). On PL plates little difference in zone size between the mutants, R1 and the wild type was seen, but a reduction in Cel and Prt produced by the mutants was evident (Fig. E9). To assess these differences more accurately the strains (including *Ecc* SCRI193 for comparison) were grown in MM PL (containing PGA, an inducer of pectate lyase) at 27°C to an optical density of approx. 4.0 (600nm). The amount of enzyme, in both the supernatant and cell fraction was then assessed by spectrophotometry (Fig. E10). No spectrophotometric results were produced for Prt since insufficient levels of this enzyme were produced. Beside plate assays no further enzyme data was obtained for R1.

5.2.5.5.a Pectate lyase

Spectrophotometric studies (Fig. E10) indicated that PL synthesis in SCRI1043 was only half that of *Ecc* SCRI193. 85% of PL was found in the supernatant of SCRI1043, which was similar to 69% found by Hinton *et al.* (1989b) and 95% by Lanham *et al.* (1991). A5/8 and A5/22 had reduced levels of PL synthesis, producing 68% and 43% of the wild type level respectively. The levels in the supernatant of A5/8 and A5/22 varied considerably, representing 61% and 31% of the wild type level respectively. As the level of extracellular PL decreased from SCRI1043 to the mutants, the level of intracellular PL appeared to increase, but to a much lesser extent. To study the levels of intracellular PL more accurately isoelectric focusing was carried out (Fig. E11). On analysis of the gel a gradual decrease in the extracellular PLs, PLc and PLd of A5/8 and A5/22 were seen compared to SCRI1043, together with a gradual increase in intracellular PLc and PLd. This result was therefore in agreement with the spectrophotometric data obtained. Levels of PLa and PLb synthesis were not investigated. Statistical analysis

Cellulase



Pectate lyase Protease

Figure E9The production of Cel, PL and Prt by Eca SCRI1043, A5/22, A5/8 and R1 on Cel, PL and Prt plates.

A = Eca SCRI1043
B = A5/22
C = A5/8
D = R1

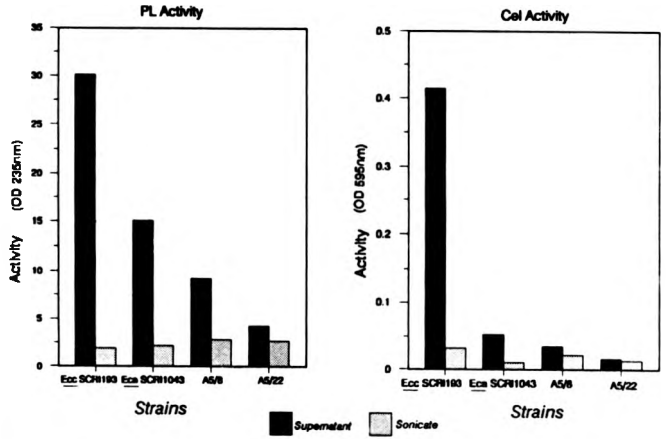


Figure E10Activities of PL and Cel from supernatants and sonicates of Ecc SCRI193, Eca SCRI1043, A5/8 and A5/22.
Activities expressed as change in OD/ml/min.

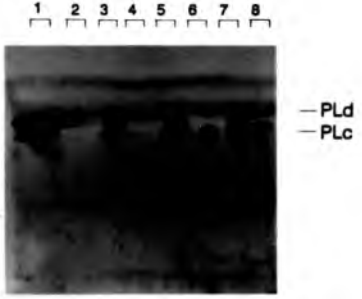


Figure E11 Isoelectric focusing of the PL isozymes of Ecc SCRI193, Eca SCRI1043, A5\8 and A5/22, followed by incubation with a PL overlay for 90 min.

1 = Ecc SCRI193 supernatant
2 = Ecc SCRI193 sonicate
3 = Eca SCRI1043 supernatant
4 = Eca SCRI1043 sonicate
5 = A5/8 supernatant
6 = A5/8 sonicate
7 = A5/22 supernatant
8 = A5/22 sonicate

using t-tests suggested that the level of enzyme activity in the supernatants of all the strains was significantly different. No statistical differences were seen between the intracellular PL levels however.

5.2.5.5.b Cellulase

Spectrophotometric assays (Fig. E10) indicated that SCRI1043 produced only 15% of the cellulase level of *Ecc* SCRI193, when the strains were grown in PL minimal medium. This probably represented constitutive production of Cel (Hinton *et al.* 1987). In SCRI1043 81% of the cellulase appeared in the supernatant although this is in disagreement with both Hinton *et al.* (1989b) and Lanham *et al.* (1991), who found cellulase to be almost exclusively cytoplasmic. While cellulase assays were performed in this case using OBR-cellulose (Biely *et al.* 1985), those of Hinton and Langham were performed using CMC (carboxymethylcellulose). Recent studies in our laboratory, however, using OBR-cellulose to determine levels of cellulase in SCRI1043 indicate an 80% level within the supernatant (Mulholland pers. comm.). The level of cellulase production in A5/8 and A5/22 was 89% and 46% of that found in SCRI1043. The level of cellulase in the supernatants of A5/8 and A5/22 represented 66% and 32% of that found in SCRI1043.

5.2.5.6 Analysis of the cell surface

Although, from the above studies, a number of alterations were found which may have accounted for the Rvi- phenotype of the mutants (such as reduced enzyme levels and an inability to grow on galactose) the nature of the mutant isolation technique (phage resistance) suggested that an alteration in the cell surface of the mutants may have taken place. This possibility was therefore investigated.

5.2.5.6.a Phage adsorption

The resistant nature of mutants A5/22 and A5/8 may have been due to either a failure of phage A5 to adsorb to the mutants or to replicate within them. The ability of phage A5 to adsorb to A5/22 and SCRI1043 was investigated (Fig E12). From this study it was clear that phage A5 was unable to adsorb to the A5/22 cell wall suggesting an alteration in the adsorption site of the phage, and therefore in the cell wall of the strain. A5 adsorption to SCRI1043 was rapid, with 99% adsorbing in 60 minutes. The adsorption of phage A5 to A5/8 was not investigated.

5.2.5.6.b Motility

Several studies on the virulence of phytopathogenic bacteria have noted alterations in motility (see Section 1.3.3). To investigate this the wild type, revertant and mutant strains were stabbed into motility agar and their swarming ability observed (Fig. E13). After an incubation period of 24 hours SCRI1043 and R1 had spread from their original inoculation site to produce swarms. No swarm was detected with either A5/22 or A5/8. After 48-72 hours, however, "blebs" around the inoculation sites of A5/22 and A5/8 were visible and were larger in the case of A5/8. On examination by light microscopy both the wild type and revertant strains were clearly motile. The majority of mutant cells appeared non-motile although a small percentage in both cases were.

On examination by electron microscopy the wild type and revertant strains possessed clearly visible peritriceous flagella (usually between 1 and 4 per cell) (Fig. E14a). Only a very small percentage of both did not possess any flagella (Fig. E14d). A5/22 cells were almost entirely without flagella, although on occasion short single flagella were visible (Fig. E14c). A very small percentage (<0.1%)

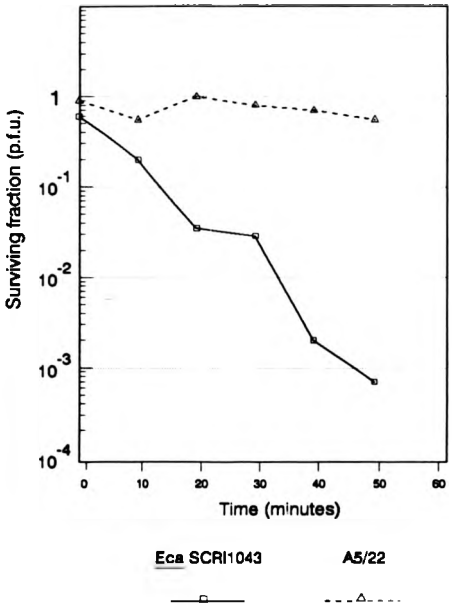


Figure E12 Adsorption of phage A5 to Eca SCRI1043 and A5/22 at 27°C.

Figure E13

Motility of (A) *Eca* SCR11043, (B) A5/22, (C) A5/8 and (D) R1 on 0.3% motility agar, after 24 hours (Plate 1) and 48 hours (Plate 2) incubation.

Plate 1

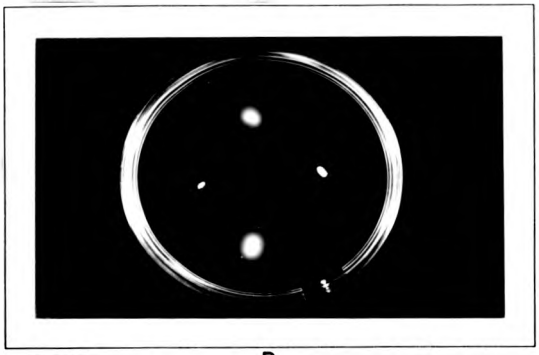
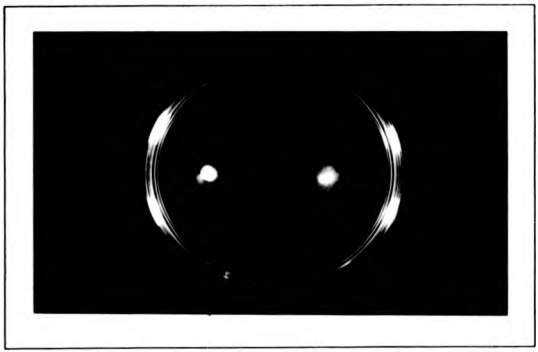


Plate 2



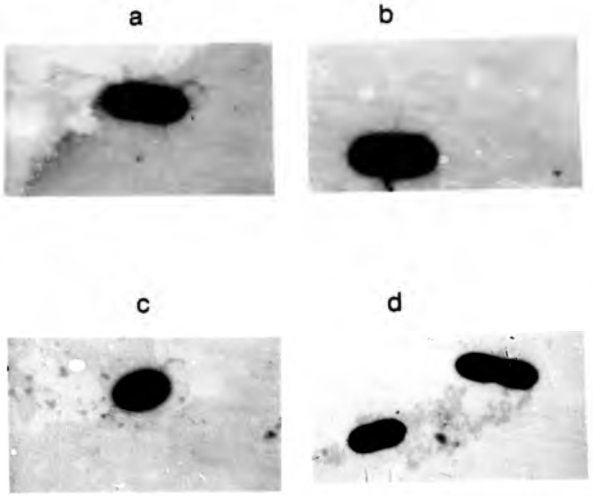


Figure E14. Electron micrographs of Eca SCRI1043 a) wild type, b) A5/8 possessing reduced number of flagella, c) A5/22 possessing structurally incomplete flagella, and d) A5/22 lacking flagella. a, c and d are magnified 8,000 x whereas b is magnified 10,000x.

possessed 1-2 flagella which appeared similar to those of SCRI1043. A5/8 showed similar results to A5/22 but a higher percentage (>0.1%) possessed 1-2 intact flagella (Fig E14b).

5.2.5.6.c Sensitivity to surface active agents

Further support for a cell surface alteration in A5/22 came from the increased sensitivity of this mutant to a number of surface active agents. These included ethyl diamine tetraacetic acid (EDTA), sodium dodecyl sulphate (SDS), Triton-X100 and Deoxycholate (DOC) (Table E4).

Triton-X100, at concentrations of 0.1% and 0.01%, had no observable effect on the stability of the A5/22 compared to SCRI1043. At 0.01% no difference in sensitivity to DOC was observed, but at 0.1% a 2 log drop in the number of c.f.u. of A5/22 compared with SCRI1043 was seen. At 0.1% both EDTA and SDS prevented growth of SCRI1043 and A5/22. This was also true of EDTA at 0.01%. At 0.001% however, a two log reduction in A5/22 sensitivity was noted. SDS at 0.01% led to a 6 log decrease in the number of A5/22 c.f.u. compared to the wild type. Surface active agents were not tested on A5/8 or R1.

Previous studies in *Erwinia*, using phage and bacteriocin resistance to select for Rvi- mutants, showed the mutants had alterations in LPS structure (Schoonejans *et al.* 1987) and in one case alterations in OM proteins (Expert and Toussaint 1985). Both structures were therefore investigated.

	Eca SCRI1043	A5/22
Nutrient Agar	6.8×10^7	4.1×10^7
0.1% DOC	2.3×10^7	2.0×10^5
0.1% SDS	NG	NG
0.01% SDS	2.0×10^7	3.6×10^1
0.1% EDTA	NG	NG
0.01% EDTA	NG	NG
0.001% EDTA	6.8×10^7	7.6×10^5

Table E4. Plating efficiencies of *Eca* SCRI1043 and A5/22 on nutrient agar containing different surface active agents at various concerntrations. NG = no growth, DOC = Deoxycholate, SDS = Sodium dodecyl sulphate and EDTA = Ethyl diamine tetraacetic acid.

5.2.5.6.d Lipopolysaccharide analysis

LPS was obtained by phenol-water extraction, samples electrophoresed through an 11% polyacrylamide gel and the resultant gel silver stained. The mutants produced a different profile from the wild type strain (Figs E15). SCRI1043 showed three darkly stained regions of low molecular weight (bands A, B and C) and a ladder of increasing molecular weight, a similar pattern to that observed by Murray *et al.* (1990). The ladder consisted of doublets, the upper band of each pair staining grey and the lower staining red. This doublet ladder is thought to represent the core oligosaccharide with increasing numbers of covalently bound O-antigenic side chains (Hitchcock *et al.* 1986). Band A almost certainly represents the core oligosaccharide, while bands B and C probably represent the core with one to two covalently bound O-antigen units. The LPS profile of A5/22 (Fig. E15 gel 1) appeared to be lacking all but band A, ie. the core oligosaccharide. This mutant therefore appeared to have lost all the O-antigen side chains but retained an intact core. If core sugars had been removed band A would have been expected to migrate further down the gel (as seen with the LPS of the R mutant Fig. E16), but this was not the case with A5/22. It should be noted here that a direct comparison between *Erwinia* and *E.coli* LPS molecules needs to be viewed with caution. It is recognised that in wild type organisms that have predominantly smooth LPS (O-antigen containing LPS) there is considerable size heterogeneity of the individual LPS molecules. A high proportion (up to 40 to 50%) of these molecules are of the Ra (core oligosaccharide lacking O-antigen) and SR (semi rough LPS possessing a core and one covalently bound O-antigen unit) chemotype (Hitchcock *et al.* 1986). From Fig. E15a the darkly stained bands B and C of the wild type suggest that this could be the case. Figure E15b shows the LPS structure of SCRI1043, A5/8 and A5/22. While this gel lacks the clarity of the previous one it can be seen that A5/8 appears to contain an unaltered core (band A) and may contain a small amount of semi rough LPS (band B).

Figure E15

Electrophoresis and silver staining of lipopolysaccharide in 11% polyacrylamide gels. Bands A, B and C represent the core region and the core region with 1 to 2 covalently bound O-antigen side chains respectively. The ladder of increasing molecular weight represents the core region with increasing numbers of O-antigen side chains.

Lane 1 = A5/22

Lane 2 = *Eca* SCRI1043

Lane 3 = *E.coli* F583 (Rd2 mutant -purchased from Sigma)

Lane 4 = *Eca* SCRI1043

Lane 5 = A5/8

Lane 6 = A5/22

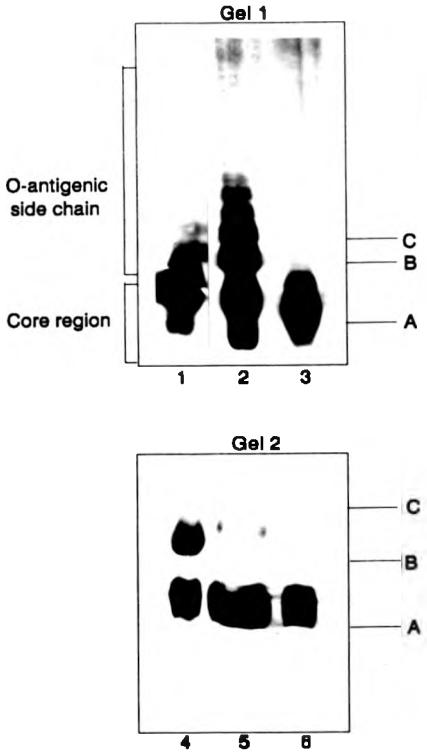
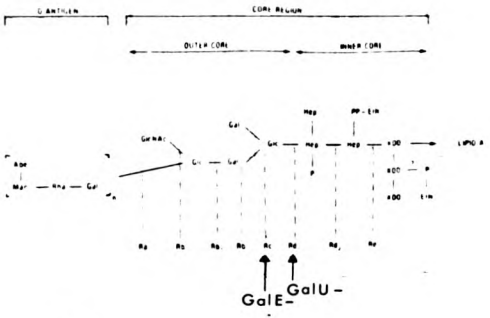

Gel 1
O-antigenic side chain
Core region
C
B
A
1
2
3
Gel 2
C
B
A
4
5
6

Figure E16.

A. Structure of *S.typhimurium* lipopolysaccharide. Abbreviations; Abe, abequose; Man, D-mannose; Rha, L-rhamnose; Gal, D-galactose; GlcNAc, N-acetyl-D-glucosamine; Glc, D-glucose; Hep, L-glycero-D-mannoheptose; KDO, 3-deoxy-D-mannooctulosonic acid; EtN, ethanolamine; P, phosphate. The LPS structures and corresponding chemotypes of mutants defective at various stages of core biosynthesis are indicated by the brocken lines and associated letter designations (from Rick 1989). The positions of chemotypes produced by GalE$^-$ and GalU$^-$ mutations are also indicated.

B. Electrophoresis through a polyacrylamide gel of LPS structures from *S.typhimurium*. S, represents smooth LPS, Ra to Re represent LPS from rough mutants of various types, and LA represents lipid A only (from Hitchcock *et al.* 1986).

A



B

5.2.5.6.e Effects of osmolarity on motility

Earlier studies on *Rhizobium* and *Agrobacterium* led to the isolation of avirulent mutants which had reduced motility, were phage resistant, had increased sensitivity to surface active agents and exhibited reduced growth on low osmolarity media (see Section 1.3.2.b). These mutants were found to be affected in β-(1-2) glucan synthesis. It was noted by Dylan *et al.* (1990a) that on increasing the medium osmolarity the phenotypic changes associated with the cell surface (including motility) of *ndv* mutants of *Rhizobium* could be substantially suppressed. The ability of A5/22 and A5/8 to return to a motile phenotype in such conditions was therefore studied. On MM containing from zero to 0.5 M sodium chloride the mutants remained non-motile, suggesting that a mutation similar to the *ndv* mutation in *Rhizobium* had not taken place (data not shown).

5.2.5.6.f Outer membrane protein analysis

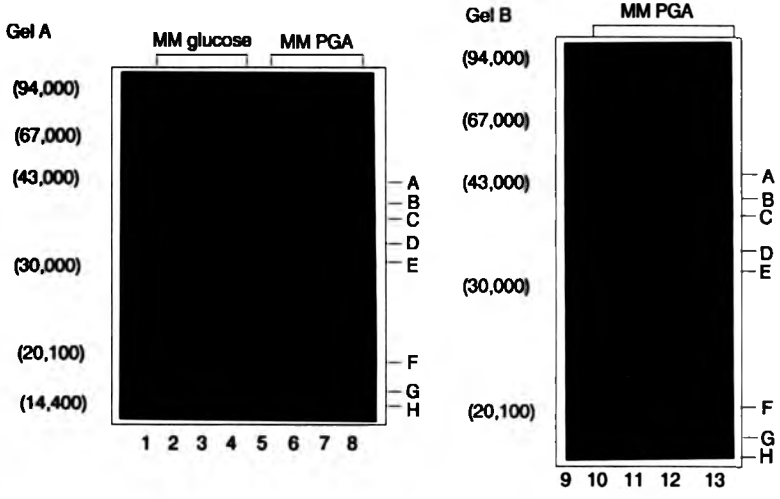
The OM protein profiles of R1, A5/22 and A5/8 were compared with SCRI1043 after growth in both MM PL and MM glucose. The extracted proteins were electrophoresed through an 11% polyacrylamide gel and stained with Coomassie blue (Fig E17). An alteration in banding patterns of the mutants compared to the wild type was seen, whether grown in MM glucose or MM PL (Fig. E17A). Two protein bands of molecular weight 38,000 and 36,000 predominated in each case, with little, if any, difference in intensity between them. These bands are of similar size to those observed in other *Eca* strains where they are thought to represent an OmpA-like protein and a porin-like protein (Saarilahti and Palva 1986). From the MM PL grown cells a band of 42,500 appeared stronger in both mutants than in SCRI1043, although this result was not consistently obtained on similar gels. The most consistent alteration in banding pattern of the mutants in both MM glucose and

Figure E17 Outer membrane proteins extracted from both MM glucose and MM PGA grown cells, electrophoresed through an 11% polyacrylamide gel. Molecular weight of bands are A = 42,000 , B = 38,000, C = 36,000, D = 32,000, E = 30,500, F = 18,000 , G = 15,000 and H = 14,000. Molecular weight standards are Phosphorylase b (94,000), Albumin (67,000), Ovalbumin (43,000) , Carbonic Anhydrase (30,000), Trypsin Inhibitor (20,100) and α-Lactalbumin (14,000).

Molecular weights in Daltons (Da).

1, 5 and 9 = molecular weight standards
2 = Eca SCRI1043 MM glucose grown cells
3 = A5/22
4 = A5/8

6 and 10 = Eca SCRI1043 MM PGA grown cells
7 and 11 = A5/22
8 and 12 = A5/8
13 = R1


Gel A
MM glucose
MM PGA
(94,000)
(67,000)
(43,000)
(30,000)
(20,100)
(14,400)
A
B
C
D
E
F
G
H
1 2 3 4 5 6 7 8
Gel B
MM PGA
(94,000)
(67,000)
(43,000)
(30,000)
(20,100)
A
B
C
D
E
F
G
H
9 10 11 12 13

MM PL grown cells was the disappearance of a 32,000 band, clearly visible in SCRI1043, and the appearance of a 30,500 band in the mutants. This latter band was present in SCRI1043 but at much reduced quantities. Another consistent alteration appeared in a band of approximately 18,000, which in the mutants appeared to migrate slightly further down the gel than in SCRI1043. In all strains bands of 15,000 and 14,000 appeared in the MM PL grown cells, suggesting that they were induced by PGA. From Fig. E17 the protein profile of R1 appeared similar, if not identical to SCRI1043.

5.2.5.6.g Iron utilization and siderophore production

Alterations in OM protein profiles in *Echr* were previously found in mutants deficient in iron uptake (Expert and Toussaint 1985; Enard *et al.* 1988). The ability of both wild type *Eca* SCRI1043 and mutants A5/22 and A5/8 to produce siderophores was tested, together with their ability to "scavenge" for iron in an iron depleted medium. The production of siderophores would indicate a similar system of iron "scavenging" (in iron depleted medium) to *Echr*.

The ability of SCRI1043, A5/22 and A5/8 to produce siderophores was tested by streaking on siderophore assay plates. The presence of siderophores lead to a colour change from blue to orange by breakdown of an iron(III)-Dye complex. A known producer of siderophore was used as a control (*Echr* strain 3937). The resultant plates indicated that no siderophore like compounds were released by SCRI1043 or the mutants (Fig. E18). The slight orange colour observed may have been due to a pH change over the 2-3 days incubation (Expert pers. comm.).

To examine the ability of the wild type and mutants to grow in iron depleted medium they were inoculated, with controls, into a Tris based medium. All strains

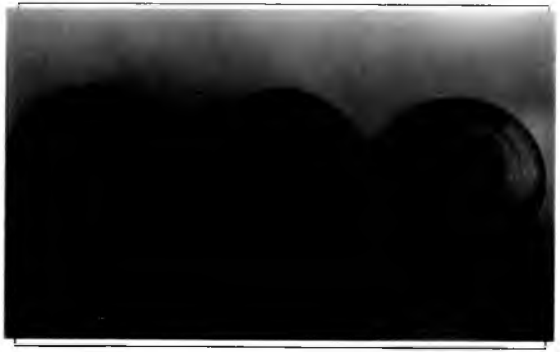


Figure E18 Siderophore production by Eca SCRI1043 and A5/22 on siderophore assay medium.

AN193-60 = negative control (see text)
Echr 3937 = positive control.

except the negative control were able to grow, suggesting an ability to "scavenge" iron. Since no quantification of available iron was undertaken however, these results should be viewed with caution.

5.2.6 Complementation and cloning

In order to locate the gene responsible for the pleiotropic phenotype, a gene library of the *Eca* genome was made. The library was characterised to ensure it was representative of the entire *Eca* genome by complementing a number of auxotrophs and by placing the cosmids into *E.coli* in the search for *pel*, *cel* and *prt* structural genes. Tryptophan, adenine, uracil, cysteine, nicotinic acid and arginine auxotrophs were complemented at a frequency of approximately 1% (data not shown). From a total of 1350 *E.coli* colonies (containing the cosmid library) tested, 50 produced small amounts of PL, giving a ratio of 1 in 27. No Cel or Prt producing colonies were isolated (data not shown).

A5/22 colonies containing the cosmid library were stab inoculated into motility agar. 7 out of 700 colonies tested became motile. Plasmid preps were performed on these colonies. On restriction of the cosmids with *Eco*RI four similar profiles were obtained (Fig. E19). The complemented mutants were sensitive to phages A1, A5, A2 and A3 to varying degrees, A5/22.cos4 appearing most sensitive, resembling that of the revertant (data not shown). From plate assays, PL, Cel and Prt production appeared as wild type and A5/22.cos4 appeared fully virulent when tested *in planta* (data not shown). From preliminary studies cosmid4 failed to complement A5/8 to a motile phenotype.

Figure E19. Four cosmids (pHC79) each containing a fragment of the Eca SCRI1043 chromosome capable of complementing the *Rvl-* mutant A5/22.

Cosmids restricted with EcoRI.

Lane 1 = Lambda HindIII
Lane 2 = cosmid 1
Lane 3 = cosmid 2
Lane 4 = cosmid 3
Lane 5 = cosmid 4
Lane 6 = Lambda HindIII

5.3 Discussion

Hinton *et al.* (1989a) used Tn*5* insertion mutagenesis to investigate the nature of virulence in *Eca* SCRI1043. This work identified 9 mutants exhibiting Rvi- phenotypes, which included 5 auxotrophs, 1 growth rate mutant (altered in both *in vitro* and *in vivo* growth) and 3 enzyme mutants, termed *pep*, reduced in both PL and PG synthesis and secretion, but unaltered in Cel and Prt. From this study Hinton *et al.* (1989a) concluded that either virulence in *Eca* simply reflected its ability to multiply and produce large amounts of pectolytic enzymes or another bacterial function existed but was not detected by the mutagenesis or screening procedure. The investigation in this report is therefore an extension of this work, using another means of obtaining Rvi- mutants in an attempt to identify other bacterial functions involved in the virulence of this organism.

A number of phage resistant mutants of SCRI1043 were obtained (Table E1) and their rotting abilities tested in potato plants. Of these mutants A5/8, A5/12 and A5/33 showed an intermediate rot of value 3, while A5/22 showed a much reduced rot of value 1-2 (Fig. E2, see Section 2.17). A5/22 and one mutant of intermediate virulence (A5/8) were investigated further. Once these mutants had been identified as *Eca*, they were inoculated into tubers to observe whether similar results were obtained with a more crude plant test. With high inoculum levels SCRI1043 was clearly more virulent than A5/8, which in turn appeared more virulent than A5/22 (Fig. E3). Statistically the rotting abilities of A5/8 and A5/22 were not significant. At low initial inocula however, although a rot did occur in each case, little difference between the strains was seen, suggesting that cell numbers may have been an important factor in virulence.

The mutants were characterised, firstly, along similar levels to those of Hinton *et al.* (1989a). The mutants were able to grow on MM containing glucose which

suggested they were prototrophic. When grown on MacConkey agar plates containing a range of other carbon sources, with the exception of galactose, similar results were obtained for both SCRI1043 and the mutants. On galactose the mutants grew less well and were less red (indicating inefficient fermentation) than SCRI1043. A more accurate experiment was carried out to study the utilization of carbon sources by the mutants in relation to their growth rates. The strains, together with the revertant R1, were grown in liquid MM containing glucose, xylose, lactose, glycerol, galactose, PGA, PGA plus glycerol or glucose plus galactose (Figs. E5 and E6). On the first five carbon sources little if any difference between the growth rates of the strains was seen.

In planta growth was studied in potato plants from initial inocula of 1×10^3 and 1×10^5 cells. In general SCRI1043 grew slightly faster than A5/8, which in turn appeared to grow slightly faster than A5/22 (Fig. E8). The first signs of rotting by SCRI1043 were in one case at a population of approximately 8×10^5 (Fig. E8, Table E3) , although in general a significant rot was seen only at cell densities of 5×10^7 or higher. While SCRI1043 reached cell densities of up to 2×10^9 and A5/8 up to 7×10^8, A5/22 failed on all occasions to obtain values above 1×10^8. While populations of 5×10^7 to 1×10^8 were able to cause a degree of rotting, this may have been a critical level where rotting began, but where higher levels were needed to obtain full virulence. At these critical levels other factors, such as a decrease in enzyme production, may have prevented rotting taking place. Statistically different cell densities were seen between SCRI1043 and A5/22 on the final day of only 2 of the 4 replica experiments (Fig. E8b/c). Since there appears to be no correlation between these differences and inoculum density, or between these differences and incubation times, the validity of these differences is open to question.

On galactose a significant reduction in growth rate of A5/8 was evident as was the inability of A5/22 to utilize galactose (Fig. E6c). This difference in galactose

utilization could have been a major factor in the altered virulence of the mutants, especially since their galactose utilization paralleled their relative differences in virulence. In glucose plus galactose, glucose exhibited catabolite repression over galactose utilisation in all strains until this carbon source was depleted, followed by growth of all strains except A5/22 on galactose (Fig. E6d). Jayaswal *et al.* (1985a) isolated a mutant (AH1028) of *Ecc* strain AH2 altered in galactose utilization. Growth in glycerol plus galactose led to a cessation of growth of AH1028 (bacteriostasis) which on addition of glucose led to resumed growth. This mutant was affected in the production of UDP glucose-pyrophosphorylase, a mutation in the *galU* gene (Fig. E20). A similar mutation has recently been reported in *Ecc* SCC3193 (Pirhonen *et al.* 1991). In glycerol plus galactose mutant A5/8 showed little difference in growth rate to SCRI1043 and R1 (Fig. E7), suggesting that galactose was not blocking glycerol utilization. Such a response could, possibly, result from a "leaky" mutation in a gene such as *galU*, but more likely from a mutation in a different gene, possibly within the Gal operon. A5/22 on the other hand, grew initially at a slightly slower rate than the other strains followed by rapid bacteriostasis. On addition of glucose growth was resumed. This result is therefore very similar to that of Jayaswal *et al.* (1985a) and may reflect a mutation in galactose utilization similar to that of AH1028. *galU* mutations have also been shown to lead to bacteriostasis in *E.coli* (Koplow and Goldfine 1974) and *S.typhimurium* (Ames *et al.* 1974). Another mutation in *E.coli* leading to bacteriostasis is in the *galT* gene involved in the synthesis of Gal-1-P-Uridyl-transferase (Fukasawa *et al.* 1962; Kalckar *et al.* 1959; Yarmolinsky *et al.* 1959). Mutations in both *galU* and *galT* cause bacteriostasis by build up of the toxic intermediate Gal-1-P. Mutations in the galactose operon which are probably not involved in the phenotypic changes seen in mutant A5/22 are in *galK* (coding for Galactokinase) (Kalcker *et al.* 1959; Nikaido 1961), which leads to mutations that are unable to utilize galactose down to Gal-1-phosphate (the galactose intermediate responsible for bacteriostasis in GalU mutants) and *galE* (coding for UDP-Gal-4-

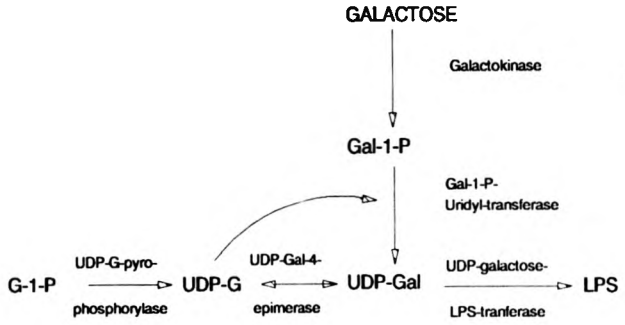


Figure E20 Schematic representation of the incorporation of exogenous galactose into the LPS of galE mutants of S.typhimurium (Billing 1984)

epimerase) (Fukasawa *et al* 1962; Fukasawa and Nikaido 1961; Nikaido 1961) which leads to cell death (bacteriolysis) in the presence of galactose, due to a build up of UDP-galactose (Fig. E20). In addition to bacteriostasis in the presence of galactose, *galU* mutants have also been shown to have alterations in LPS biosynthesis (Fukasawa *et al.* 1962; Sandararajan *et al.* 1962), EPS biosynthesis (Billing 1984) and flagella formation (Komeda *et al.* 1977).

The enzymes PL, Cel and Prt were clearly affected in their production in both mutants (PG production was not investigated). From enzyme plates the production of PL and Cel appeared slightly reduced, whereas Prt production appeared to have ceased (Fig. E9). On more accurate analysis of PL and Cel production by spectrophotometric assay, synthesis (and in the case of PL, secretion) was clearly reduced (Fig. E10). The assay for Cel did not allow accurate analysis of secretion, although it may have been affected. Isoelectric focusing of PLs clearly showed a reduction in the alkaline PLs, PLc and PLd (Fig. E11). Such mutants have not previously been isolated in *Eca*. However, similar global effects on enzyme production in *Ecc* SCC3193 have been reported (Pirhonen *et al.* 1991) and in *Ecc* SCRI193 are presently under investigation in our laboratory (Salmond pers. comm.) (see Section 1.2.2.1.6).

Alkaline PLs appear to be essential for tissue maceration and disease development (see Section 1.2.2.1.3), with the induction of large numbers of such enzymes controlling success or failure of infection (Zucker and Hankin 1970; Zucker 1972). Hinton *et al.* (1989a) and Lei *et al.* (1985a) also showed a strong correlation between synthesis and secretion of PLs and virulence. While PLs play a major role in virulence they have also been shown to trigger plant host defence mechanisms ie. phytoalexins (Davis 1986a, 1986b). Induction of a large number of PLs would lead to the rapid breakdown of substances like oligogalacturonides to smaller molecules which could no longer trigger host defences. Such an over production can be

achieved by suitable regulation and amplification of *pel* genes in *Ecc* (Plastow *et al.* 1986; Willis *et al.* 1987) and in *Eca* (Lei *et al.*1985a). A number of regulatory genes have been found which control enzyme production either in a positive or negative manor (see Sections 1.2.2.1.2 and 1.2.2.1.6). A5/22 and A5/8 appear to be altered in a regulatory loci which have a global positive regulatory affect on a number of plant cell wall degrading enzymes. *In planta* this reduced enzyme production , particularly of the alkaline PLs, may lead to a competition between growth of the bacterium and phytoalexin production. It would appear, in the case of A5/22, that a "stale mate" is reached *in planta* where cell numbers reach a value below the threshold for disease development, are not able to exceed this value, but equally are not "overwhelmed" by the plant defence mechanisms. A5/8 on the other hand, while showing a very similar phenotype to A5/22 produces twice the amount of enzymes, which is sufficient to reach threshold numbers for tissue maceration and disease development. While the above can be theoretically postulated, the *in planta* growth data obtained in this study is insufficient, however, to support this idea practically. The global regulatory effect seen in A5/8 and A5/22 may indicate a second regulatory mechanism involved in the production of cell wall degrading enzymes of *Eca*. This regulation appears to affect enzyme production on a larger scale than the *pep* mutants of *Eca* isolated by Hinton *et al.* (1989a).

The nature of both the isolation technique (phage resistance) and the galactose effect on the mutants suggested an alteration of the cell surface. Initial investigations using surface active agents suggested that A5/22 at least, was affected in its cell surface by nature of its increased susceptibility to the surface active agents SDS and EDTA (Chatterjee *et al.* 1977; Sanderson *et al.* 1974). This was then verified by showing that phage A5 was unable to adsorb to the mutant (Fig. E12). In order to examine the cell surface more closely both LPS and OM proteins were analysed.

GalU mutants lack all sugars beyond the heptose region of the inner core of their

LPS, leading to an Rd_1 phenotype (Fig. E16a) (Rick 1989), which shows a significant increase in electrophoretic mobility through polyacrylamide gels (Fig. E16b) (Hitchcock *et al.* 1986). Mutant A5/22 appeared to have lost the O-side chains (Fig. E15) but since no increase in electrophoretic mobility was seen in the core oligosaccharide (band A) this suggested that this structure remained intact. The LPS profile of A5/8 appeared similar to that of A5/22 but possibly contained a small amount of semi rough LPS (band B). Since other defects in galactose utilization also lead to altered LPS structures, ie. a lesion in *galE* results in the synthesis of an Rc LPS (Fig. E16b) lacking all sugars distal to the first galactose residue (Fig. E16a), it seemed highly likely that the inability of both mutants to fully utilize galactose was responsible for their alteration in LPS. The exact structure of the LPS is not known however. A number of workers have found that such alterations in LPS can affect virulence (see Sections 1.2.2.2.a and 1.3.2.c) although the results of Schoonejans *et al.* (1987), which showed that only mutants affected in the core LPS of *Echr* led to reductions in virulence, would indicate that the LPS alterations in A5/22 and A5/8 would not affect virulence.

Pirhonen *et al.* 1988 have shown reductions in protease production in heptose deficient LPS mutants, and suggest a single mutation is responsible for both the phenotypes. These mutants did not show a reduction in virulence however. Although such a phenomenon has been noted in EPS mutants of *Xanthomonas campestris* (Daniels *et al.* 1984a) this is the only evidence available which shows a relationship between LPS deficiency and the lack of production of a cell wall degrading enzyme.

OM protein profiles were clearly altered in the mutants compared to both SCRI1043 and R1 (Fig. E17). While no alterations in OM protein profiles have been directly associated with *galU* or *galT* mutants, small reductions in the number of OM proteins have been noted in *galE* mutants (Ames *et al.* 1974). Many workers have

observed alterations in the OM protein profiles of LPS mutants in phytopathogens (see Sections 1.2.2.2.b and 1.3.4) but only Expert and Toussaint (1985) were able to relate these alterations to reductions in virulence (iron acquisition). Our data suggest that A5/22 and A5/8 are unaltered in their iron "scavenging" ability. LPS mutants of *E.coli* (Koplow and Goldfine 1974) and *S.typhimurium* (Ames *et al.* 1974) altered in OM protein profiles have, however, been analysed in particular detail. These mutants which resemble *galU* mutants by virtue of their heptose reduced or deficient LPS show, in the case of *E.coli*, reduced amounts of up to 4 OM proteins, 3 between molecular weights 33,000 and 36,000 and 1 of 44,000, and in the case of *S.typhimurium* reduced amounts of proteins of 37,500, 38,500 and 40,500, as well as alterations in protein bands below 30,000. The mutants in this study were affected in proteins of similar molecular weight to the *E.coli* and *S.typhimurium* mutants, although alterations in band intensity, rather than an overall reduction in band intensities, was seen. In *E.coli* and *S.typhimurium*, revertants restored to wild type levels of OM proteins regained resistance to surface active agents and returned to phage sensitivity (sensitivity of the *E.coli* mutants to phage C21 was dependent on the percentage heptose present in the core LPS) as in the mutants of this study. The variation in phage sensitivity led to both clear and turbid plaques on the revertant compared to only clear plaques on the wild type, a result also found in A5/22. In both *E.coli* and *S.typhimurium* alterations in LPS and OM proteins were found to be the result of a single mutation (Ames *et al.* 1974; Koplow and Goldfine 1974). Koplow and Goldfine (1974) suggested that genes coding for polypeptide synthesis and for LPS synthesis could be part of the same operon, with a mutation in a regulatory locus leading to a polar mutation affecting these genes. Alternatively a mutation in either protein or LPS synthesis, leading to a deficiency in this structure, could cause a deficiency in the other structure, ie. by a need for LPS to anchor proteins, or a need for proteins to allow LPS to incorporate properly into the outer membrane (Ames *et al.* 1974; Koplow and Goldfine 1974). Koplow and Goldfine (1974) also showed that the LPS mutants were reduced in phosphorus,

believed to be involved in cross-linking and rigidity of LPS (Rick 1989), which may affect spatial distribution of LPS in the outer membrane. As well as LPS effects, differences in OM porins have been observed in *E.coli* mutants altered in MDO (Fiedler and Rotering 1988). Again tests on varying osmolarity media (see Section 5.2.5.6.e) suggest that A5/22 and A5/8 are unaffected in MDO.

Reductions in motility have been noted commonly in phytopathogens with reduced virulence, including mutants altered in ß-(1-2)glucan (see Section 1.3.2.b), LPS (see Section 1.3.2.c), and in *E.coli* mutants with a lesion in *galU*, a mutation shown to affect virulence in *Ecc* strains AH2 (Jayaswal *et al.* 1985a) and SCC3193 (Pirhonen *et al.* 1991). The exact nature of the non-motile phenotypes of LPS mutants of phytopathogens is not clear, however *S.typhimurium* mutants altered in LPS (Ames *et al.* 1974) and *E.coli* mutants altered in MDO (Fiedling and Rotering 1988) together with *Rhizobium* and *Agrobacterium* mutants altered in ß-(1-2)glucan (Dylan *et al.* 1990a; Geramia *et al.* 1987; O'Connell and Handelsman 1989) and EPS (Cangelosi *et al.* 1987; Uttaro *et al.* 1990) all show deficiencies in flagella formation (fla-), and not in flagella rotation (mot-) or chemotaxis (che-). Similarly mutants with lesions in *galU* show deficiencies in flagella formation (Komeda *et al.* 1977). In this study A5/22 and A5/8 were also unable to form complete flagella (Fig. E14). In all cases this appears to be a consequence of deficiencies in polysaccharide biosynthesis. The role that this flagella deficiency plays in virulence is probably a minor one, if in fact it plays any role at all. Besides a minor role in the attachment of *Rhizobium* to plant cells (Dylan *et al.* 1990b), and the requirement of motility for invasion of plants from external sources (Bayot and Ries 1986; Hattermann and Ries 1989; Panopoulos and Schroth 1974) little further evidence exists for the role of motility in virulence or symbiosis of plant associated bacteria (Pirhonen *et al.* 1991). It would seem in this study therefore, that this phenotype is a consequence of an LPS deficiency and is unlikely to play a role in virulence.

Complementation of A5/22 was achieved by production of an *Eca* SCRI1043 gene library. 1 out of 7 complementing clones, which returned A5/22 to a motile phenotype, was investigated more closely. On enzyme plates A5/22 containing the clone appeared to produce PL, Cel and Prt at the same levels as the wild type strain and had also returned to phage sensitivity, although clear plaquing phages produced turbid plaques similar to the revertant (data not shown). This suggested that a full return to a wild type phenotype was not achieved. The ability of A5/22.cos4 to cause tissue maceration *in planta* however, equalled that of SCRI1043 and R1. An attempt to complement A5/8 to a motile phenotype using A5/22.cos4 appeared to be unsuccessful although this study would have to be carried out in more detail before any conclusions could be drawn, as to the genotype similarity of the two mutants.

In summary A5/22 and A5/8 are phage resistant mutants altered in a number of other phenotypes which include reduced virulence, reduced enzyme production, LPS deficiencies, OM protein alterations, increased sensitivity to surface active agents, reduced motility and an altered ability to utilize the carbon source galactose. Alterations in A5/22 are in general more severe than in A5/8, which seems to correlate with its decreased maceration abilities *in planta*. The role of motility and outer membrane proteins in the virulence of these mutants is not clear but previous studies suggest that they may not be of particular importance (see Sections 1.3.3 and 1.3.4). The importance of alterations in the bacterial outer membrane (specifically LPS), the production of cell wall degrading enzymes (particularly the alkaline PLs) and the altered ability to utilize galactose, have all been shown to be important in virulence. Due to the pleiotropic nature of the mutants it is not possible however, to assign a role in virulence to just a single phenotype. Mutant A5/22 appears to be mutated in a single locus (suggested by the ability of the mutant to readily revert), whose product may be involved in the regulation of a wide range of phenotypes. This regulatory gene may be in or related to the galactose operon, since mutations in this region have been shown to lead to many of the phenotypes

exhibited by A5/22. The down regulation of enzyme production may be a consequence of a mutation in this locus or in a locus which affects both enzyme production and galactose utilization independently.

In planta, a reduced enzyme production, a bacteriostatic effect caused by galactose and a weakened cell surface could all play a role in reducing virulence. With A5/22 a "stale mate" may be reached between enzyme production and the action of plant defence mechanisms, preventing bacterial numbers reaching threshold levels sufficient to lead to disease development. A5/8, which appears to be less severely affected in enzyme production, galactose utilization and LPS biosynthesis may be able to reach such a threshold and cause a significant rot, although to a lesser extent than the wild type strain. If A5/8 is affected in the same locus as A5/22, which seems unlikely from complementation data, its defects may be less severe, leading to less severe alterations in regulation of a number of phenotypes.

More work is needed to determine whether A5/22 and A5/8 are affected in the same locus or two closely related loci. Further complementation analysis will determine whether this is the case. Sequencing will determine whether the genes have been previously analysed and if so whether their product(s) are involved in regulation or are directly involved in galactose utilization (eg. *galU*). Alternatively the gene(s) may represent an important locus responsible for the control of a wide range of phenotypes some of which are involved in the virulence of *Eca*.

6 Final Discussion

The aim of this work was to obtain phage resistant mutants which, when tested on potato plants, exhibited a reduced virulence (Rvi-) phenotype. Previous workers using phages in this way have isolated phage resistant mutants possessing alterations in their cell surface (see Section 1.2.bii). In order to try this approach a number of phages were isolated. Of the *Eca* SCRI1043 phage resistant mutants that exhibited an Rvi- phenotype, two were chosen for further study. These mutants exhibited a range of phenotypes including; 1) a reduction in the extracellular enzymes PL, Cel and Prt, 2) an inability to utilize galactose, 3) reduced motility, 4) increased sensitivity to surface active agents, 5) an alteration in LPS profiles and 6) an alteration in outer membrane protein profiles, as well as reduced virulence and phage resistance. Equating the responsibility of a single phenotype to reduced virulence is difficult in this situation, since any one or more of the phenotypic alterations could effect virulence. From previous studies (see Introduction) it seems likely that alterations in enzyme production, galactose utilization and LPS structure are the most likely candidates to effect virulence. Other phenotypes however cannot be ruled out. The most probable explanation for the reduced virulence observed is that a mutation in a regulatory gene (which appears to be different in each mutant) effects both enzyme production and galactose utilization. The effect of an alteration in regulation of the galactose operon leads to cell surface alterations in LPS. A combination of these phenotypes *in planta* may lead to a decreased ability of the cell to breakdown the plant cell wall, and may also lead to the cell becoming more susceptible to plant defence mechanisms. This is supported by the observation that one mutant is more severely affected in enzyme production and LPS structure than the other, and in turn is more severely affected in virulence. The mutations obtained in this report are the first examples of regulatory genes affecting both global regulation of enzymes and regulation of the galactose operon (the latter leading to a number of cell surface alterations).

Future work on the mutants would involve reducing the size of complementing cosmids and sequencing the genes of interest. From the DNA sequence the protein product of the gene could be predicted, and this product computer screened for homology with other, known protein products. The protein could also be expressed in a T7 expression system, and protein localization studied within the cell.

A by-product of the above investigation was the isolation of a number of phages to *Eca* SCRI1043. During the isolation of these phages a number of other phages were isolated to *Ecc* SCRI193 and *Echr* NCPPB1066 (only AL1 of the latter phages being used in this report). The phages were characterised using a number of criteria and divided into groups, based predominantly on structural morphology and RE analysis (Fig. C16). The *Eca* phages fell into two groups based on structural morphology, groups A and C. All but one of the group A phages showed close genomic relationships based on RE patterns. The group C phages fell into three subgroups (I, II and III) based on RE pattern variations. The forth subgroup (IV) was derived from phages which appeared unrelated to each other, or for which complete characterisation was not undertaken. The *Ecc* phages all fell into group B, based on structural morphology. The characterisation of this group was not sufficiently detailed to divide the phages into groups, but on the basis of the results obtained all the phages appeared to differ in some respects. The host ranges of the *Eca* phages were much wider than those of the *Ecc* phages, and this allowed them to be used in a phage typing system. Using this system two strains, originally classified *Ecc*, were re-classified as *Eca*, and a number of *Eca* strains re-classified as *Ecc*. Although *Ecc* phages have been characterised in the past (see Section 1.4.5 and Table A3), this is the first example of the characterisation of *Eca* phages.

Eca SCRI1043 and Ecc SCRI193 are used extensively in our laboratory, and are amenable to a wide range of genetic techniques. One very important technique

previously unavailable to these strains is generalised transduction. The isolated phages were therefore screened for this event and two phages ϕKP and ϕM1 were found to transduce in *Ecc* SCRI193 and *Eca* SCRI1043 respectively. The transducing abilities the phages were investigated and attempts made to improve transduction frequencies. ϕKP has a very narrow host range, and of the strains tested for susceptibility to this phage, only one other showed susceptibility. This strain *Ecc* ATCC39048, which is being used in our laboratory is now also amenable to generalised transduction. This is the first example of a transducing phage for *Eca* and only the second for *Ecc* (Mukvick *et al.* 1987). Both phages are now being used extensively in our laboratory.

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