Identification of Pathogenicity Determinants of *Erwinia carotovora* subsp. *carotovora* by Transposon Mutagenesis

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Transposon mutagenesis and large-scale virulence screening of the isolated mutants on axenic tobacco seedlings were employed to identify pathogenicity determinants of the soft rot pathogen *Erwinia carotovora* subsp. *carotovora*. Screening of 6,200 prototrophic transposon mutants resulted in the isolation of several classes of strains exhibiting reduced virulence or avirulence. Characterization of these mutants showed that the majority of the strains were defective in motility and showed a reduced virulence phenotype. The mutants in the other main category were avirulent and were affected in exoenzyme production and growth on pectin.

Some of these mutants appeared to produce reduced amounts of pectin lyase (Pnl⁻), but most of the strains were defective in either secretion (Out⁻) or production (Exp⁻) of several extracellular enzymes. The Exp⁻ mutants represent a novel class of exoenzyme-negative mutants showing a pleiotropic defect in production and secretion of pectic enzymes, cellulase, and protease. The wild-type allele for one class of Exp⁻ mutants was cloned by complementation after bacteriophage T4GT7 transduction of a genomic library.

Additional keywords: bacterial soft rot, T4 transduction.

Erwinia carotovora subsp. *carotovora* (Jones) Bergey *et al.* is a phytopathogenic enterobacterium causing soft rot, blackleg, or stem rot on many economically important crops (Pérombelon and Kelman 1980). The disease is characterized by extensive maceration of the affected tissue caused by a variety of plant cell wall-degrading enzymes secreted by the pathogen. The exoenzyme arsenal of *E. c.* subsp. *carotovora* includes pectase lyase (Pel), pectin lyase (Pnl), polygalacturonase (Peh), cellulase (Cel), and protease (Prt), which are thought to be involved in pathogenicity (Chatterjee and Vidaver 1986; Collmer and Keen 1986; Kotoujansky 1987).

Random mutagenesis with transposable elements has been widely used to identify genetic determinants of pathogenicity in many gram-negative phytopathogens (Mills 1985; Daniels *et al.* 1988). Genetic analysis of *E. carotovora* and the related soft rot pathogen *E. chrysanthemi* Burkholder *et al.* by transposon mutagenesis has resulted in the isolation of a variety of auxotrophs and strains unable to utilize a specific carbohydrate or strains that are defective in one or more enzymatic activities (Chatterjee *et al.* 1983, 1985; Thurn and Chatterjee 1985; Zink *et al.* 1984; Jayaswal *et al.* 1984; Diolez and Coleno 1985; Salmond *et al.* 1986; Hinton and Salmond 1987; Ellard *et al.* 1989). However, only in a few studies have transposon mutants of *E. carotovora* been isolated that are affected in virulence, possibly due to problems encountered in the screening of a large number of mutants. Handa *et al.* (1987) described the isolation of Mu-induced mutants of *E. c.* subsp. *carotovora* that were impaired in their ability to rot cut potato tubers. In addition to auxotrophs, the researchers obtained mutants defective in the secretion of either Pel or Peh or both, a galactose-sensitive mutant, and prototrophic mutants able to synthesize and secrete

pectolytic enzymes but apparently affected in other yet uncharacterized function(s) required for virulence. Hinton *et al.* (1989) characterized *E. c.* subsp. *atroseptica* Tn5-induced mutants unable to cause blackleg in potato plants. They identified a mutant with a reduced growth rate and strains that synthesized and secreted reduced levels of both Pel and Peh.

In this study, we describe the identification of reduced virulence mutants after screening 6,200 prototrophic transposon-induced mutants on axenic tobacco plants for virulence. The mutants showing reduced virulence were characterized and assigned to four phenotypic categories according to their motility and production of exoenzymes. Novel pleiotropic mutations affecting synthesis and secretion of several exoenzymes were identified, and one of the corresponding wild-type alleles was isolated by complementation cloning.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. The generalized transducing phage T4GT7 (Wilson *et al.* 1979) and the transposon delivery vehicle λ 1105 (Way *et al.* 1984) were kindly provided by G. G. Wilson (University of California, Davis) and N. Kleckner (Harvard University, Cambridge), respectively. The *E. c.* subsp. *carotovora* strains were cultured in L medium or M9 minimal medium (Miller 1972) supplemented with 0.4% sugar (arabinose, fructose, fucose, galactose, glucose, lactose, maltose, rhamnose, sucrose, trehalose, or xylose), 0.5% sodium polypectate (PGA; Sigma P-1879; Sigma Chemical Co., St. Louis, MO) or 1% Citrus pectin (partially methylesterified, Sigma P-9135) at 28° C and *Escherichia coli* (Migula) Castellani and Chalmers strains in L medium at 37° C. For Pel, Peh, Cel, and Prt assays, the strains

were grown to stationary phase ($A_{600nm} = 4$) in L medium with 0.5% PGA according to Hinton and Salmond (1987). Pnl activity was induced by adding mitomycin C to a final concentration of 1 μg/ml to exponentially growing culture (about 3×10^8 cells per milliliter in M9 minimal medium) followed by incubation in the dark until the cells lysed (10–12 hr). For detection of exoenzymes, indicator media were used as described by Diolez and Coleno (1985) for pectolytic enzymes, by Andro et al. (1984) for cellulases, and by Hankin and Anagnostakis (1975) for proteolytic activity. Antibiotics were added to media as appropriate, kanamycin (Km) at 25 μg/ml and ampicillin (Ap) at 150 μg/ml.

Transposon mutagenesis. Phage λ 1105 described by Way et al. (1984) was employed for transposon mutagenesis. λ 1105 carries a mini-kan transposon that contains a Km resistance (Km^r) marker from Tn903 between the outermost inverted repeats of Tn10 (Way et al. 1984). To obtain derivatives of E. c. subsp. carotovora with a functional λ receptor protein (LamB$^+$), the lamB expression vector pAMH62 (Harkki and Palva 1985) was introduced into SCC3193 by T4GT7 transduction (Pirhonen and Palva 1988). For transposon mutagenesis, λ 1105 was propagated in E. coli TG1 and used to infect an overnight culture of SCC3193(pAMH62) at a multiplicity of infection of 0.4 in the presence of 10 mM MgSO$_4$. After phage adsorption (3 hr, 37° C), the cells were collected by centrifugation, resuspended into fresh L medium, and incubated for 1 hr at 28° C to allow expression of the Km^r marker before plating onto L Km medium. Selection (Ap) for the lamB expression plasmid pAMH62 was omitted at this stage and the plasmid was rapidly lost.

Transduction of plasmids and chromosomal markers with T4GT7. The transducing mutant derivative of bacteriophage T4, T4GT7 (Wilson et al. 1979), was used to transfer plasmids from E. coli to E. c. subsp. carotovora (Pirhonen and Palva 1988) and plasmids as well as chromosomal genes between E. c. subsp. carotovora strains. T4GT7 was first propagated in the E. coli supE host strain TG1. This phage stock was subsequently propagated in the E. c. subsp. carotovora or E. coli donor strain and used to infect the recipient E. c. subsp. carotovora grown overnight. Phages were allowed to adsorb for 30 min at 37° C. The cells were collected by centrifugation, resuspended into fresh medium, and incubated for 1 hr at 28° C to allow marker gene expression before plating onto selective medium. Using this procedure, transduction frequencies of approximately 10^{-4} per plaque-forming unit (pfu) were obtained both when plasmids and chromosomal markers were transduced between E. c. subsp. carotovora strains.

Pathogenicity tests. The pathogenicity of the isolated transposon insertion mutants was tested on axenic tobacco seedlings (Nicotiana tabacum L. 'Samsun') propagated in 24-well tissue culture plates on MSP medium (Flow Laboratories, McLean, VA) supplemented with 2% sucrose and solidified with 0.3% gellan gum. The seedlings (about five per well) were grown for 3–6 wk in a Conviron growth chamber at 26° C and with a 16-hr daylight regime (2 W/m^2). The E. c. subsp. carotovora strains to be tested were first grown to single colonies on M9 medium plates

containing sucrose and Km and then patched onto L medium plates containing Km and grown overnight. The patches were transferred with a sterilized multiprong device to 96-well microtiter plates containing 100 μl of M9 medium with sucrose per well and grown for 12–24 hr. The tobacco seedlings were surface-inoculated by pipetting bacterial suspensions (10^8 bacteria per well) onto the plants without wounding. The inoculated plants were incubated in plastic growth chambers to maintain high humidity, and the development of soft rot symptoms was followed for 2 days.

The ability of the strains to macerate potato stem was assessed by using tuber-grown potato (Solanum tuberosum L. 'Bintje') plants grown in the greenhouse until 50 cm high. The plants were inoculated by stabbing the stem with a toothpick and transferring a loopful of bacteria from a fresh L plate into the wound. The symptoms were scored essentially as described by Hinton et al. (1989).

Assay of bacterial motility. Motility was assayed on L medium solidified with 0.3% agar. After inoculation, these semisolid plates were incubated at 28° C until SCC3193 produced a halo of swimming bacterial cells.

Cell fractionation and enzyme assays. Bacterial cultures were chilled on ice and the culture supernatants collected by removing the cells through centrifugation. The scaled-down osmotic shock procedure (Palva 1978) of Heppel (1971) was used to obtain a periplasmic fraction. This method was further modified to suit E. c. subsp. carotovora by increasing the concentration of MgCl$_2$ in the last step to 20 mM. If less than 10 mM MgCl$_2$ was used, pectic enzymes remained largely cellbound. A cell lysate was obtained by disruption of washed cells by a brief ultrasonic treatment. The cell lysate, the periplasmic fraction, and the culture supernatant were stored at $-20°$ C until assayed. Pel activity was assayed by the thiobarbituric acid method

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	Reference or source
Escherichia coli K12		
HB101	F$^-$ proA2 leu-6 thi galK2 lacY1 recA13 rpsL20 supE44 hsdD hsdM	Boyer and Roulland-Dussoix 1969
TG1	Δ(lac-pro) supE thi hsd5 (F' traD36 proA$^+$B$^+$ lacI^q lacZΔM15)	Carter et al. 1985
Erwinia carotovora subsp. *carotovora*		
SCC3193	Wild-type	Pirhonen et al. 1988
SCC3060	ExpI$^-$	This study
SCC3065	ExpII$^-$	This study
SCC3111	Nonmotile	This study
SCC3112	Reduced motility	This study
SCC3113	Reduced motility	This study
SCC3125	Out$^-$	This study
SCC3127	Pnl$^-$	This study
Plasmids		
pUC18		Norrander et al. 1983
pUC4		Vieira and Messing 1982
pTK806	ExpII$^+$, 2.7-kb insert complementing the ExpII$^-$ mutation	This study

[a] Exp, deficiency in exoenzyme production; Out, deficiency in secretion of exoenzymes.

(Sherwood 1966), and the activity was expressed as A_{548nm}. Peh and Cel activities were determined by measuring the release of reducing groups as described by Collmer et al. (1982) and Hinton and Salmond (1987), respectively. One unit of Peh corresponds to the amount of enzyme that produced 0.1 μmol of glucose equivalent per minute at 30° C. One unit of Cel was defined as the amount of enzyme that produced 1 μmol of glucose equivalent per hour at 30° C. Pnl activity was assayed by measuring the increase in A_{235nm} according to Zink et al. (1985), except that partially methylesterified Citrus pectin was used as a substrate. One unit of Pnl activity corresponds to the amount of enzyme that produced an increase in A_{235nm} of 1.0 per minute at 30° C. Prt was assayed by the azocasein method (Ji et al. 1987), and one unit was defined as the amount of enzyme that produced an increase in absorbance of 1.0 per hour at 30° C and 436 nm. β-Lactamase assay was done as described by O'Callaghan et al. (1972).

Isoelectric focusing (IEF). Ultrathin IEF of Pel isoenzymes was done by using an LKB Multiphor II apparatus and LKB Ampholines (pH ranges 3.5–10 and 9–11) essentially as described in Willis et al. (1987) or in the manufacturer's instructions.

Characterization of cell envelope proteins and lipopolysaccharides (LPS). A cell envelope fraction containing primarily cell wall material was isolated as previously described (Palva 1978), and the proteins were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970). Characterization of LPS was as described by Hitchcock and Brown (1983).

DNA techniques. Plasmid DNA was isolated as described by Holmes and Quigley (1981) or Birnboim and Doly (1979). Standard cloning techniques were as described in Maniatis et al. (1982). Restriction enzymes and T4 ligase were used according to the specifications given by the manufacturer (IBI, New Haven, CT). The genomic library from SCC3193 was constructed by partial Sau3A digestion of the isolated chromosomal DNA and ligation of the fragments into pUC18 opened with BamHI. Ap^r transformants of E. coli HB101 were pooled and used to generate T4GT7 stocks.

Gel hybridization and Southern analysis. Chromosomal DNA digested with XhoI and HindIII was separated by electrophoresis in 0.8% agarose gels. Gel hybridization was done as described by Silhavy et al. (1984). The gels were dried and hybridized to a labeled pUC4 probe (Vieira and Messing 1982) carrying the Km^r gene from Tn903. Southern transfer and hybridization was done as described by Maniatis et al. (1982).

RESULTS

Transposon mutagenesis and isolation of LPS-defective mutants. E. c. subsp. carotovora SCC3193(pAMH62) was mutagenized with λ 1105 (Way et al. 1984). After mutagenesis Km^r colonies were obtained with a frequency of 10^{-7}/ pfu. To test for the presence of transposon-induced mutants, we screened the Km^r colonies for auxotrophs and bacteriophage T4-resistant mutants. Of a total of 10,000 Km^r colonies isolated, 0.4% were auxotrophs. Screening

for T4 resistance detected 30 mutants, and subsequent characterization of their LPS by SDS-PAGE showed that all of them were defective in their LPS structure (data not shown). Only three of the LPS mutants were avirulent in the tobacco virulence assay. These strains showed a galactose-sensitive phenotype, similar to that described earlier for galU mutations in E. c. subsp. carotovora (Jayaswal et al. 1985).

Isolation of mutants affected in virulence. The virulence of the transposon mutants was assayed on axenic tobacco seedlings grown in 24-well tissue culture plates. Surface inoculation of these seedlings with the wild-type E. c. subsp. carotovora strain resulted in the appearance of the first soft rot symptoms after 12 hr, and after 2 days all the plant material in the well was totally macerated. To exclude mutants that might exhibit reduced virulence because of trivial reasons such as auxotrophy or a general growth defect, the Km^r colonies were pooled and grown twice to stationary phase in M9 media supplemented with Km and sucrose before screening the mutants for virulence. The virulence of 6,200 prototrophic transposon mutants was tested by this assay, and the symptom development was compared to that of the wild-type strain. The mutants that infected the plants more slowly than the wild-type or not at all were retested several times before further characterization. A total of 298 mutants reproducibly showed reduced virulence, and the symptoms produced ranged from avirulence (30 strains) to a slightly reduced spread of the infection.

E. c. subsp. carotovora mutants affected in motility exhibit reduced virulence. Motility of the pathogen has been shown to affect the virulence in E. amylovora, Pseudomonas phaseolicola, and P. syringae (Bayot and Ries 1986; Panopoulos and Schroth 1974; Hattermann and Ries 1989). To clarify the role of motility in the virulence of E. c. subsp. carotovora, we determined the motility of both SCC3193 and the 298 reduced-virulence mutants on semisolid L medium. The motility of 268 of these mutants was altered; thus, they constitute the largest category of mutants affected in virulence. The majority of the mutants were completely nonmotile (Mot⁻), but mutant strains with intermediate motility could also be identified (Fig. 1). Subsequently, the motility of one third of the screened 6,200 transposon mutants was tested. All of the motility mutants obtained also exhibited reduced virulence. None of the motility mutants were, however, fully avirulent, but rather showed a reduced spreading of the soft rot symptoms as compared to the wild-type (Fig. 2). The enzyme production of 35 motility mutants showing different motility phenotypes was tested on enzyme-indicator plates (data not shown), and a representative Mot⁻ strain was assayed for enzyme production and secretion (Table 2). The motility mutants resembled the wild-type parent in all the other characteristics tested except motility and virulence.

Avirulent mutants are affected in the production and secretion of exoenzymes. In addition to the motility mutants showing reduced virulence, 30 avirulent but motile mutants were isolated. To rule out siblings and the presence of multiple transposon insertions, the strains were characterized by gel hybridization with the Km^r gene as a probe.

The results of this analysis indicated that four of the 30 strains analyzed were siblings, but 26 of the mutants contained unique single insertions (data not shown) and were characterized further. These 26 independent avirulent mutant strains were tested for production of pectic enzymes, cellulase, and protease on indicator plates. Based on these results, the mutants could be divided into three phenotypic categories. Two to three mutants of each class were assayed for Pel, Peh, Cel, and Prt production and localization. The strains in each category appeared identical; as a summary, the results from one representative strain from each class are shown in Table 2. The three mutants in the first group (e.g., SCC3125; Table 2) resembled previously described *E. c.* subsp. *carotovora* and *E. chrysanthemi* Out⁻ mutants that are unable to secrete pectinases and cellulases but accumulate these enzymes in the periplasm (Chatterjee *et al.* 1985; Andro *et al.* 1984). The next mutant category contained 20 pleiotropic mutant strains that showed Cel⁻ and Prt⁻ phenotypes on indicator media and synthesized reduced levels of pectic enzymes (e.g., SCC3065; Table 2). Furthermore, the residual Pel and Peh activities were periplasmic in these mutants as they were in Out⁻ mutants (Table 3). As these mutant strains appeared to have a pleiotropic defect in the production of several exoenzymes, the mutants have been designated as Exp⁻ (exoenzyme production). The last mutant class included three avirulent strains (e.g., SCC3127) that were motile and appeared to synthesize and secrete wild-type levels of the exoenzymes when grown in the presence of PGA. However, when Pnl activities were assayed from mitomycin C-induced cultures, these strains showed reduced activity, and were therefore designated as Pnl⁻.

The Pel isoenzymes of two or three strains from each category of mutants shown in Table 2 were characterized
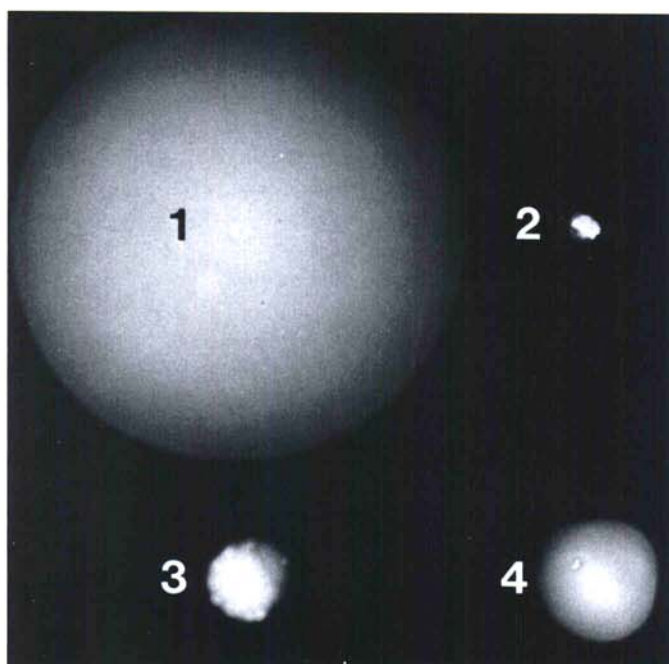

Fig. 1. Motility test of the *Erwinia carotovora* subsp. *carotovora* wild-type strain SCC3193 and mutants showing reduced virulence and altered motility on semisolid L medium (Miller 1972) 30 hr after inoculation. Strains are 1, SCC3193; 2, SCC3111; 3, SCC3112; and 4, SCC3113.
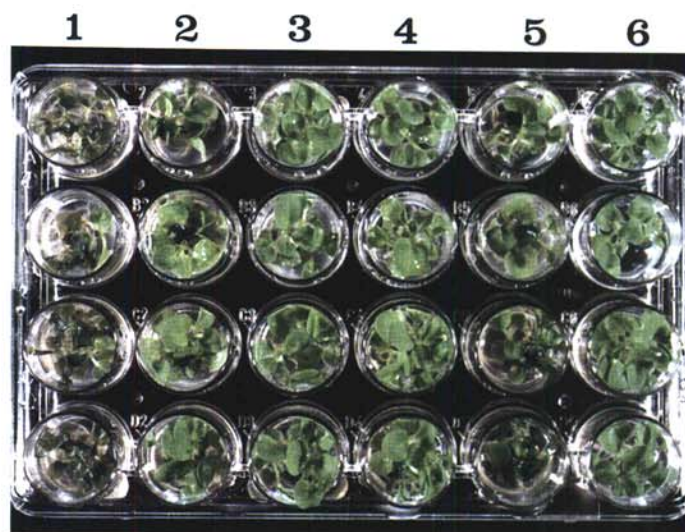

Fig. 2. Virulence assay of the *Erwinia carotovora* subsp. *carotovora* wild-type strain SCC3193 and mutants showing reduced virulence and complementation of Exp⁻ phenotype by a genomic clone. Four-week-old tobacco seedlings were surface-inoculated with 10⁸ bacterial cells and incubated for 48 hr in high humidity in a plant growth chamber. Row 1, parental strain SCC3193; row 2, SCC3111 (Mot⁻); row 3, SCC3025 (Out⁻); row 4, SCC3065 (Exp⁻); row 5, SCC3065(pTK806); and row 6; SCC3127 (Pnl⁻).

Table 2. Exoenzyme production by mutants with reduced virulence

| | | | Enzyme activity[a] | | | | | | | | |
| | | | Pel | | Peh | | Cel | | Prt | | Pnl |
Strain	Phenotype	Number of mutants	Total	Sn	Total	Sn	Total	Sn	Total	Sn	Total
SCC3193	Wild-type		0.71	88	8.5	87	0.56	84	0.30	94	2.4
SCC3111	Mot⁻	268	0.51	75	10.7	73	0.71	84	0.86	93	2.1
SCC3125	Out⁻	3	0.73	1	9.1	2	0.61	27	0.55	91	2.0
SCC3065	Exp⁻	20	0.46	3	4.3	2	0.04	29	0.09	2	0.1
SCC3127	Pnl⁻	3	0.53	98	8.5	96	0.60	80	0.34	92	0.3

[a] Pel refers to pectate lyase, Peh to polygalacturonase, Cel to cellulase, Prt to protease, and Pnl to pectin lyase. Total refers to the total enzyme activity in cells and culture supernatant expressed as units per milliliter of culture. Sn refers to the percentage of total activity found in the culture supernatant. For Pel, Peh, Cel, and Prt assays the bacteria were grown to stationary phase ($A_{600nm} = 4$) in L medium (Miller 1972) supplemented with 0.5% sodium polypectate, and Pnl was assayed from mitomycin C-induced cultures growing in M9 minimal medium (Miller 1972). Pel activity was assayed by the thiobarbituric acid method of Sherwood (1966), and the results were expressed as A_{548}. Peh, Cel, Prt, and Pnl assays were as described by Collmer *et al.* (1982), Hinton and Salmond (1987), Ji *et al.* (1987), and Zink *et al.* (1985), respectively. The experiment was done at least three times; the values shown are from a single representative experiment.

by isoelectric focusing followed by activity staining from both culture supernatant and periplasmic fractions. Differences in pIs, relative intensity, or number of Pel isoenzymes were not detected in these mutants as compared to the wild-type strain (data not shown). Characterization of outer membrane protein and LPS profiles of the mutants by SDS-PAGE showed that the mutants were indistinguishable to the wild-type in this respect (data not shown).

To confirm that the mutant phenotypes were caused by the insertion only, the transposon insertions from the characterized strains (Fig. 1; Table 2) were transduced by T4GT7 to a clean genetic background (SCC3193). Cotransduction of the previously characterized mutant phenotype (reduced virulence and Mot⁻, Out⁻, Exp⁻, or Pnl⁻, respectively) with the selected Km^r phenotype was 100% in each case.
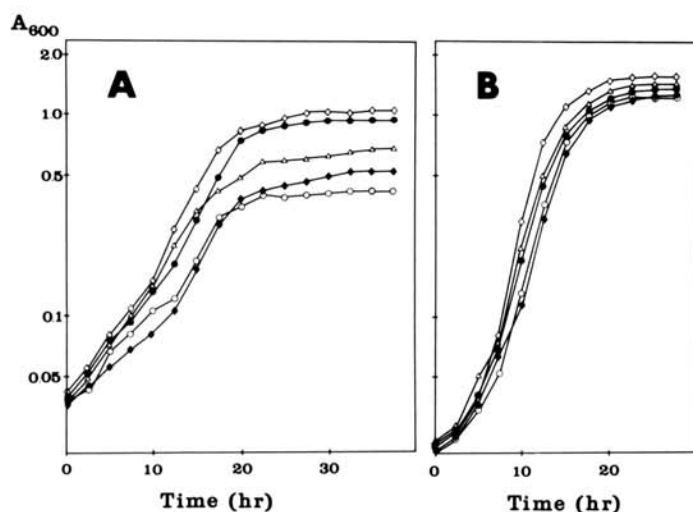

Fig. 3. Growth of *Erwinia carotovora* subsp. *carotovora* wild-type strain SCC3193 and mutants affected in virulence in M9 minimal medium (Miller 1972) with 1% Citrus pectin (**A**) or 0.4% sucrose (**B**) as the sole carbon source. Cells were cultured overnight in the respective medium and diluted 1:100, and the growth was followed by determining the optical density at A_{600nm} at 2-hr intervals. Symbols indicate strains as follows: ●- - -●, SCC3193; ◇- - -◇, SCC3111 (Mot⁻); △- - -△, SCC3125 (Out⁻); ○- - -○, SCC3065 (Exp⁻); and ◆- - -◆, SCC3127 (Pnl⁻).

Carbohydrate utilization of the reduced virulence mutants. The carbohydrate utilization of the mutants in the four categories was examined by cultivating them in M9 minimal medium with separate additions of 10 common sugars. All of the mutants grew normally on the sugars tested (data not shown). The *in vitro* growth rates of the mutant strains were studied in L medium and M9 medium with 0.4% sucrose, 0.5% PGA, or 1% Citrus pectin as the sole carbon source. No clearcut differences in the growth rates of any mutant could be seen as compared to the wild-type (data not shown). However, when grown on pectin, only the Mot⁻ mutants reached the wild-type cell density in stationary phase, while the growth of the other mutants ceased earlier (Fig. 3).

Virulence of the mutants on potato. The virulence of the mutants was tested on tuber-grown potato plants (cv. Bintje) by stem inoculations as suggested by Lapwood and Read (1986). The wild-type strain SCC3193 produced a black lesion in the stem that spread in both directions from the inoculation point (data not shown). When the scale of Hinton *et al.* (1989) was applied and the wild-type parent given a value of five, the Mot⁻ mutant SCC3111 and the Pnl⁻ mutant SCC3127 produced symptoms rated at 3. The Out⁻ and Exp⁻ mutants made the stem slightly soft and were scored as 1.

Isolation of clones complementing Exp⁻ mutants. To obtain plasmid clones that would complement the Exp⁻ mutants, a genomic library of the wild-type strain SCC3193 was established in pUC18 and transformed to *E. coli* HB101. The library was transduced en masse to the Exp⁻ mutants by T4GT7, and the plasmid transductants were screened for enzyme production on indicator plates. Several clones that appeared to complement the Exp⁻ phenotype were obtained. These clones were transduced to the 20 Exp⁻ mutant strains and the complementation of the enzyme phenotype was tested on indicator plates. The results suggested that the 20 Exp⁻ mutants could be divided into two complementation groups called ExpI (19 strains) and ExpII (1 strain). However, Southern hybridization analysis with the inserts in plasmid clones and the Km^r marker as probes showed that the clones apparently complementing ExpI mutants did not correspond to the mutants, suggesting that these clones were suppressing rather than complementing the mutant phenotype. In contrast, ExpII clones (e.g., pTK806; Fig. 4) showed true complementation in restoring both the production and secretion of exoenzymes (Table 4) as well as virulence to mutant SCC3065 (Fig. 2).

DISCUSSION

The use of random transposon mutagenesis to identify pathogenicity determinants of *E. c.* subsp. *carotovora* requires the screening of large numbers of transposon-induced mutants for virulence. To facilitate this screening work, a rapid and reproducible small-scale pathogenicity test was a necessity. We have shown earlier that *N. tabacum* is susceptible to the *E. c.* subsp. *carotovora* strain SCC3193 and can be utilized as a test plant in virulence assays (Pirhonen *et al.* 1988). Axenic tobacco seedlings propagated on tissue culture plates were considered as the best alterna-

Table 3. Cellular localization of pectic enzymes in Exp⁻ and Out⁻ mutants

Strain	Phenotype	Fraction	Enzyme activity[a]		
			Pel	Peh	Bla
SCC3193(pUC18)	Wild-type	Sn	89	87	7
		Pf	6	8	69
		Cl	5	5	24
SCC3060(pUC18)	ExpI⁻	Sn	6	15	10
		Pf	64	60	67
		Cl	30	25	23
SCC3065(pUC18)	ExpII⁻	Sn	5	11	11
		Pf	71	69	73
		Cl	24	20	16
SCC3125(pUC18)	Out⁻	Sn	13	27	14
		Pf	74	44	71
		Cl	13	29	15

[a] Percentage of pectate lyase (Pel), polygalacturonase (Peh), and β-lactamase (Bla) activities found in culture supernatant (Sn), periplasmic fraction (Pf), and cell lysate (Cl). Pel and Peh assays were as given in Table 2, and the Bla assay was as defined in O'Callaghan (1972).

tive for virulence screening of a large number of mutants. Comparison of the results obtained by the tobacco virulence assay with those from stem-inoculated potato suggested that the tobacco assay gave a good estimate of the virulence of *E. c.* subsp. *carotovora* and the mutants isolated.

Transposon mutagenesis with a modified Tn*10* element proved efficient in generating mutants in strain SCC3193. Isolation of several different types of mutants affected in virulence suggested that the target site preference of Tn*10* (Kleckner *et al.* 1979) did not hamper this analysis.

Bacteriophage T4 provides a useful tool for genetic analysis of *E. c.* subsp. *carotovora*. T4 is an effective selec-

tive agent for LPS-defective mutants of *E. c.* subsp. *carotovora* and *E. c.* subsp. *atroseptica* (Pirhonen *et al.* 1988). Isolation of bacteriophage T4-resistant, LPS-defective insertion mutants that were fully virulent confirmed our earlier results (Pirhonen *et al.* 1988) that LPS is not a pathogenicity factor in *E. c.* subsp. *carotovora*. More importantly, the transducing derivative of T4, T4GT7 (Wilson *et al.* 1979), can be used to transduce plasmids from *E. coli* to *E. c.* subsp. *carotovora* (Pirhonen and Palva 1988) and, as we show here, also to transduce plasmids and chromosomal markers between different *E. c.* subsp. *carotovora* strains. The transduction frequencies are sufficient (10^{-4}) to allow the use of T4GT7 as a generalized transducing phage in *E. c.* subsp. *carotovora*.

The transposon-induced *E. c.* subsp. *carotovora* mutants isolated after virulence screening fell into two broad categories: motility mutants that exhibited reduced virulence and avirulent strains having defects in the production of exoenzymes and showing reduced growth on pectin. The first group was by far the largest (268 of 298 mutants affected in virulence) and included nonmotile (Mot⁻) isolates as well as strains with reduced motility. The motility mutants were unaffected in every other physiological parameter tested, except virulence. Motility has been shown to affect the virulence of *E. amylovora*, *P. phaseolicola*, and *P. syringae*, but only when the inoculum is applied externally (Bayot and Ries 1986; Panopoulos and Schroth 1974; Hattermann and Ries 1989). In contrast, our *E. c.* subsp. *carotovora* Mot⁻ mutants showed reduced spread of the infection also in stem-inoculated potato plants. Similar results have also been obtained with wound-inoculated tobacco plants (data not shown). These results suggest that the bacterial motility is essential not only during the onset of infection but also for the rapid spreading of *E. c.* subsp. *carotovora* inside the plant tissue.

The second category of mutants, avirulent strains impaired in the production of enzymes, could be further divided into three classes. The first one includes three strains similar to previously characterized *E. c.* subsp. *carotovora* and *E. chrysanthemi* Out⁻ mutants that are defective in secretion of pectic enzymes and cellulases (Chatterjee *et al.* 1985; Andro *et al.* 1984).

The second class of avirulent mutants showed a pleiotropic phenotype having defects in the production of several exoenzymes. These Exp⁻ mutants synthesized reduced levels of pectic enzymes and negligible amounts of Cel and Prt. Furthermore, the residual Pel and Peh activities were mainly periplasmic (SCC3060, SCC3065; Tables 2 and 3).
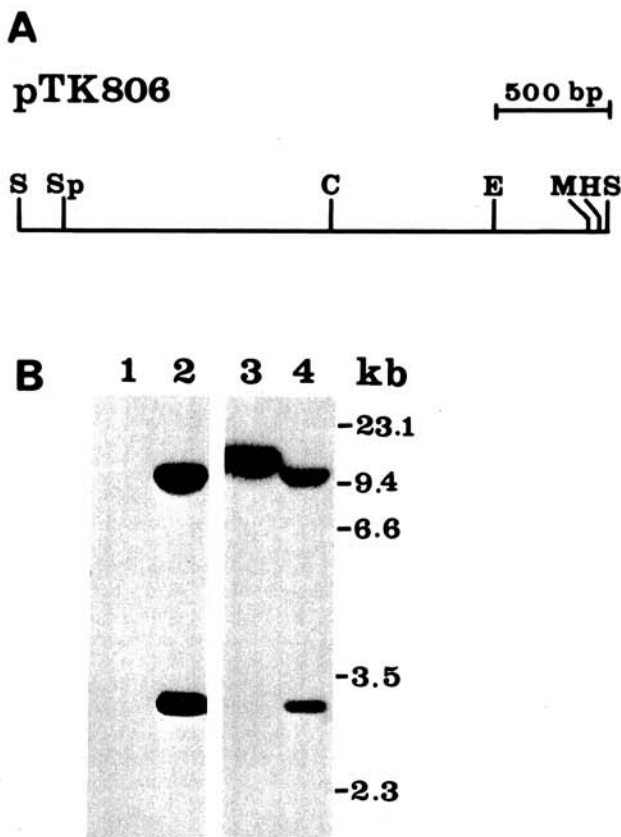
A

pTK806

500 bp

B

Fig. 4. A, Restriction map of the insert in the clone pTK806 complementing ExpII⁻ mutants. The symbols C, E, H, M, S, and Sp correspond to *Cla*I, *Eco*RI, *Hin*cII, *Mlu*I, *Sau*3A, and *Sph*I, respectively. Only the terminal *Sau*3A sites used in the cloning are indicated. **B,** Southern analysis of the ExpII⁻ mutant SCC3065. Chromosomal DNA from SCC3193 (lanes 1 and 3) and SCC3065 (lanes 2 and 4) was digested with *Hin*dIII and *Xho*I and separated in 0.8% agarose gel before transfer and hybridization. DNA in lanes 1 and 2 was probed with the kanamycin resistance gene from Tn*903*, and in lanes 3 and 4 with the chromosomal insert of pTK806.

Table 4. Complementation of ExpII⁻ mutant SCC3065 by the Exp⁺ clone pTK806

	Enzyme activity[a]							
	Pel		Peh		Cel		Prt	
Strain	Total	Sn	Total	Sn	Total	Sn	Total	Sn
SCC3193(pUC18)	0.8	84	12.0	80	0.72	92	0.61	94
SCC3065(pUC18)	0.23	10	4.1	1	0.08	13	0.04	2
SCC3065(pTK806)	0.44	62	16.6	58	0.30	84	0.47	87

[a] Pectate lyase (Pel), polygalacturonase (Peh), cellulase (Cel), and protease (Prt) activities were assayed as given in Table 2. Total refers to the total enzyme activity expressed as units per milliliter of culture; Sn refers to the percentage of total activity found in the culture supernatant.

The molecular basis for the Exp⁻ phenotype is not known. It is possible that there is a regulatory system that controls the production of several classes of exoenzymes as well as their secretion. An alternative explanation for this phenotype is that the reduced production of enzymes is a secondary effect caused by defective export, possibly at an earlier stage as in Out⁻ mutants. Isolation of a plasmid clone pTK806 that complemented the ExpII⁻ mutant SCC3065 but not ExpI⁻ mutants suggests that the Exp⁻ mutants can be divided into at least two complementation groups. The ExpII clone should be useful in clarifying the molecular basis of the Exp⁻ phenotype.

The Exp⁻ phenotype resembles that of the Out⁻ mutants in that the exoenzymes are retained in the periplasm. However, in Out⁻ mutants, enzyme production is not affected; this shows that Out⁻ strains are clearly distinct from Exp⁻ mutants. Hinton *et al.* (1989) have isolated reduced virulence mutants of *E. c.* subsp. *atroseptica* that are affected in secretion and synthesis of pectic enzymes, but in these Pep⁻ strains the Prt activity remained unaffected, which differentiates them from the Exp⁻ phenotype. Hinton and Salmond (1987) and Ellard *et al.* (1989) reported Prt⁻ and Cel⁻ mutants of *E. c.* subsp. *carotovora*, but more detailed analysis of enzyme activities, localization, and virulence would be needed to establish whether these mutants are identical with Exp⁻.

The three mutants included in the third class (Pnl⁻) were particularly interesting because they are avirulent but are apparently unaffected in the production and secretion of Pel and Peh. When Pnl activities of these strains were assayed from mitomycin C-induced cultures, they were found to produce reduced levels of Pnl. This suggests that the reduced growth on pectin observed in these mutants could be caused by a decrease in Pnl activity. Similar growth reduction seen in Exp⁻ and Out⁻ mutants could also be a consequence of a defect in production or secretion of Pnl.

The range of phenotypes among the mutants demonstrates the importance of motility and efficient enzyme production and secretion as determinants of virulence and pathogenicity in *E. c.* subsp. *carotovora*. Our results, which show that all the 26 completely avirulent mutants obtained were impaired in their enzyme production and growth on pectin, suggest that the ability to degrade and catabolize plant cell wall pectin is a crucial feature for the pathogenicity of *E. c.* subsp. *carotovora*. This is in accordance with the demonstrated importance of the pectolytic enzymes in soft rot (Collmer and Keen 1986).

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