The Major Secreted Cellulase, CelV, of *Erwinia carotovora* subsp. *carotovora* Is an Important Soft Rot Virulence Factor

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CelV is the major secreted β-1,4-endoglucanase of the phytopathogen *Erwinia carotovora* subsp. *carotovora*. Its role in virulence was investigated by comparing the ability of a cellulase-deficient mutant to cause soft rot of potato tubers with that of the wild type. A significant role was demonstrated despite the inability of CelV to effect soft rot when expressed in *Escherichia coli*. This established the major cellulase of *E. carotovora* subsp. *carotovora* as a virulence factor and provided evidence for a synergistic relationship between the various classes of exoenzyme in attack of potato tubers.

The soft rot erwinias are enterobacterial phytopathogens that exert their effect via maceration of host tissue. *Erwinia carotovora* subsp. *carotovora* causes soft rot in a range of economically important crops—potatoes and carrots in particular (Perombelon and Kelman 1980). The major pathogenicity determinants are an arsenal of extracellular pectinases (including several pectate lyase isozymes, pectin lyase, pectin methylesterase, and polygalacturonase), which are secreted. In addition, a range of other degradative enzymes are secreted, such as cellulase and protease, but their role in virulence is equivocal. The exoenzymes are secreted into the external medium by two distinct pathways: one-step and two-step (reviewed in Salmond and Reeves 1993; Wandersman 1992; Pugsley 1993). Protease is secreted by a one-step, Sec-independent pathway, functionally similar to that of hemolysin secretion in *Escherichia coli*. Cellulase and pectinases are secreted by a two-step mechanism. Passage across the inner membrane is Sec-dependent, whereas translocation across the outer membrane is effected by proteins encoded by the *out* cluster (Reeves *et al.* 1993). Out mutants, therefore, are severely deficient in their ability to macerate potato tubers because of their inability to translocate pectinases (and perhaps cellulases) across the outer membrane into the surrounding host tissue.

The present study concerns the cellulase CelV, a β-1,4-endoglucanase that is responsible for at least 95% of the detectable carboxymethyl cellulase (CMCase) activity of *E. carotovora* subsp. *carotovora*. The *celV* gene has been cloned and sequenced (Cooper and Salmond 1993). In common with most other cellulases, the protein consists of several functionally distinct domains. At the amino terminus there is a classical signal sequence, followed by a large catalytic domain that shows homology to catalytic domains of family A (Gilkes *et al.* 1991), including that of endoglucanase Z of *E. chrysanthemi* (Guiseppi *et al.* 1988). The carboxy-terminal domain shows homology to the cellulose-binding domains of several *Bacillus* cellulases as well as that of CelZ of *Clostridium stercorarium,* for which cellulose binding has been demonstrated (Jauris *et al.* 1990), and there is evidence for such a function in CelV (Cooper and Salmond 1993). The two functional domains are connected by a linker of extended structure, rich in threonine and proline, which is a common feature of cellulases.

Pectate lyases have been demonstrated to be important determinants of the ability of *Erwinia* species to cause soft rot (Hinton *et al.* 1989; Collmer and Keen 1986). Indeed, expression of pectate lyases by *E. coli* (Zink and Chatterjee 1985; Keen and Tamaki 1986) or inoculation with acellular enzyme preparations (Barras *et al.* 1987) has been demonstrated to result in significant maceration of potato tubers. It therefore seems to be the consensus view that the other classes of secreted macerating enzymes, such as proteases and cellulases, play no role or only a very small role in virulence; and it is perhaps for this reason that such a large proportion of work on *Erwinia* focuses on pectate lyases.

Cellulases and proteases have been cloned from various phytopathogenic bacteria in addition to *Erwinia*, such as *Xanthomonas* and *Pseudomonas*. In *X. campestris* pv. *campestris*, both cellulase (Gough *et al.* 1988) and protease (Dow *et al.* 1990) have been demonstrated to exert slight effects on virulence, and there is good evidence that the major extracellular endoglucanase of *Pseudomonas solanacearum* plays a significant role in pathogenesis of tomatoes (Roberts *et al.* 1988). The roles of proteases and cellulases in pathogenicity of *E. chrysanthemi* and *E. carotovora*, however, have not been extensively investigated. Dahler *et al.* (1990) showed that mutants deficient in one or all of the proteases of *E. chrysanthemi* were not impaired in virulence, as determined by potato disk assays or inoculation of chrysanthemum plant stems. Conversely, Aymeric *et al.* (1989) carried out studies on a marker exchange mutant that appeared to show that the absence of endoglucanse Z of *E. chrysanthemi* had a profound effect on virulence of the pathogen on *Saintpaulia* plants. However, it appears that the mutant used in this study is also

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deficient in its ability to secrete cellulase and pectate lyases (Py *et al.* 1993; F. Barras, personal communication), indicating a secondary mutation in the *out* cluster that would itself have a profound effect on virulence.

The aim of this study, then, was to investigate the effect of cellulase deficiency on the ability of *E. carotovora* subsp. *carotovora* to cause soft rot, in order to determine unequivocally whether or not this enzyme is a significant virulence factor.

RESULTS

Isolation and characterization of a cellulase mutant.

Strain SCRI193 was mutagenized with methane sulfonic acid ethyl ester (ethylmethylsulfonate, EMS), to give a 3.5% survival rate. Survivors were screened on cellulase, pectinase, and protease assay plates for exoenzyme defects. This resulted in a very low occurrence of cellulase minus mutants (0.05%, compared with 2.4% auxotrophs) (Reeves 1992). A mutant, GS7000, was isolated that exhibited a complete loss of ability to degrade carboxymethyl cellulose (CMC). Figure 1 shows the results of cellulase activity assays of cytoplasmic, periplasmic, and culture supernatant fractions from GS7000 compared with the wild type. The results indicate that this Cel$^-$ phenotype is not due to cellular accumulation of cellulase activity and that other extracellular enzymes (pectate lyase, protease) are synthesized and secreted as normal (data not shown). Figure 1 also shows that the cellulase deficiency of GS7000 is complemented by expression of *celV* on a vector of copy number less than five per chromosome (pDEE339; Table 1). The figure shows how this small increase in copy number results in a slight decrease in the efficiency with which CelV is secreted. As previously reported (Py *et al.* 1991b), overexpression of cellulase by *E. carotovora* subsp. *carotovora* results in some accumulation in the periplasm, presumably as a result of overloading of the secretory apparatus. Even a copy number of about five has a very slight overloading effect.

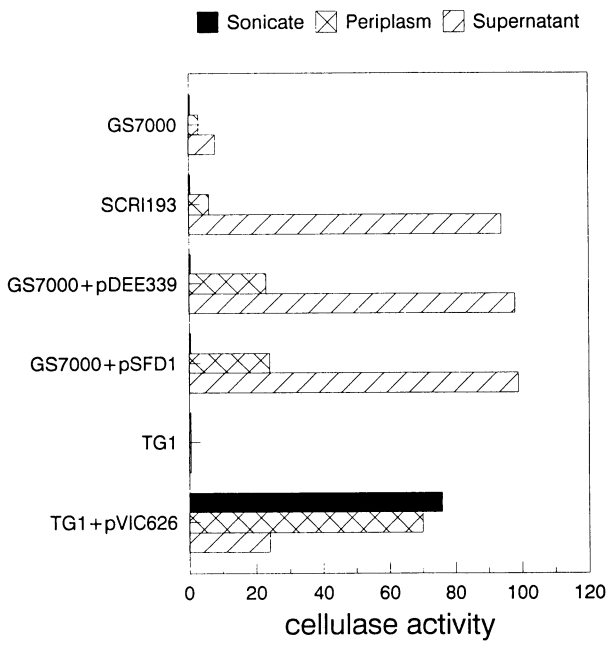

Fig. 1. Localization of cellulase activity for strains used in this study. The graph shows comparison of wild-type SCRI193 with the Cel$^-$ mutant GS7000, complemented derivatives of GS7000, and *Escherichia coli* TG1 with and without pVIC626, a pBR322 derivative carrying *celV*. Mid-stationary phase cultures were fractionated into supernatant, periplasm, and cytoplasm fractions and then assayed for cellulase activity as described in Materials and Methods. Activity is expressed in arbitrary units based on a standard curve constructed using serial dilutions of SCRI193 whole culture sonicates, so that total SCRI193 activity is 100.

Ideally, investigation of the role of cellulase in virulence should be carried out with a marker exchange mutant, in which *celV* is interrupted, so the precise nature of the mutation responsible for the cellulase-deficient phenotype is known. However, marker exchange at the *celV* locus in strain SCRI193 has proved difficult, so we chose instead to investigate the role of CelV in *E. carotovora* subsp. *carotovora* virulence with GS7000. Efforts were made to ensure that the mutation responsible for the observed phenotype resided in the *celV* locus and affected only the synthesis or activity of cellulase.

The nature of GS7000 was investigated by complementation using a pSF6-based cosmid library of SCRI193 DNA packaged into phage lambda. Screening of 1,000 transductants on cellulose assay plates resulted in the isolation of nine clones exhibiting restoration of the Cel$^+$ phenotype, and cosmids (pSFD1-pSFD9) isolated from these were prepared, analyzed by agarose gel electrophoresis, and introduced into *E. coli* TG1 by transformation. All nine of the cosmids isolated encoded cellulase activity in *E. coli* and had several restriction fragments in common with cosmids characterized during the cloning of *celV* (data not shown; V. Cooper, personal communication) indicating the presence of the struc-

Table 1. Strains and plasmids used in this study

Strains/plasmids	Relevant information	Reference/source
Erwinia carotovora subsp. *carotovora*		
SCR1193	Wild type	Forbes and Perombelon (1985)
GS7000	SCR1193-derived Cel$^-$	This study
Escherichia coli		
TG1	*supE, thi, Δ(lac-pro), ecoK, hsd5/F', traD36, proA$^+$B$^+$, lacIq, lacZ*ΔM15	Sambrook *et al.* (1989)
K38	K12, HfrC	Tabor and Richardson (1985)
Plasmids		
pLG339	*ori* SC101, kanamycin resistance, tetracycline resistance	Stoker *et al.* (1982)
pDEE339	*Sph*I/*Eco*RI 2-kb fragment carrying *celV* cloned into the same sites of pLG339	This study
pSFD1-9	Cosmids isolated from a pSF6-based library of SCRI193 DNA, all able to complement GS7000	This study
pVic626	*Sph*I/*Eco*RI 2-kb fragment carrying *celV* cloned into *Eco*RI site of pBR322	Cooper and Salmond (1993)
pT7-6	Ampicillin resistance, T7 g 10p, *ori*pMB1	Tabor and Richardson (1985)
pGP1-2	Kanamycin resistance, *ori*P15A, *cI857, pL*-T7 gene 5	Tabor and Richardson (1985)

tural gene. This provides indirect evidence that GS7000 can only be complemented by the structural gene and therefore strongly suggests that the mutation responsible maps to the *celV* locus.

The question as to whether the phenotype of GS7000 results solely from a mutation within *celV* was investigated by transduction analysis. A derivative of SCRI193 containing transposon Tn*5* linked within about 10 kilobases (based on cotransduction frequencies) to *celV* was used as the donor strain. A lysate of this strain was prepared with the generalized transducing phage ΦKP (Toth *et al. 1993*), and the resulting lysate was used to transduce GS7000. Of the resultant kanamycin-resistant transductants, 62% were Cel^+ and the remainder Cel^- when picked onto CMC assay plates. The phenotypes of the Cel^+ and Cel^- transductants were exactly those of SCRI193 and GS7000, respectively, with regard to synthesis and secretion of cellulase, pectate lyase, and protease (data not shown). This supports the complementation data, providing evidence that the mutation responsible is in the region of the *celV* locus. The clean reversion of the phenotype in all respects in Cel^+ transductants provides reasonable evidence against the existence of a second site mutation (unless it is very close to the *celV* locus and affects only cellulase) or a regulatory mutant with pleiotropic effects.

Western analysis of GS7000 and complemented derivatives.

As Figure 2 shows, Western analysis of GS7000 with polyclonal antibodies against CelV failed to identify either a full-size product or a degradation or truncation derivative that was recognized by the polyclonals used. On the other hand, when GS7000 was complemented by either one of the pSF6-based cosmids, or by pDEE339, CelV was synthesized and secreted to the same extent as in the wild-type strain SCRI193 (although there was a slight increase in synthesis and therefore a very slight decrease in efficiency of secretion).

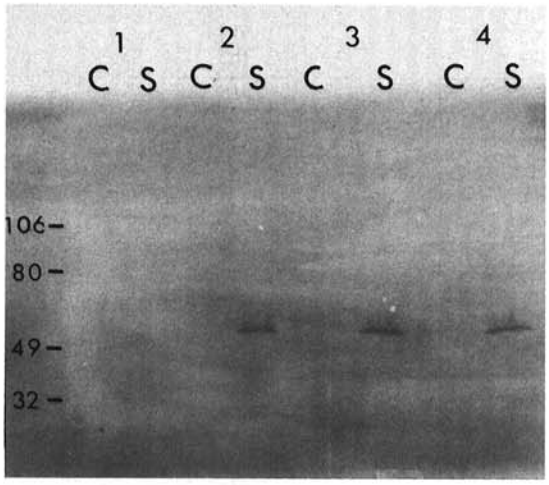

Fig. 2. Western analysis using polyclonal antibodies against CelV. The gel shows comparison of SCRI193 and GS7000, together with complemented derivatives of GS7000. 1 = GS7000; 2 = SCRI193; 3 = GS7000 + pDEE339; 4 = GS7000 + pSFD1. Mid-stationary phase cultures were fractionated into supernatant and cell sonicate, then precipitated with trichloroacetic acid and used in Western analysis, as described in Materials and Methods. S = supernatant; C = cell sonicate (i.e., cytoplasm + periplasm). Positions of standards are shown on the left in kilodaltons.

The role of cellulase in virulence.

The importance of CelV in soft rot was assessed by comparing the ability of GS7000 and the wild-type SCRI193 to macerate potato tubers. Tubers were inoculated with cell suspensions in stab holes; the stab sites were sealed to increase anaerobiosis, and the potatoes were incubated wrapped in alternate layers of wet paper towel and plastic cling wrap to increase humidity. The conditions therefore mimicked those under which *Erwinia* species attack potatoes in storage, i.e., decreased oxygen tension and high humidity.

Figures 3 and 4 show the significant difference between the abilities of GS7000 and SCRI193 to macerate new potato tissue of cv. Cyprus. This deficiency is restored by complementation with pDEE339 and with pSFD1, although in some cases (particularly a lower inoculum concentration of GS7000 + pSFD1) the restoration is not completely to wild-type levels. This is despite the observation from Fig. 1 that cellulase activity is restored to wild-type levels and localization when GS7000 is complemented with either pDEE339 or pSFD1. The most likely explanation is that the lack of antibiotic selection during growth in potato tissue results in significant plasmid loss, thereby reducing the levels of cellulase activity compared with those seen in liquid culture. However, it remains clear that there is a significant difference in virulence between GS7000 and either of the complemented strains.

We further investigated the possibility that lack of complete complementation of virulence was due to plasmid loss in the absence of selection. Samples of macerated tissue from tubers inoculated with GS7000 + pDEE339 and GS7000 + pSFD1 and incubated for 2 days were serially diluted in LB and spread onto LB plates in the absence of selection. After incubation for 18 hr, isolated colonies replicated on relevant selective media were scored for antibiotic resistance, the rate of survival indicating the frequency of plasmid loss. Approximately 13% of the resulting colonies had lost pDEE339 after 2 days' growth, while 47% had lost pSFD1 following the same treatment. This provides good evidence that incomplete complementation of the virulence deficiency of GS7000 is at least partly due to plasmid instability in the absence of antibiotic selection.

By comparison, *E. coli* TG1 did not exhibit any degradative ability, even when it expressed CelV at a relatively high level. The amount of rotted tissue resulting from inoculation with TG1 either with or without the *celV* gene was no different from that resulting when a stab hole was not inoculated. Comparison with the graph of cellulolytic activity (Fig. 1) shows that this inability to rot occurs despite the high levels of activity measured in TG1 + pVIC626. This effect seems unlikely to be due to the inability of *E. coli* to secrete CelV, because as Figure 1 indicates, high-level expression of CelV in TG1 results in the appearance of some cellulase activity in the supernatant sample, as a result of leakage into the external milieu.

Tubers were inoculated with two different densities of cells, as described in Materials and Methods. Comparison of Figure 3A and B indicates that the general effects were the same regardless of the inoculum used. In both cases, the role of cellulase is clear; GS7000 was well complemented by both plasmids used, and *E. coli* failed to cause significant maceration. Interestingly, inoculation with lower cell numbers, although initially causing very little rot, by day 4 actually

caused a greater level of maceration than resulted from the higher inoculum concentration.

DISCUSSION

Our results show that the CelV⁻ mutant GS7000 of *E. carotovora* subsp. *carotovora* has a significantly reduced ability to macerate potato tissue compared with the wild type, SCRI193. GS7000 has a growth rate equal to SCRI193 (data not shown), makes wild-type levels of other extracellular enzymes, and is deficient only in the production of cellulase. This establishes that cellulase must contribute to the ability of *E. carotovora* subsp. *carotovora* to macerate potato tuber.

In contrast, there is no evidence for even the slightest ability of *E. coli* to macerate tuber tissue when expressing CelV at a high level. These results contrast with those found for the pectolytic enzymes expressed in *E. coli,* as discussed in the introduction. Although unable to independently macerate host tissue, cellulase nevertheless seems important in the collaborative action of the range of secreted degradative enzymes.

There appears to be a synergistic relationship between the various classes of exoenzyme in bacterial soft rot pathogenesis, which makes sense when one considers the complexity of the plant tissue being attacked. A pathogen might be able to degrade a particular host tissue component efficiently, but without the ability to degrade the other major components, a scaffold of pathogen-resistant host tissue structures would remain, severely inhibiting further invasion. Given the abundance of cellulose and its structurally robust nature, it is clear that secretion of an arsenal of powerful pectinases, even combined with other degradative enzymes, would not be sufficient for efficient invasion if a cellulose scaffold remained to stifle further penetration by the pathogen through host tissue. Thus cellulase may well act in concert with the pectinases to assist ingression.

The results indicated that inoculation with fewer cells in-

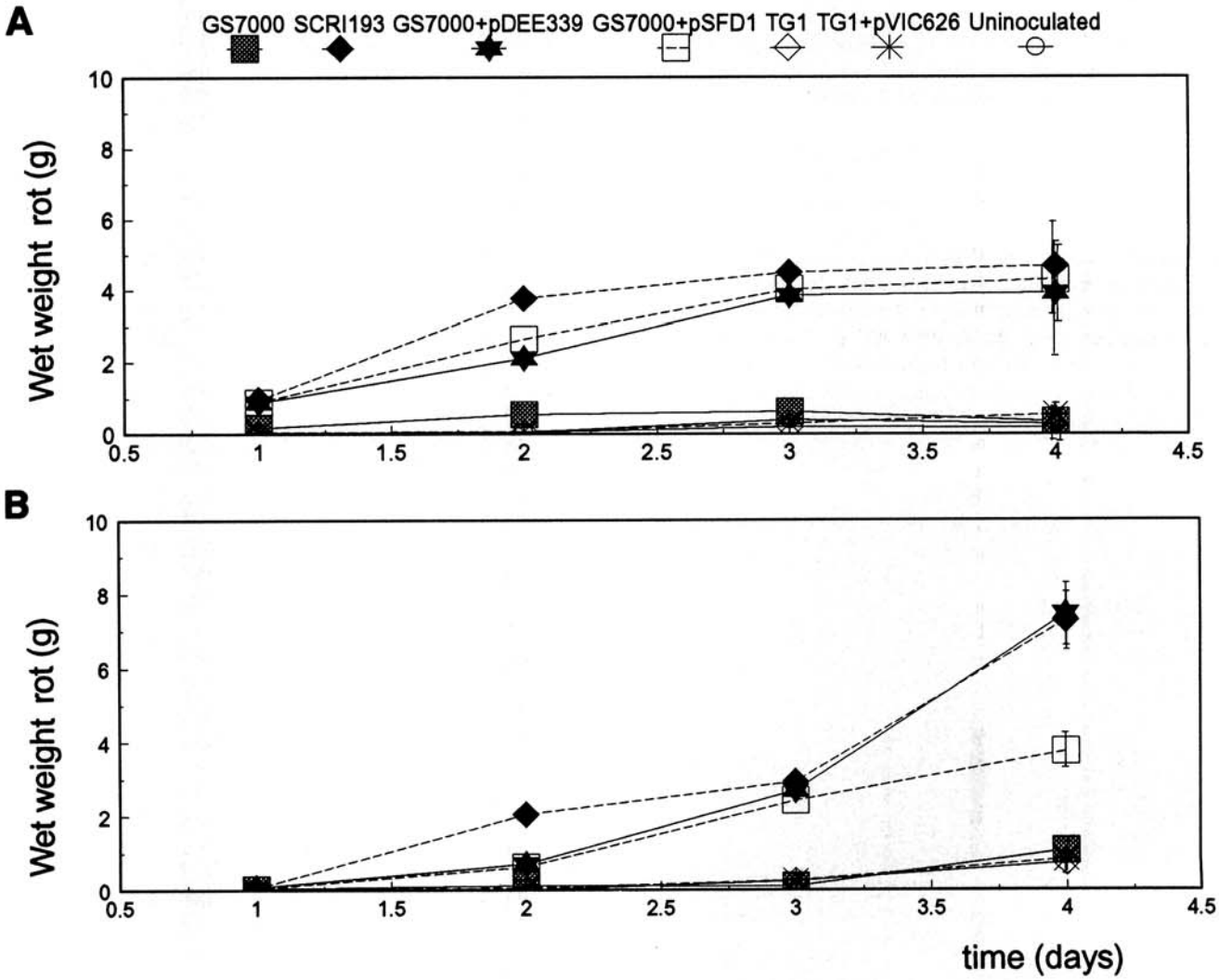

Fig. 3. Virulence assays on potato tubers. Cyprus new potatoes were inoculated with liquid cultures and incubated for up to 4 days, at which time macerated tissue was removed and weighed. Results shown are means for six replicas of each strain, for each time point and each inoculum size. **A,** Results for inoculation with approximately 10^7 colony-forming units (CFU); **B,** inoculation with approximately 10^3 CFU. For simplicity, 95% confidence bars are given above the relevant key only for the final time point. Similar effects were seen for other new potato cultivars; Cara baking; and Romano red, although average rot varied considerably.

itially showed little maceration activity but resulted in a greater level of maceration by day 4. This unexpected effect could result from several factors. For example, the richness of LB compared with potato tissue could mean that a low cell-to-medium ratio is a considerable advantage in terms of growth potential, resulting in higher cell numbers and greater levels of maceration. Alternatively, the high initial levels of maceration caused by more inoculum could initiate a plant response that slows further invasion, and the lower-density inoculum might be able to invade further into the host tissue, attaining higher cell numbers before such a response could be initiated.

MATERIALS AND METHODS

Culture media.

Erwinia species and *E. coli* were grown at 30 and 37° C, respectively, in LB (Miller 1972) or nutrient broth (when using spectinomycin) and on corresponding solid plates (15 g/L of agar). Antibiotics were added to give a final concentration of 50 µg/ml.

Recombinant DNA techniques were performed as described by Sambrook *et al.* 1989.

EMS mutagenesis was as described by Reeves *et al.* 1993.

Enzymatic activity assays.

The cellulolytic activity of colonies was assessed on CMC agar plates (Andro *et al.* 1984). Activity in cellular fractions was assessed with Ostazin brilliant red cellulose (Sigma, Poole, U.K.) (Biely *et al.* 1985), and using well assays on CMC agar plates, by comparing halo diameters with a standard curve constructed from serial dilutions of SCRI193 whole culture sonicate. Pectate lyase and protease activity of colonies was assessed on agar-based plates (Andro *et al.* 1984; Reeves *et al.* 1993).

Polyclonal antibodies against CelV.

For preparation of antigen, *celV* was overexpressed using the T7 g 10p system of Tabor and Richardson (1985). *celV* was cloned into pT7-6 , and the resulting plasmid was introduced into K38 already containing pGP1-2. High-level exclusive production of CelV was induced at 42° C using the method described by Tabor and Richardson (1985), and a periplasmic fraction was taken by spheroplasting (Sambrook *et al.* 1989). The band corresponding to CelV was cut from a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, which had been loaded with periplasmic fractions that were concentrated using a Centriprep 30 (Amicon, Stonehouse, U.K.), and stained with 0.1 M KCl. The gel band was dried, homogenized in minimum phosphate buffered saline, and used to raise polyclonal antibodies from a rabbit as described by Ausubel *et al.* (1987). The resulting serum was affinity-purified to eliminate nonspecific binding as described by Sambrook *et al.* (1989).

Fig. 4. Example of potato soft rot in a tuber used in the virulence assays represented in Figure 3, incubated for 2 days following inoculation with undiluted cell suspensions. The potato is sliced through the center of inoculation sites and macerated tissue is removed for the assay.

Cell fractionation.

Strains were grown in LB to mid-stationary phase. For Western analysis, supernatant and cell sonicate samples were prepared (Hinton and Salmond 1987). For enzyme assays, supernatant fractions were prepared in the same way. Periplasmic fractions were prepared by spheroplasting (Sambrook *et al.* 1989), and after washing and resuspending the spheroplasts, the cytoplasmic fraction was prepared by sonicating in the same way.

Western analysis.

Samples were concentrated with trichloroacetic acid (Py *et al.* 1993), resuspended in 10% of the volume of water, and incubated for 10 min with a half volume of TSTD (Py *et al.* 1991a). SDS-PAGE was carried out using the Phast system (Pharmacia, St. Albans, U.K.), on a 10–15% gradient gel. Proteins were transferred to nitrocellulose filter (Hybond; Amersham, Little Chalfont, U.K.), using the Pharmacia Phast Transfer apparatus, as described by the manufacturer. The nitrocellulose filter was incubated with anti-CelV polyclonal antiserum, then with alkaline phoshatase-conjugated anti-rabbit antibodies (Promega, Southampton, U.K.), and alkaline phosphatase activity was detected using 5-bromo-4-chloro-3-indolyl phosphate, and nitroblue tetrozolium (Sigma).

Cosmid complementation.

pHCP2, a pBR322-based plasmid that carries *E. coli* LamB (Salmond *et al.* 1986), was introduced into GS7000 by electroporation (Py *et al.* 1991b), rendering the strain susceptible to infection by lambda. A pSF6 library of SCRI193 chromosomal DNA in the form of a phage lambda lysate was then used to infect GS7000, as described by Reeves *et al.* 1993, and transductants were selected with NBA spectinomycin plates. Minipreparations of cosmid DNA were done using a Magic Miniprep kit (Promega), and transformation of *E. coli* was as described by Chung and Miller (1988).

Transduction analysis.

SCRI193 was electroporated with pHCP2, allowing infection but not replication by λ (Salmond *et al.* 1986). A high titer lysate of λ::Tn*5* was prepared (de Bruijne and Lupski 1984) and used to infect SCRI193 + pHCP2 (Ellard *et al.* 1989). Transductants were pooled and used to prepare a heterogeneous culture in LB and 10 mM $MgSO_4$, and this was infected with the generalized transducing phage ΦKP (Toth *et al.* 1993). To prepare a high-titer lysate, 200-µl aliquots of culture were incubated with serial dilutions of phage at room temperature for 10 min, added to 4 ml of LB, 10 mM $MgSO_4$, and 0.05% agar, and poured onto LB plates before incubation at 28° C for 16 hr. Plates showing confluent lysis were harvested by removing the top agar, rinsing the plate with 2 ml

of phage buffer (10 mM Tris, 10 mM $MgSO_4$, and 0.01% gelatin; pH 7.4). The mixture was vortexed with 0.5 ml of chloroform for 15 min, then centrifuged, and the supernatant was removed and stored over chloroform).

The lysate was used to transduce GS7000. An overnight culture grown in LB and $MgSO_4$ was incubated with approximately 10^8 plaque-forming units of lysate for 30 min at 26° C; the cells were washed in phage buffer and then incubated, with shaking, for 30 min at 30° C in LB before spreading onto appropriate plates and incubating for 48 hr at 30° C.

Any Cel^+ transductants were investigated further by infection with ΦKP to prepare a lysate and transduction of several Cel^- mutants, then scoring for the percentage of cotransduction of kanamycin resistance and cellulase activity. This resulted in the identification of strain DW102, which exhibited between 44 and 62% cotransduction, depending on the mutant transduced. Representative Cel^+ and Cel^- transductants were characterized for growth rate and exoenzyme production and secretion.

Virulence tests.

The strains to be assessed were grown to mid-stationary phase in LB. Potatoes were washed and surface-sterilized by soaking for 10 min in 5% hypochlorite solution, rinsed in tap water for 10 min, and air-dried. Sterile Gilson Pipetteman tips (0–200 µl size) were used to make uniform bores in the tubers, and these were inoculated with 10-µl samples of either an overnight culture (approximately 10^7 cells) or a 10^{-4} dilution of this (approximately 10^3 cells). The site of inoculation was sealed with high-vacuum silicone grease (BDH, Lutterworth, U.K.), and the tuber was wrapped in alternate layers of wet paper towel and plastic cling wrap, then incubated in a sealed plastic box at 25° C for 24, 48, 72, or 96 hr (adapted from Murata *et al.* 1990; Reeves 1992).

NOTE ADDED IN PROOF

While the present work was in press, M. Boccara and coworkers reported that, in contrast, cellulase does not appear to play a very significant role in the pathogenesis of *Saintpaulia ionantha* by *Erwinia chrysanthemi* (Boccara, M., Aymeric, J. L., and Camus, C. 1994. Role of endoglucanases in *Erwinia chrysanthemi* 3937 virulence on *Saintpaulia ionantha.* J. Bacteriol. 176:1524-1526.)

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