Mutation in a Gene Required for Lipopolysaccharide and Enterobacterial Common Antigen Biosynthesis Affects Virulence in the Plant Pathogen *Erwinia carotovora* subsp. *atroseptica*

I. K. Toth,² C. J. Thorpe,¹ S. D. Bentley,¹ V. Mulholland,¹ L. J. Hyman,² M. C. M. Perombelon,² and G. P. C. Salmond¹

¹Department of Biochemistry, University of Cambridge, Tennis Court Road, The Downing Site, Cambridge CB2 1QW, U.K.; ²Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, U.K.
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Spontaneous bacteriophage-resistant mutants of the phytopathogen *Erwinia carotovora* subsp. *atroseptica* (*Eca*) SCRI1043 were isolated and, out of 40, two were found to exhibit reduced virulence in planta. One of these mutants, A5/22, showed multiple cell surface defects including alterations in synthesis of outer membrane proteins, lipopolysaccharide (LPS), enterobacterial common antigen (ECA), and flagella. Mutant A5/22 also showed reduced synthesis of the exoenzymes pectate lyase (Pel) and cellulase (Cel), major virulence factors for this pathogen. Genetic analysis revealed the pronounced pleiotropic mutant phenotype to be due to a defect in a single gene (*rffG*) that, in *Escherichia coli*, is involved in the production of ECA. We also show that while other enteric bacteria possess duplicate homologues of this gene dedicated separately to synthesis of LPS and ECA, *Eca* has a single gene.

Erwinia carotovora subsp. *atroseptica* (*Eca*) is the etiological agent of the potato disease blackleg, and is a member of the soft rot group of erwinias. This group, which also includes *Erwinia carotovora* subsp. *carotovora* (*Ecc*) and *Erwinia chrysanthemi* (*Echr*), is responsible for substantial crop losses worldwide (Perombelon and Kelman 1980). These soft rot pathogens cause plant tissue maceration and necrosis mediated by several secreted enzymes: pectate lyases (Pel), polygalacturonases (Peh), cellulases (Cel), and proteases (Prt). Most of these enzymes have been shown to be important in virulence, since mutations affecting their synthesis or secretion lead to a reduction or loss of virulence (Kotoujansky 1987).

Corresponding author: G. P. C. Salmond; Telephone: 01 223 333 650; Fax: 01 223 333 345; E-mail: gpcs@mole.bio.cam.ac.uk

Present address of C. J. Thorpe: DuPont UK Ltd., CIC, Block B, The Mill Site, 40, Station Road, Cambridge, CB1 2UJ, U.K.

Present address of V. Mulholland: Diagnostics and Molecular Biology Section, Scottish Agricultural Science Agency, East Craigs, Edinburgh, EH12 8NJ, Scotland.

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Other factors have also been shown to be involved in the expression of virulence in plant pathogens. Random transposon mutagenesis has been used to identify factors affecting pathogenicity and virulence in a number of plant pathogens including *Ecc*, *Echr*, and *Eca* (Andro et al. 1984; Enard et al. 1988; Handa et al. 1987; Hinton and Salmond 1987; Hinton et al. 1989; Mulholland et al. 1993; Murata et al. 1991; Pirhonen et al. 1991.) In *Eca*, this random mutagenesis approach has been used to identify a number of factors affecting virulence, including auxotrophy, growth rate, and motility (Hinton et al. 1989; Mulholland et al. 1993).

As an alternative to a random screen for mutants affected in pathogenicity, there have been a few studies specifically directed at investigating the role of the bacterial cell surface in virulence by the analysis of mutants selected as spontaneously resistant to bacteriocins and bacteriophages. Schoonejans et al. (1987) used bacteriophages ϕ EC2 and Mu to isolate lipopolysaccharide (LPS)-defective mutants of *Echr* that also exhibited reduced virulence when inoculated into *Saintpaulia ionantha* plants. Expert and Toussaint (1985) also used resistance to ϕ EC2, as well as a number of bacteriocins, to isolate reduced virulence mutants of *Echr* altered in their outer membrane protein (OMP) profiles under iron-limiting conditions. Some of these alterations in OMP profiles were due to the loss of polypeptides corresponding to siderophore receptors. Finally, phage T4 has been used to screen for LPS-defective mutants of *Ecc* after transposon mutagenesis. Most of these mutants remained virulent but three exhibited a galactose-sensitive phenotype and were impaired in virulence due to the failure to survive in the galactose-rich environment of the plant (Pirhonen et al. 1991).

We reasoned that the use of phage T4 as a selection for cell surface mutants of *Ecc* might create a bias for selection of specific mutant types. Consequently, we have isolated a spectrum of bacteriophages that infect *Eca* (Toth 1989) and have used some of them in this study to select for phage resistant mutants that could then be screened for effects on virulence in planta. The principal aim was to define novel bacterial genes that play an essential role in plant disease. The products of these genes could be involved in the pathogen's assault on the host or its survival in planta. A bacteriophage, ϕ A5, isolated

on *Eca* SCRI1043 (Toth 1989), has been used to isolate phage-resistant mutants that exhibit a reduced virulence phenotype when inoculated into potato plant stems. These ϕ A5-resistant mutants have been phenotypically characterized and found to be highly pleiotropic. One of these mutants has been complemented and shown to be affected in a gene involved in the production of the enterobacterial common antigen (ECA) and LPS.

RESULTS

Isolation of mutants and testing of virulence-associated phenotypes.

Bacteriophage-resistant mutants of *Eca* SCRI1043 (wild type) were isolated after exposure to bacteriophage ϕ A5. Forty independently isolated mutants were tested for reduced virulence in planta by stem inoculation into potato plants. After incubation for 5 to 7 days, blackleg symptoms were rated on a scale of 0 to 5 (see Materials and Methods). One mutant, A5/22, showed significantly reduced blackleg symptoms, to a level of 1 to 2.

As a more quantitative test of virulence, the ability of SCRI1043 and mutant A5/22 to rot potato tuber tissue was also compared. Tubers were inoculated with 20- μ l samples of bacterial culture and incubated at 25°C. The weight of rot produced was determined over a 7-day period. The ability of A5/22 to rot the tuber was reduced, compared with that of the wild-type strain (Fig. 1).

In vitro and in planta growth characteristics were also tested. In vitro studies showed A5/22 to be prototrophic, unaffected in growth rate, and unable to utilize galactose, though

not a galactose-sensitive mutant of the type described by Pirhonen et al. (1991) (data not shown). In planta growth rates of A5/22 and SCRI1043 were assayed by inoculation of potato plants in duplicate and monitoring of in planta viable cell counts over a 4- to 6-day period (see Materials and Methods). The mutant growth rate was found to be similar to that of SCRI1043 (data not shown). Therefore, A5/22 is capable of surviving and growing in the plant environment and its reduced virulence phenotype is not a reflection of the mutant being killed.

The levels of exoenzyme production of A5/22 and SCRI1043 were assessed in two ways. Bacterial colonies were picked onto enzyme assay plates for Pel, Cel, and Prt. All indicator plates showed a reduction in the amount of enzymes produced by A5/22, compared with SCRI1043, as determined by halo size. Spectrophotometric assays were then used to assess activities of the main virulence factors, Pel and Cel, in supernatant and intracellular fractions (Fig. 2). A5/22 produced 15.9% Pel activity and 32.6% Cel activity, compared with the wild type, indicating significantly reduced production of these virulence factors in the mutant. It is also notable that, for both Pel and Cel, a disproportionately large amount of the total activity was found in the sonicate fraction of the A5/22 mutant. This suggests a reduction in the efficiency of secretion of these enzymes from the cell, possibly due to disruption of the secretion apparatus.

Unlike the motile, wild-type SCRI1043, A5/22 was found to be nonmotile in motility agar (data not shown). Scanning electron microscopic examination showed that SCRI1043 pos-

Fig. 1. Tuber rotting ability. Columns represent the mass of rotten tissue produced around the inoculation point by wild-type *Erwinia carotovora* subsp. *atroseptica* (*Eca*) (SCRI1043), mutant A5/22, and complemented mutant (A5/22+pKT80). Data presented represent a single experiment though tests were carried out on at least two separate occasions giving similar patterns of rotting.

Fig. 2. Spectrophotometric assay for pectate lyase (PEL) and cellulase (CEL) in the supernatants and sonicates of cultures of SCRI1043, A5/22, and A5/22 harboring a complementing clone (pKT80). Samples were taken from stationary phase cultures (approximately 1.2 OD₆₀₀ units). Pel and Cel assays were carried out in triplicate on the same sample for each strain and expressed as a percentage of wild-type activity per OD unit.

sesses clearly visible peritrichous flagella (usually between 1 and 4 per cell) while A5/22 has none, although, on occasion, a short, single flagellum was visible (data not shown).

Analysis of cell surface characteristics.

Previous studies of bacteriophage- and bacteriocin-resistant, reduced-virulence mutants of *Echr* showed the mutants had alterations in LPS structure (Schoonejans et al. 1987). The LPS profile of A5/22 was therefore investigated. A5/22 produced a profile significantly different from that of the wild-type strain (Fig. 3). 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 was observed by Murray et al. (1990). The ladder consisted of doublets, the upper band of each pair staining gray and the lower staining red. A5/22 retained band A but lacked all others, demonstrating a severe alteration in LPS structure.

Alterations in OMPs have also been observed in bacteriophage-resistant, reduced virulence mutants of *Echr* (Expert

and Toussaint 1985). The OMP profile of A5/22 was compared with that of SCRI1043 after growth in minimal broth containing glucose (Fig. 4). Various differences were seen between the OMP profiles of the A5/22 and SCRI1043. e.g. proteins of approximately 20, 35, and 40k Da from wild type are less abundant in, or absent from, the mutant, whereas proteins of approximately 16, 18, 25, and 31 kDa are present in the mutant but not in the wild type. Some of these alterations may relate to the defects in secretion and motility.

Complementation and sequence analysis.

A genomic library of *Eca* SCRI1043 DNA cloned into the cosmid pHC79 was transferred en masse to mutant A5/22 and recombinants screened for restoration of motility. From a total of 700 colonies screened, four complementing cosmids were isolated that had similar restriction patterns (data not shown). All four cosmids had an insert of around 40 kb. One of these cosmids, pIKT4, was tested and shown to restore wild-type exoenzyme production, bacteriophage sensitivity, motility, and virulence in planta (data not shown). Subclones of pIKT4 were constructed in pBR322 and tested for complementation, as shown by the restoration of motility and the bacteriophage-sensitive phenotype. A complementing clone, pKT80, consisting of an *NheI-EcoRI* fragment of approximately 2 kb cloned into pBR322, was isolated and found to restore mutant A5/22 to the wild-type or near-wild-type phenotype for the following: in planta virulence; bacteriophage sensitivity; ga-

Fig. 3. Electrophoresis and silver staining of lipopolysaccharides in 11% polyacrylamide gel showing the banding patterns of wild-type *Erwinia carotovora* subsp. *atroseptica* (*Eca*) (SCRI1043), mutant A5/22, and *Escherichia coli* F583 (Rd2 mutant purchased from Sigma, St. Louis, MO). Bands A, B, and C represent (A) the core region, and the core region with (B) one or (C) two covalently bound O-antigen side chains. The ladder of increasing molecular weight represents the core region with increasing numbers of O-antigen side chains.

Fig. 4. Outer membrane protein profile of wild-type *Erwinia carotovora* subsp. *atroseptica* (*Eca*) (SCRI1043), mutant A5/22, and complemented mutant (A5/22+pKT80). Molecular mass markers (M) are shown with values (kDa) indicated.

lactose utilization, motility, and sensitivity to surface active agents (data not shown); ability to rot tubers (Fig. 1); exoenzyme production (Fig. 2); and OMP profile (Fig. 4). The apparently incomplete restoration of Pel activity (to 52.4% of wild type; Fig. 2) could be due to various effects such as the copy number of the complementing gene, expression levels of the translationally coupled genes, and the generally complex regulation of this set of exoenzymes.

The nucleotide sequence of the insert from pKT80 was determined (accession number AF044332). Analysis of this 2,058-bp *NheI-EcoRI* fragment predicted the presence of one complete open reading frame (ORF), *orf2*, of 1,071 bp and a second, incomplete ORF at the beginning of the fragment (*orf1*), the 3' end of which overlaps the predicted translational start of *orf2* by 7 bp, presumably indicating translational coupling. BLAST searches performed via the ISREC data base showed *orf1* and *orf2* to have a high degree of similarity to *Escherichia coli* ORFs o355 (85%) and o379 (81%), respectively. These ORFs were first identified as part of the *E. coli* genome sequencing project (Daniels et al. 1992). They have since been designated *rffG* (o355) and *rffD* (o379) as part of an operon concerned with the synthesis of the ECA (Meier-Dieter et al. 1990). Using the colony immunoblotting procedure of Meier-Dieter et al. (1989), we showed that A5/22 is deficient in surface-exposed ECA (data not shown). Computer data base searches with the ORF2 polypeptide sequence also revealed multiple homologues, from different gram-negative and gram-positive bacterial genera and species, thought to encode nucleotidiphosphosugar dehydratases. Most are likely to be involved in LPS synthesis (Metzger et al. 1994; Stevenson et al. 1994; Jennings et al. 1993; Jiang et al. 1991; Morona et al. 1994; Freiberg et al. 1997) though some homologues from *Streptomyces* spp. are required for polyketide antibiotic synthesis (Bechthold et al. 1995; Linton et al. 1995; Merson-Davies and Cundliffe 1994; Lombó et al. 1997; Pissowotzki et al. 1991).

To confirm that *orf2* is solely responsible for the complementation, a series of deletion derivatives of the 2.0-kb insert of pKT80 were constructed (Fig. 5). The ability of these constructs to restore a motile and bacteriophage-sensitive phenotype in mutant A5/22 was tested. The results (Fig. 5) demonstrated that the loss of any part of *orf2* abolishes complementation and also that only *orf2* is required for complementation. Further confirmation that *orf2* is responsible for the complementation of mutant A5/22 to a wild-type phenotype was obtained with a cloned *orf2* gene carrying a frameshift mutation (introduced by digestion, end-filling, and re-ligation of a *Clal* site). This frameshift mutation leads to the production of a truncated protein lacking 21 amino acids from its C terminus. The plasmid produced by the re-ligation was named pKT90 and was unable to complement A5/22. We also utilized the T7 expression system devised by Tabor and Richardson (1985) to demonstrate that *orf2* encodes the predicted protein of 40 kDa (data not shown).

Southern blot analysis of chromosomal DNA of enterobacterial species.

It was shown by Marolda and Valvano (1995) that *E. coli rffG* is a functional homologue of *rffB* in the same strain and that both of these genes code for dTDP-glucose hydratases. Based on this, chromosomal DNA from various enteric bacteria was hybridized with an internal fragment of *orf2* in an attempt to assess the occurrence of gene duplication in other strains. A low stringency regime was employed in an attempt to allow hybridization of the probe to both *rffG* and *rffB* genes in the strains analyzed. Digestion with *BglII* was chosen because there are no target sites for this enzyme within the *rffG* gene of *E. coli* or its *Eca* homologue or within the *rffB* gene of *S. typhimurium*. Obviously, this does not exclude the existence of *BglII* sites within homologues of unknown sequence, so the resulting hybridizing bands must be treated as suggestive. Interestingly, the resulting autoradiogram (Fig. 6) revealed a hybridizing band in the *Echr* lane. This shows that,

Fig. 5. Physical and genetic map of pKT80 insert DNA. Arrows represent the partial ($\Delta orf1$) and complete (*orf2*) open reading frames. Also represented are the various subclones created for complementation (pKT82, 84, 86, 88, 90) or T7 protein expression (pCJ22, 44) and their ability to complement the A5/22 mutant phenotype; +, complements; -, unable to complement; nd, not determined. * indicates the point of truncation due to a frameshift mutation (see text).

although negative for ECA, *Echr* contains a homologue of at least one of the genes needed for ECA synthesis. There is a single band in the *Eca* lane (confirmed by shorter exposure; data not shown), corresponding to the *orf2* gene, suggesting that there is no additional *rfbB* homologue present. It seems this may also be true for *Ecc* (lane 2). Two bands are present in both the *S. typhimurium* and *E. coli* lanes. In the case of *S. typhimurium*, one of the bands represents the 3.2-kb *Bgl*I fragment that contains *rfbB* (Jiang et al. 1991). The other band probably represents *rffG*. In the case of *E. coli*, one of the bands represents the 4.3-kb *Bgl*I fragment that contains *rffG* (Daniels et al. 1992). The second band probably represents *rfbB*.

DISCUSSION

We have used the *Eca* bacteriophage ϕ A5 to select spontaneous mutants of *Eca* with cell surface alterations. Most ϕ A5 resistant mutants were unaffected in virulence but two mutants exhibited a reduction in their ability to cause the symptoms of blackleg. One of these mutants, A5/22, was characterized further and was shown to be particularly pleiotropic in that it exhibited multiple phenotypic differences from the wild-type strain, including changes in OMP profile, LPS structure, and presence of flagella. The disruption of the cell surface of the mutant was further indicated by cross resistance to a range of *Eca* bacteriophages and by an increased sensitivity to the action of surface active agents. Perhaps the most interesting phenotype displayed by A5/22 is the reduced levels of production of the exoenzymes Pel and Cel, major virulence determinants in this strain (Mulholland et al. 1993).

Complementation and the subsequent subcloning and sequencing of the complementing locus showed that the mutant phenotype of A5/22 is due to disruption of a gene (*orf2*) encoding a dTDP-glucose dehydratase. This protein is homologous to RffD from *E. coli*, part of an operon concerned with production of the ECA. The function of ECA remains unclear and any role in virulence has not been previously demonstrated. The similarities in DNA sequence and physical arrangement, and the ECA-negative phenotype of A5/22, strongly imply that *orf1* and *orf2* are direct functional homologues of *rffD* and *rffG*.

Data base searches also showed ORF2 to be a member of a large family of sugar dehydratases. These isozymes are present in a range of species and genera, including gram positives (Bechthold et al. 1995; Linton et al. 1995; Merson-Davies and Cundliffe 1994; Lombó et al. 1997; Pissowotzki et al. 1991). The majority are thought to be primarily involved in the synthesis of cell surface LPSs. In many cases, these structures have been proposed to be virulence determinants (Köplin et al. 1993; Metzger et al. 1994; Rajakumar et al. 1994; Robertson et al. 1994; Jiang et al. 1991; Jennings et al. 1993; Morona et al. 1997; Muñoz et al. 1997; van den Bosch et al. 1997). Interestingly, data base searches reveal a subgroup of sugar dehydratases that have been shown to be required for the synthesis of polyketide-based antibiotics in Actinomycetes (Bechthold et al. 1995; Linton et al. 1995; Merson-Davies and Cundliffe 1994; Lombó et al. 1997; Pissowotzki et al. 1991). As well as the obvious evolutionary link between the sugar dehydratases, this also suggests a possible common ancestry for the operons for LPS and polyketide antibiotic synthesis, the separate

pathways having been created by the "shuffling" of biochemical modules (Liu and Thorson 1994; Linton et al. 1995).

Other results in this study suggest that while *E. coli* and *S. typhimurium* possess two homologues of *orf2*, *Eca* and *Echr* have only one. The enzyme dTDP-glucose dehydratase is essential for the production of both ECA and LPS. The synthesis of these two products in *E. coli* is controlled by two separate regulons, hence the physiological advantage in duplication of the dTDP-glucose dehydratase encoding gene. Since *Eca* has only one gene encoding a dTDP-glucose dehydratase (*orf2*), and a disruption of this gene results in loss of both LPS and ECA, it seems certain that the product of *orf2* functions in both the LPS and ECA pathways. *Echr* also carries only one gene encoding dTDP-glucose dehydratase but is unique among the Enterobacteriaceae in that it does not produce ECA. *Echr* is positive, however, for LPS production, suggesting that its *orf2* homologue functions within the LPS pathway. Presumably, as well as lacking a duplicate dTDP-glucose dehydratase encoding gene, *Echr* must lack another essential part of the ECA regulon.

A5/22 also showed reduced production and secretion of the exoenzymes Pel and Cel. Several such enzymes have been shown to be important in the virulence of various soft rot *Erwinia* spp. (Hinton et al. 1989; Murata et al. 1991; Reeves et al. 1993; Walker et al. 1994; Babitskaya et al. 1995; Chatter-

Fig. 6. Autoradiogram of a Southern blot of chromosomal DNA hybridized with a radiolabeled probe comprising a DNA fragment internal to *orf2*. Chromosomal DNA was prepared from (lane 1) *Erwinia carotovora* subsp. *atroseptica* SCRI1043, (lane 2) *E. carotovora* subsp. *carotovora* SCRI193, (lane 3) *E. chrysanthemi* NCPPB1066, (lane 4) *Salmonella typhimurium* (laboratory stock), and (lane 5) *Escherichia coli* DH1, digested with *Bgl*I, electrophoresed on a 0.7% agarose gel before blotting onto a nylon filter. DNA fragment sizes are given in kilobases.

jee et al. 1995; Murata et al. 1994). The secretion of these exoenzymes requires a number of proteins, some of which are known to be present in either the outer or inner membrane of the cell envelope. Pel and Cel are secreted via the Type II Out secretion system (Pugsley 1993; Reeves et al. 1993). The disruption of the cell surface of mutant A5/22 could have an effect on the insertion and function of one or more of the secretory apparatus proteins, thereby leading to a reduction in the levels of enzymes secreted into the extracellular environment. Previous studies of mutants of *Ecc* affected in the Out secretory apparatus protein products demonstrated a decrease in the level of enzyme secretion with a consequent buildup of enzyme within the mutant cells (Reeves et al. 1993). This could explain the disproportionately large amount of exoenzyme activity detected in the mutant sonicate sample. However, it is less easy to explain the decrease in levels of actual production of the extracellular enzyme types tested. Further study of A5/22 is needed before the cause of the decrease in the level of exoenzyme synthesis is elucidated. One possible model that could explain the results would be that inability to assemble one or more cell surface structures with fidelity is “sensed” by the pathogen and the information relayed back to the level of exoenzymes synthesis, leading to its down modulation. This hypothetical sensing system might monitor insertion fidelity of an OMP or even a nonproteinaceous surface component and may involve an extracytoplasmic sigma factor (Missiakas and Raina 1998). The observation of OMP profile alterations of mutant A5/22 would be consistent with this model.

Given the importance of exoenzymes in the pathogenic processes of soft rot *Erwinia* spp. (Murata et al. 1994), it is likely that the decreased levels of production/elaboration of these enzymes by A5/22 are at least one of the causes of the reduction in blackleg symptoms shown by the mutant. However, as we have shown before (Mulholland et al. 1993), the loss of motility could also be a contributory factor since the mutant strain will be unable to spread through the plant tissue at the same rate as the wild-type strain. ECA is unlikely to be

involved in virulence, as the ECA-deficient *Echr* is an effective pathogen. Disruption of LPS may also appear to be unlikely to have a direct effect upon virulence, but a growing body of research implicates such cell surface structures in the infection processes of plant and animal pathogens—with disruption of these structures resulting in reduced virulence (Köplin et al. 1993; Metzger et al. 1994; Rajakumar et al. 1994; Robertson et al. 1994; Jiang et al. 1991; Jennings et al. 1993; Morona et al. 1997; Muñoz et al. 1997; Titarenko et al. 1997). A common theme in the above studies is that LPS provides protection from host defenses. A5/22, however, is capable of survival and growth within the plant yet seems unable to mount a full attack on its host. It is conceivable that this is due to the absence of some currently undefined host-pathogen interaction required for triggering a fully aggressive attack by this blackleg pathogen.

MATERIALS AND METHODS

Strains and media.

Bacterial strains, bacteriophages, and plasmids are listed in Table 1. Media composition, antibiotics, and auxotroph analysis have been described previously (Hinton et al. 1989; Lanham et al. 1991). Motility agar was as described by Wolfe and Berg (1989) with incubation conditions of 25°C for 24 h. Bacteriophage methods have been described previously (Toth et al. 1997; Toth 1991).

Production of bacteriophage-resistant mutants.

Bacteriophage-resistant mutants were produced as described by Schoonejans et al. (1987) with overnight incubation at 27°C. Colonies that grew were tested for bacteriophage resistance by spotting 10 µl of a high-titer lysate of bacteriophage onto a lawn of cells. To confirm that this bacteriophage resistance was not due to lysogeny, each colony was grown overnight in nutrient broth at 27°C and its supernatant spotted onto a lawn of wild-type bacteria. Lack of bacteriophage release indicated resistance not lysogeny.

Table 1. Bacterial strains, bacteriophage, and plasmids

Designation	Relevant details	Source/reference
<i>Escherichia coli</i>		
F83	Lipopolysaccharide (LPS) Rd2 mutant	Sigma, St. Louis, MO
DH1	F ⁻ , <i>recA1</i> , <i>endA1</i> , <i>thi1</i> , <i>hsdR17</i> , <i>gyrA96</i> , ($r_k^-m_k^-$), <i>supE44</i> , <i>relA1</i>	Hanahan 1983
<i>Erwinia carotovora</i> subsp. <i>atroseptica</i>		
SCRI1043	Wild type	Hinton et al. 1989
A5/22	SCRI1043, Rvi ⁻ , ϕ A5 ^r	This study
<i>Erwinia carotovora</i> subsp. <i>carotovora</i> (<i>Ecc</i>)		
SCRI193	Wild type	Forbes and Perombelon 1985
<i>Erwinia chrysanthemi</i> (<i>Echr</i>)		
NCPB1066	Wild type (ECA ⁻)	Gilbert et al. 1986
<i>Salmonella typhimurium</i>		
ϕ A5	Wild type	Laboratory stock
pT7-5	Phage isolated on SCRI1043	Toth 1989
pT7-6	Protein expression vector	Tabor and Richardson 1985
pIKT43	Protein expression vector	Tabor and Richardson 1985
pKT80	SCRI1043 pHCT79 library clone that complements A5/22	This study
pKT82	pBR322::4.5kb <i>NheI/EcoRI</i> fragment from pIKT43	This study
pKT84	pKT80 Δ <i>SaI</i>	This study
pKT86	pKT80 Δ <i>AccI</i>	This study
pKT88	pKT80 Δ <i>EcoRV</i>	This study
pKT90	pKT80 Δ <i>NruI</i>	This study
pCJ44	pKT80 <i>rffG</i> frameshift derivative	This study
pCJ22	pT7-5, <i>rffG</i>	This study
	pT7-6, Δ <i>rffG</i>	This study

Virulence assays.

Both plant and tuber virulence assays were as described by Hinton et al. (1989). Blackleg symptoms were scored on the following numerical index: 0 = no reaction; 1 = slight browning around inoculation site; 2 = slight blackening around inoculation site; 3 = small black rot spreading from inoculation site; 4 = medium black rot spreading from inoculation site; and 5 = large black rot accompanied by complete stem collapse (Hinton et al. 1989). Bacteriophage-resistant mutants that gave symptoms of 1 or 2 were retested on several further occasions on both micropropagated plants and on tuber-grown plants (approximately 40 cm tall). Mutants that consistently gave such symptoms were termed reduced virulence (Rvi⁻) mutants.

In planta growth rates.

In planta growth rates were determined as described by Hinton et al. (1989), except that viable counts were assayed with crystal violet pectate (CVP) agar (basal layer: 15 g of Bacto agar, 5.5 g of CaCl₂ · 6H₂O, 2 g of NaNO₃, 1 g of Oxoid tryptone, 2 ml of crystal violet [0.075% solution], pH 7.0 to 7.4, made up to 1 liter; overlay: 25 g of sodium polypectate, 2.5 ml of EDTA [5% solution]) in place of Stewart's medium.

Exoenzyme assays.

Pel and Cel assays were performed as described by Hinton and Salmond (1987) except that the cellulase assay substrate used was Ostazin brilliant red hydroxymethylcellulose (Biely et al. 1985).

Extraction, electrophoresis, and staining of OMPs.

OMPs were extracted by the method of Beher and Schnaitman (1981) and electrophoresed through 11% sodium dodecyl sulfate-polyacrylamide gels according to the method of Laemmli (1970). Before electrophoresis, samples were incubated at 100°C for 5 to 10 min. Protein molecular mass markers (Pharmacia, Uppsala, Sweden) were in the range 200,000 to 6,000 Da. Staining was carried out in 400 ml of Coomassie blue (Bio-Rad, Hercules, CA) (25% propan-2-ol, 10% glacial acetic acid, 0.1% Coomassie blue) overnight, followed by several changes of destain (25% propan-2-ol, 10% glacial acetic acid) until the background cleared.

Extraction, electrophoresis, and staining of LPSs.

LPS extraction (including further purification to remove nucleic acids) and electrophoresis were as described by Westphal and Jann (1965). Silver staining of the gel was carried out as described by Hitchcock and Brown (1983).

DNA manipulation and sequence analysis.

All molecular biological techniques have been described previously unless otherwise stated (Maniatis et al. 1982). A chromosomal DNA library of *Eca* SCRI1043 was prepared and packaged into phage λ as previously described by McGowan et al. (1995). DNA sequences were determined by the dideoxynucleotide chain termination method of Sanger et al. (1977). Data base searches with DNA and protein sequences were performed with BLAST on-line at the ISREC web site.

Colony immunoblot for the ECA.

Cells from a 10-ml nutrient broth overnight culture were re-suspended in 500 µl of NaCl (0.5%) and spotted onto a Hybond C (Amersham, Rainham, U.K.) filter. Assays for ECA were then carried out as described by Meier-Dieter et al. (1989) with a specific antibody, mAb898.

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