

Role of RpoS in virulence and stress tolerance of the plant pathogen *Erwinia carotovora* subsp. *carotovora*

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The plant-pathogenic bacterium *Erwinia carotovora* subsp. *carotovora* causes plant disease mainly through a number of extracellular plant-cell-wall-degrading enzymes. In this study, the ability of an *rpoS* mutant of the *Er. carotovora* subsp. *carotovora* strain SCC3193 to infect plants and withstand environmental stress was characterized. This mutant was found to be sensitive to osmotic and oxidative stresses *in vitro* and to be deficient in glycogen accumulation. The production of extracellular enzymes *in vitro* was similar in the mutant and in the wild-type strains. However, the *rpoS* mutant caused more severe symptoms than the wild-type strain on tobacco plants and also produced more extracellular enzymes *in planta*, but did not grow to higher cell density *in planta* compared to the wild-type strain. When tested on plants with reduced catalase activities, which show higher levels of reactive oxygen species, the *rpoS* mutant was found to cause lower symptom levels and to have impaired growth. In addition, the mutant was unable to compete with the wild-type strain *in planta* and *in vitro*. These results suggest that a functional *rpoS* gene is needed mainly for survival in a competitive environment and during stress conditions, and not for effective infection of plants.

Keywords: extracellular enzymes, pathogenicity, sigma factor, catalase, competition

INTRODUCTION

The phytopathogenic bacterium *Erwinia carotovora* subsp. *carotovora* causes soft rot on many economically important plants. The pathogen produces a number of plant-cell-wall-degrading enzymes, cellulases (Cel), pectate lyases (Pel), pectin lyase (Pnl), polygalacturonase (Peh) and proteases (Prt). The production and secretion of these enzymes is tightly regulated by several regulatory genes that have been identified and characterized in a number of studies (Andersson *et al.*, 1999; Chatterjee *et al.*, 1995; Cui *et al.*, 1995, 1996; Eriksson *et al.*, 1998; Frederick *et al.*, 1997; Harris *et al.*, 1998;

Jones *et al.*, 1993; Liu *et al.*, 1993, 1998, 1999; Mukherjee *et al.*, 1996; Murata *et al.*, 1991, 1994; Pirhonen *et al.*, 1991, 1993; Thomson *et al.*, 1997, 1999). These genes encode various types of regulators (both positive and negative) that act on transcription, transcript stability and secretion of virulence factors.

During recent years, the function of the alternative sigma factor RpoS (σ^{38}) has been extensively studied in *Escherichia coli* and other Gram-negative bacteria. RpoS is a regulator of stationary-phase-induced genes and is required for survival during stress and starvation (for reviews, see Hengge-Aronis, 1996; Loewen & Hengge-Aronis, 1994; Loewen *et al.*, 1998). Recently, the *rpoS* gene from *Er. carotovora* subsp. *carotovora* strain 71 was cloned and an *rpoS* mutant strain was constructed and characterized (Calcutt *et al.*, 1998; Mukherjee *et al.*, 1998). It was shown that RpoS is needed to withstand various environmental stresses *in vitro*, and that the *rpoS* mutant had increased maceration capability and produced increased amounts of extracellular enzymes *in*

Abbreviations: Amp, ampicillin; Cel, cellulase; Cm, chloramphenicol; EAI, *Erwinia* autoinducer; Km, kanamycin; Peh, polygalacturonase; Pel, pectate lyase; PGA, polygalacturonic acid; Pnl, pectin lyase; Prt, protease; SA, salicylic acid.

The GenBank accession number for the sequences determined in this work is AJ238884.

vitro. This effect was found to be mediated by lower expression of the negative regulator *rsmA* in the *rpoS* mutant (Mukherjee *et al.*, 1998). We have previously found that a mutant that overproduces RpoS due to a mutation in a gene designated *expM* has reduced virulence and is affected in extracellular enzyme production and secretion (Andersson *et al.*, 1999). The overproduction of RpoS was found to partly cause the phenotype of the *expM* mutant. The ExpM protein is a response regulator homologous to RssB/SprE in *Es. coli* and MviA in *Salmonella typhimurium*. These proteins belong to a new group of response regulators that are involved in the control of the stability of RpoS (Andersson *et al.*, 1999; Bearson *et al.*, 1996; Muffler *et al.*, 1996; Pratt & Silhavy, 1996). In this study we set out to characterize the phenotype of an *rpoS* mutant of *Er. carotovora* subsp. *carotovora* strain SCC3193, especially with regard to its virulence on tobacco plants. The mutant caused more severe symptoms than the wild-type strain on tobacco, presumably due to higher *in planta* production of extracellular enzymes, although it did not grow to higher cell densities on these plants. However, we show that the mutant was not able to infect tobacco with reduced catalase levels as efficiently as the wild-type strain, and that the growth of the mutant was impaired on these plants. Furthermore, we found that the *rpoS* mutant was outcompeted by the wild-type strain *in planta* and *in vitro*. As expected, we found that the *rpoS* mutant was more sensitive than the wild-type strain to osmotic and oxidative stress *in vitro*.

METHODS

Strains, plasmids, basic techniques and chemicals. The bacterial strains, plasmids and phages used in this study are listed in Table 1. *Er. carotovora* subsp. *carotovora* and *Es. coli* were grown in L medium at 28 °C and 37 °C, respectively. Plasmids were isolated with Qiagen plasmid purification kits according to the instructions given by the manufacturer. Transfer of plasmids to *Er. carotovora* subsp. *carotovora* was done by electroporation using a Bio-Rad gene pulser and transfer to *Es. coli* by electroporation or standard transformation techniques. Samples for Northern and Western blot analysis were collected from cells grown in L medium supplemented with 0.4% polygalacturonic acid (PGA; P-1879, Sigma). Assays for enzymes in *in vitro* cultures were performed on cells grown both with and without PGA. Ampicillin (Amp; 150 µg ml⁻¹) or kanamycin (Km; 25 µg ml⁻¹) were added when appropriate. Km was not used during the collection of samples for Northern blots and enzyme assays in order to avoid negative antibiotic effects. Transduction in *Er. carotovora* subsp. *carotovora* was performed as described by Pirhonen *et al.* (1991) using T4GT7. Transduction in *Es. coli* was performed with P1 using standard procedures (Miller, 1972). β-Galactosidase assays were performed essentially as described by Miller (1972) and plasmid pMMKatF₂ was used as a positive control in these experiments. The PCR reactions were performed with the proof-reading polymerase *Pfu* (Stratagene). Absorbance and optical density were measured with a DU-70 spectrophotometer from Beckman.

Construction of the *Er. carotovora* subsp. *carotovora* *rpoS* mutant strain. The alignment of nucleotide sequences from *Es. coli* and *S. typhimurium* *rpoS* genes enabled us to design an

oligonucleotide primer pair, RAPSIG (5'-AATTCGTTACA-AGGGGAAATCCGTA) and VIKSIGMA (5'-TTCATATC-GTCATCTTGGCGTGGTATCTT), which was used for PCR analysis of genomic *Er. carotovora* subsp. *carotovora* DNA. The PCR-generated product of predicted size, 870 bp, was subsequently cloned into pBluescript II to make pBSIG and checked by sequencing. Next, the Km^r interposon from pHP45Ω-Km (Fellay *et al.*, 1987) was inserted into the *Dra*II site of the internal region of *rpoS* in the plasmid pBSIG. To generate a homologous exchange mutant of the *Er. carotovora* subsp. *carotovora* *rpoS* gene, the suicide vector pUT mini-Tn5 Cm was used. pUT mini-Tn5 Cm carries the λpir-dependent R6K *ori*, which does not replicate in bacteria that lack λpir, such as *Er. carotovora* subsp. *carotovora* (de Lorenzo & Timmis, 1994). The mobile unit of pUT mini-Tn5 Cm was cut out with *Eco*RI and *Xba*I and replaced with the fragment carrying the *rpoS* gene interrupted by the Km^r gene from pBSIG digested with *Eco*RI/*Xba*I. This construction, pPUSIG, was mobilized by conjugation from *Es. coli* S17-1 λpir into *Er. carotovora* subsp. *carotovora* SCC3193 as described by de Lorenzo & Timmis (1994). Transconjugants were selected for the Km^r gene used for gene inactivation on minimal medium (M9) with glycerol (0.4%) and then tested for the loss of the vector marker (Amp). The potential marker-exchange mutants were verified by PCR using primers RAPSIG and VIKSIGMA. We transduced the mutated allele back to the wild-type strain SCC3193; all transductants were found to have the same phenotype on indicator plates for extracellular enzymes. We chose one of the transductants for further studies. The transductant was named SCC8002 and a Southern blot showed that it had a single Km^r insertion in *rpoS*. A Western blot using an *Es. coli* RpoS antiserum (Jishage & Ishihama, 1995) showed that SCC8002 did not produce any RpoS protein. The mutated *rpoS* allele from SCC8002 was then cloned by cutting chromosomal DNA from SCC8002 with *Sac*I/*Sac*II and ligating these fragments to pBluescript II. The ligation was transformed into *Es. coli* and the mutated allele was isolated by direct selection for the Km^r gene of the interposon. The DNA flanking the interposon was sequenced and the sequence obtained was used to generate oligonucleotides that were used to amplify the *rpoS* gene from the wild-type strain SCC3193. The resulting PCR fragment of about 1.5 kb was cloned into the *Sma*I site of the low-copy-number vector pACYC177 to generate pRA910.

Nucleotide sequencing. Nucleotide sequence of both strands of the *Er. carotovora* subsp. *carotovora* *rpoS* gene was determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) using customized synthetic primers (15- to 22-mers; GENSET OLIGOS) and Sequenase system (version II) from US Biochemicals with [³⁵S]dATP (Amersham Life Sciences). A BLAST search was used to compare the deduced amino acid sequence from the *Er. carotovora* subsp. *carotovora* *rpoS* gene and RpoS proteins from other bacteria.

Glycogen production, catalase assays and *Erwinia* auto-inducer (EAI) measurement. Accumulation of glycogen was tested by growing cells overnight on agar plates containing 1% glucose and then staining with iodine vapour (Latil-Damotte & Lares, 1977). Catalase activity was assayed by measuring hydrogen peroxide decomposition in a spectrophotometer at 240 nm (Beers & Sizer, 1952). The amount of EAI was determined by a bioassay; *Er. carotovora* subsp. *carotovora* strain SCC3065 (Pirhonen *et al.*, 1991, 1993), unable to produce EAI and harbouring the pHV200I^r plasmid, was mixed with various dilutions of supernatant from SCC3193 and SCC8002. Light production, and hence the concentration of EAI, was then measured in a luminometer.

Table 1. Strains, plasmids and phages used in this study

Strain/plasmid/phage	Relevant genotype/property	Reference/source
Strains		
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>		
SCC3193	Wild-type	Pirhonen <i>et al.</i> (1988)
SCC8002	SCC3193 <i>rpoS</i> Km ^r	This study
SCC3065	SCC3193 <i>expI</i> Km ^r	Pirhonen <i>et al.</i> (1993)
SCC3032	SCC3193 <i>expM</i> Km ^r	Andersson <i>et al.</i> (1999)
<i>Escherichia coli</i>		
DH5 α	Strain for cloning and plasmid preparations	Hanahan (1983)
MC4100	<i>rpoS</i> ⁺	Laboratory stock
FF1112	MC4100 ϕ (<i>otsB-lacZ</i>) (λ placMu55)	Giaever <i>et al.</i> (1988)
RH90	MC4100 <i>rpoS359::Tn10</i>	Lange & Hengge-Aronis (1991)
RA100	FF1112 <i>rpoS359::Tn10</i>	This study
Plasmids		
pBluescript II	Cloning vector, Amp ^r	Stratagene
pACYC177	Cloning vector, Amp ^r Km ^r	New England Biolabs
pPUSIG	<i>rpoS::</i> Ω Km Amp ^r	This study
pRA910	<i>rpoS</i> ⁺ (<i>Er. carotovora</i> subsp. <i>carotovora</i>), Amp ^r	This study
pMMKatF ₂	<i>rpoS</i> ⁺ (<i>Es. coli</i>), Amp ^r	Mulvey <i>et al.</i> (1988)
pUT mini-Tn5 Cm	Cm ^r Amp ^r R6K <i>ori</i>	de Lorenzo & Timmis (1994)
pHV200I ⁻	<i>luxI</i> Amp ^r	Pearson <i>et al.</i> (1994)
pHP45 Ω -Km	Km ^r interposon	Fellay <i>et al.</i> (1987)
Phage		
P1	Transducing phage	Laboratory stock
T4GT7	Transducing phage	Wilson <i>et al.</i> (1979)

Enzyme assays and virulence tests. The activities of Peh, Pel, Prt and Cel *in vitro* were assayed as described previously (Pirhonen *et al.*, 1991). The levels of Peh, Pel and Prt *in planta* were measured as follows. Twenty-four plants (*Nicotiana tabacum* cv. Xanthi) were infected for each strain (about 2×10^6 c.f.u. per plant). After 48 h incubation plants were pooled in groups of six and the fresh weight was determined. Plants were then homogenized in 1 ml water. Plant material and bacteria were removed by centrifugation and the extracellular enzyme activities were determined. The results are expressed as activity per gram fresh weight (units for Peh, $0.1 \mu\text{mol glucose equivalent min}^{-1}$; for Prt, increase in $A_{436} \text{ h}^{-1} (\text{ml sample})^{-1}$; and for Pel, A_{548}). As a control, the enzyme activities in uninfected plants were determined. Symptom development and bacterial growth *in planta* were determined in wild-type plants (*N. tabacum* cv. Xanthi) and antisense catalase (Ascat1 no. 17) plants (Takahashi *et al.*, 1997). The plants were grown for 4 weeks at 22 °C in a growth chamber [16 h light regime, $150 \mu\text{E s}^{-1} \text{ m}^{-2}$ (16.7 mlx)]. Plants were then infected and bacteria extracted essentially as described by Vidal *et al.* (1998) with the exception that $2\text{--}3 \times 10^6$ c.f.u. per plant (equal numbers for SCC3193 and SCC8002) were used to infect plants. After infection, plants were incubated for 48 h under the same conditions as above, but at 100% humidity to allow efficient infection. The results were analysed statistically by means of the Student's *t*-test for which *P*-values are reported. Symptom levels were determined according to the following scale: 0 = no symptoms, 1 = clear infection of one leaf, 2 = one leaf macerated, 3 = infection spread to more

than one leaf, 4 = several leaves macerated, 5 = only a few leaves uninfected. The mean symptom level was then calculated by adding the symptom levels of the infected plants and dividing by the number of plants used in each experiment. Salicylic acid (SA) treatment was done by adding SA to a final concentration of 1 mM to the plant growth medium 24 h before infection. When competition between SCC3193 and SCC8002 was studied *in planta*, 15 plants (*N. tabacum* cv. Xanthi) were infected with a mixture of equal numbers of both strains (a total of 2.1×10^6 c.f.u. per plant). After incubation for 48 h, bacteria were extracted and serial dilutions were plated on L medium with and without Km. The total c.f.u. and the c.f.u. on Km plates (SCC8002) were calculated as c.f.u. per infected plant. As a control, 30 plants were infected by SCC3193 and SCC8002 alone (2.8×10^6 and 2.0×10^6 c.f.u. per plant, respectively). Virulence tests on potato stems were performed on greenhouse-grown potato (*Solanum tuberosum* cv. Bintje), using a toothpick to infect the plants directly into the stem as described by McMillan *et al.* (1993).

RNA isolation, Northern and Western blot analysis. RNA for Northern blot analysis was isolated by the Qiagen RNA isolation kit. Samples were collected during 24 h of growth and Northern blots were performed as described previously (Andersson *et al.*, 1999). Western blots with *Es. coli* RpoS antisera (Jishage & Ishihama, 1995) were performed according to Andersson *et al.* (1999).

Stress experiments and competition *in vitro*. Stress exper-

iments during stationary phase were performed as follows (repeated at least twice). The cells were inoculated into 5 ml L medium and grown for 16 h to stationary phase. To test osmotic stress resistance, the cells were pelleted and resuspended in fresh L medium (5 ml) with or without the addition of 1 M NaCl. The cells were then grown for 24 h at 28 °C. To test oxidative stress resistance, 10 mM H₂O₂ was added directly to an overnight culture grown for 16 h and the cells were incubated for 4 h at 28 °C. The cells were plated and counted before and after the stress treatments. Complementation of the salt stress sensitivity of *Es. coli* strain RH90 was done essentially as above. The *Es. coli* strain MC4100 and the plasmid pMMKatF₂ were used as controls in these experiments. Competition experiments between SCC3193 and SCC8002 *in vitro* were done as follows. The strains were grown separately for 16 h. They were then mixed in equal numbers and plated on L medium with and without Km to determine the c.f.u. in the inoculum. The mixture was diluted ten times in 10 mM MgSO₄ and 10 µl of this dilution (around 3 × 10⁶ total c.f.u.) was used to inoculate 5 ml L medium. The culture tubes were then grown for 48 h and again plated on L medium with and without Km. As controls, the strains were inoculated separately using the same procedure. This experiment was done four times with similar results. For stress tests during exponential phase, an overnight culture was subcultured into 5 ml fresh L medium and grown to an OD₆₀₀ of 1.0. At this point, the cells were pelleted and resuspended in 10 mM MgSO₄ with or without the addition of 2.5 mM H₂O₂. The cells were then incubated for 10 min at room temperature and serial dilutions were plated. This experiment was done twice with essentially the same results.

RESULTS AND DISCUSSION

Cloning of the *rpoS* allele of strain SCC3193 and construction of an *rpoS* mutant

The *rpoS* gene encodes a subunit of the RNA polymerase which is involved in the control of a number of genes required for stress tolerance and survival during starvation (for reviews, see Hengge-Aronis, 1993, 1996; Loewen & Hengge-Aronis, 1994; Loewen *et al.*, 1998). To characterize the *rpoS* allele in the *Er. carotovora* subsp. *carotovora* strain SCC3193 and construct an *rpoS* mutant strain we first cloned a part of the *rpoS* gene by PCR. We constructed an *rpoS* mutant by marker exchange mutagenesis and then transduced the mutated allele to a clean background (SCC3193). One of the transductants, SCC8002, was chosen for further studies. A Southern blot analysis showed that SCC8002 had a single Ω-Km insertion in *rpoS*. We also performed a Western blot with antisera to the *Es. coli* RpoS protein and found that SCC8002 did not produce any RpoS protein (data not shown). The mutated *rpoS* allele from SCC8002 was then cloned and the DNA flanking the interposon was sequenced. Oligonucleotides were generated to amplify *rpoS* from the wild-type strain SCC3193 by PCR. The resulting fragment which contains 287 bp upstream of the potential start codon was cloned into the *Sma*I site of the low-copy-number vector pACYC177 to generate pRA910. The *rpoS* gene was sequenced and found to encode a polypeptide of 330 aa with 98.5% amino acid identity to the previously reported RpoS protein (Calcutt *et al.*, 1998) from *Er. carotovora* subsp.

carotovora strain 71 (data not shown). To investigate whether the *Er. carotovora* subsp. *carotovora* *rpoS* gene was expressed from pRA910 and whether the RpoS protein encoded by the plasmid was functional in *Es. coli*, we performed complementation experiments. For this purpose we constructed the *Es. coli* strain RA100 by P1 transduction of the *rpoS*359::Tn10 allele from RH90 to FF1112, which carries a chromosomal *otsB-lacZ* fusion. The *otsB* gene in *Es. coli* encodes trehalose-6-phosphate phosphatase, which is known to be regulated by RpoS (Kaasen *et al.*, 1992). We transformed FF1112 and RA100 with pRA910 and control plasmids and assayed the β-galactosidase activity during growth in L medium. The results showed that the β-galactosidase activity in RA100 carrying the empty vector pACYC177 was almost undetectable, whilst the introduction of pRA910 into RA100 activated the *otsB-lacZ* fusion (data not shown). We also tested the ability of pRA910 to complement the salt-stress-sensitive and the catalase- and glycogen-deficient phenotypes of the *Es. coli* *rpoS* mutant RH90. We found that pRA910 complemented all these phenotypes (data not shown). The *rpoS* gene encoded by pRA910 is driven by its own promoter but does not contain the major promoter region which was found between nt -561 and -525 upstream of the *rpoS* gene in strain 71 (Mukherjee *et al.*, 1998). However, it was found in this study that a weak promoter exists between nt -166 and +19. Since the DNA sequence in this region is almost perfectly conserved between the two strains, it is likely that this minor promoter region drives the expression of *rpoS* in pRA910. Similar to the results from strain 71 (Mukherjee *et al.*, 1998), we did not detect any *rpoS* transcript smaller than about 1600 bp by Northern blot using RNA from the wild-type strain (Andersson *et al.*, 1999; data not shown) indicating that the region between nt -561 and -525 is also the major *rpoS* promoter in SCC3193.

Characterization of the stress tolerance of the *Er. carotovora* subsp. *carotovora* *rpoS* mutant *in vitro*

The sigma factor RpoS has been shown to be involved in the production of secondary metabolites, virulence and/or stress survival in many bacterial species, including plant and animal pathogens (Anderson *et al.*, 1998; Fang *et al.*, 1992; Flavier *et al.*, 1998; Loewen *et al.*, 1998; Sarniguet *et al.*, 1995; Swords *et al.*, 1997; Wilmes-Riesenberg *et al.*, 1997). We therefore decided to investigate whether RpoS has similar functions in the *Er. carotovora* subsp. *carotovora* strain SCC3193. To determine whether RpoS is needed for stress tolerance in SCC3193 during stationary phase, we analysed the phenotype of SCC8002 under osmotic and oxidative stress in L medium (Table 2). We found that SCC8002 was sensitive to these kinds of stresses as the survival of the *rpoS* mutant was significantly lower than for the wild-type strain. *Es. coli* *rpoS* mutants are deficient in the production of glycogen (Lange & Hengge-Aronis, 1991). We therefore performed a glycogen plate assay and found that SCC8002 was affected in the production of glycogen (data not shown). We also performed a

Table 2. Stress tolerance of *rpoS* mutant strain SCC8002 and wild-type strain SCC3193 during stationary phase *in vitro*

Resistance to osmotic and oxidative stresses was determined as indicated in Methods. The results shown are from a representative experiment. The experiments were repeated at least twice with similar results.

Strain	Type of stress	Numbers at start (c.f.u. ml ⁻¹)	Numbers after stress (c.f.u. ml ⁻¹)	Survival (%)
SCC3193	None	2.3 × 10 ⁹	2.5 × 10 ⁹	109
SCC8002	None	1.7 × 10 ⁹	2.0 × 10 ⁹	118
SCC3193	Osmotic	3.6 × 10 ⁹	2.3 × 10 ⁸	6.4
SCC8002	Osmotic	3.6 × 10 ⁹	4.9 × 10 ⁴	0.0014
SCC3193	Oxidative	2.8 × 10 ⁹	2.5 × 10 ⁹	89
SCC8002	Oxidative	2.8 × 10 ⁹	4.6 × 10 ⁸	16

complementation test with pRA910 and found that the plasmid complemented the stress sensitivity of the mutant as well as the deficiency in glycogen accumulation (data not shown). The complementation of these phenotypes was not complete, but nevertheless evident, perhaps due to the low expression from the minor *rpoS* promoter. We also tested stress sensitivity during exponential growth (OD₆₀₀ 1.0; 3.3–4.5 × 10⁸ c.f.u. ml⁻¹). No significant difference in tolerance towards 2.5 mM H₂O₂ between SCC3193 and SCC8002 was shown as 14% of the cells survived for both strains. However, we found that the *expM* mutant strain SCC3032, which overproduces RpoS during exponential growth (Andersson *et al.*, 1999), had enhanced tolerance as 29% of the cells survived. The fact that the *rpoS* mutant did not show higher sensitivity than the wild-type might be explained by the low levels of RpoS found in the wild-type during exponential growth (Andersson *et al.*, 1999).

Production of extracellular enzymes by the *rpoS* mutant

The ability of the *rpoS* mutant strain SCC8002 to produce extracellular cell-wall-degrading enzymes *in vitro* was characterized by growing the cells in L medium containing 0.4% PGA and determining the Peh and Pel activity in the culture supernatant. As shown in Fig. 1(a), we found these enzyme activities to be similar in SCC3193 and SCC8002. We also analysed the accumulation of transcripts from the *pehA* and *pelC* genes by Northern blot analysis; as shown in Fig. 1(b), these transcripts accumulated to similar levels in both strains. In addition, we found that the amounts of Cel and Prt in the supernatant from SCC8002 were similar to SCC3193 (data not shown). We also assayed the basal level of Peh and Pel in L medium without PGA and found similar activities from both strains (data not shown). These results were somewhat surprising since Mukherjee *et al.* (1998) found increased extracellular enzyme activities in the supernatant of an *rpoS* mutant of strain 71. They showed that RpoS acts positively on the expression of

rsmA, which encodes a negative regulator of extracellular enzyme production. RsmA has been shown to be an RNA-binding protein that most likely binds various transcripts encoding virulence determinants and expedites their degradation (Liu *et al.*, 1998). We performed a Northern blot and found that, during stationary phase, the accumulation of *rsmA* transcript was lower in our *rpoS* mutant, whilst the levels were more similar to the wild-type during exponential phase. This is to be expected since *rsmA* seems to be controlled by both σ^{70} and RpoS (Mukherjee *et al.*, 1998). Since the amount of RpoS protein is low during exponential growth (Andersson *et al.*, 1999), an *rpoS* mutation is not likely to affect *rsmA* levels until the cells reach stationary phase and RpoS starts to accumulate. This is supported by the finding that *rsmA* transcript accumulates to a higher level during exponential growth in an *expM* mutant strain which overproduces RpoS (Andersson *et al.*, 1999). Taken together, our data strongly suggest that RpoS also acts as a positive regulator of *rsmA* in SCC3193.

We also analysed the amount of the diffusible signal molecule EAI but could not find any significant difference in its production in SCC8002 compared to SCC3193 (data not shown).

Virulence of the *rpoS* mutant

To characterize the virulence of SCC8002 on plants, we performed an assay on *in-vitro*-grown tobacco. The *rpoS* mutant was found to cause more severe symptoms than the wild-type strain (see Fig. 2). This is in agreement with Mukherjee *et al.* (1998) who showed that an *rpoS* mutant strain caused increased maceration of celery petioles. We also performed a virulence test on potato stems as described by McMillan *et al.* (1993). The virulence of SCC8002 was found to be similar to SCC3193 (data not shown). The reason why the *rpoS* mutant appears to be more virulent on tobacco but not on potato is not known, but it may be due to the infection starting in different parts of the host plants

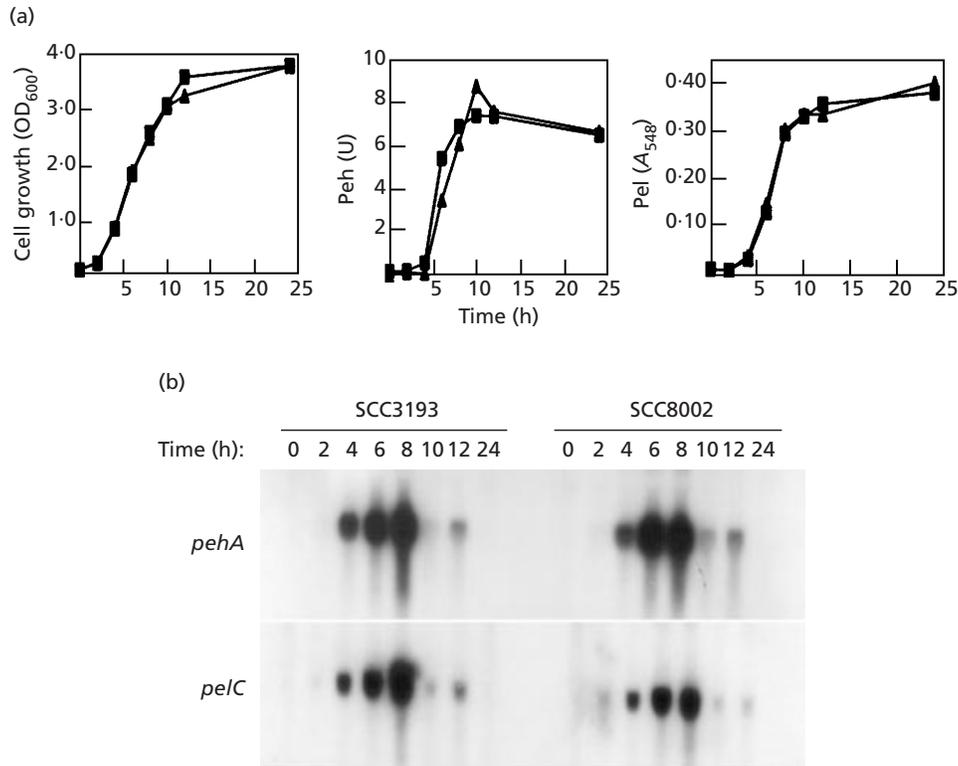


Fig. 1. Enzyme activities and mRNA accumulation in the *rpoS* mutant strain SCC8002 and wild-type strain SCC3193. (a) Cell growth and activities of Peh and Pel in culture supernatants. Cells were grown in L medium containing 0.4% PGA. ■, SCC3193; ▲, SCC8002. (b) Northern blot showing the accumulation of *pehA* and *pelC* transcripts. Five micrograms of RNA was loaded in each lane. The results are from a representative experiment.

(stem inoculation in potato, leaf inoculation in tobacco). However, our general experience is that the tobacco assay is more sensitive and recognizes smaller differences in virulence (R. A. Andersson & M. Pirhonen, unpublished).

The increased virulence of SCC8002 on tobacco seedlings prompted us to investigate the growth of the *rpoS* mutant on tobacco in more detail. We therefore performed a number of experiments on *in-vitro*-grown tobacco plants in order to investigate if the enhanced symptoms caused by the *rpoS* mutant strain SCC8002 allowed it to grow better on tobacco when compared to the wild-type strain. Since the *rpoS* mutant was found to be more sensitive to oxidative stress *in vitro* we also tested the growth and the symptom development on transgenic tobacco (Ascat plants) with severely reduced catalase activity (about 90%) due to an antisense construction (Takahashi *et al.*, 1997). It has been postulated by Takahashi *et al.* (1997) that this reduction in catalase activity results in higher levels of reactive oxygen species in these plants. The results (Fig. 2), showed that SCC8002 caused more severe symptoms than the wild-type strain on control plants although the amount of bacteria extracted from the plants infected by the mutant was slightly, but not significantly, lower after 48 h incubation as compared to control plants

infected by SCC3193. Interestingly, we found that the *rpoS* mutant caused significantly lower levels of symptoms on transgenic Ascat plants compared to control plants. Similarly, significantly lower numbers of bacteria could be extracted from the Ascat plants infected by the mutant strain as compared to control plants (P -value = 0.064). This is in contrast to the growth of the wild-type strain, which was not affected on Ascat plants as compared to control plants. These results suggest that the *rpoS* mutant strain is more sensitive to the higher levels of reactive oxygen species produced in Ascat plants. However, since 3×10^6 cells were used to inoculate the plants, multiplication clearly occurred in both hosts, showing that the Ascat plants only partially restricted the growth of the *rpoS* mutant. Interestingly, Hassouni *et al.* (1999) recently reported that an *Erwinia chrysanthemi msrA* (methionine sulf-oxide reductase) mutant exhibited reduced virulence. The *msrA* gene, which is not RpoS-regulated in *Es. coli* (Moskovitz *et al.*, 1995), encodes a protein which repairs oxidized proteins. The *Er. chrysanthemi msrA* mutant was found to have increased sensitivity to oxidative stress (Hassouni *et al.*, 1999). This study indicates, as in our work, that resistance to oxidative stress may be important for the virulence of soft-rot erwinias.

It has previously been shown that Ascat plants, as used

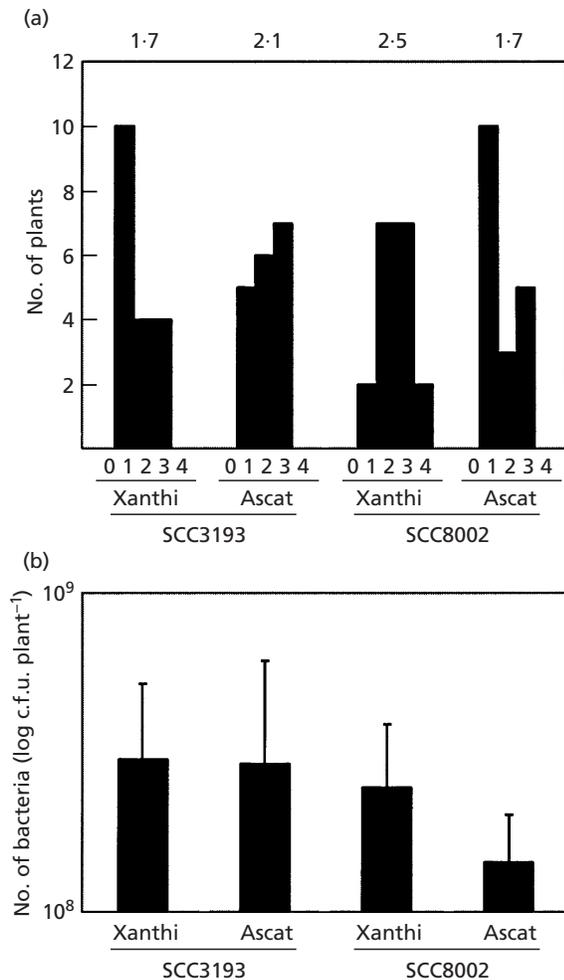


Fig. 2. Symptom development and bacterial growth in wild-type and transgenic Ascot tobacco plants. The *rpoS* mutant SCC8002 causes lower symptom levels on tobacco plants carrying an antisense construction to catalase (Ascot) when compared to control plants (Xanthi), whilst the wild-type strain SCC3193 is not affected. (a) Symptom development. Symptom levels were determined on a scale of 0–5 as described in Methods and mean values are shown above the bar diagrams. (b) Numbers of bacteria extracted from these plants. Results are means \pm SD of 18 infected plants incubated for 48 h.

in this study, are more resistant to tobacco mosaic virus (TMV) infection and that this effect is mediated through SA (Du & Klessig, 1997; Takahashi *et al.*, 1997). In tobacco, SA has been shown to induce resistance towards *Er. carotovora* subsp. *carotovora* (Palva *et al.*, 1994; Vidal *et al.*, 1998). To find out if SCC8002 was more sensitive to plant defence responses induced by SA and to rule out the possibility that the results obtained with the transgenic Ascot plants were due to higher levels of SA, we infected SA-treated tobacco plants with SCC3193 and SCC8002. On the control plants we found similar results as shown in Fig. 2, whilst the SA-treated plants showed increased resistance at the level of both symptom development and bacterial growth *in planta*.

Table 3. Extracellular enzyme activities in plants infected with *rpoS* mutant strain SCC8002 and wild-type strain SCC3193

Enzyme activities were determined and are expressed as indicated in Methods. The results shown are means \pm SD of four samples per strain. *P*-values for the difference between SCC8002 and SCC3193 are 0.011 for Peh, 0.73 for Pel and 0.0086 for Prt. Six pooled uninfected plants were used as a control in order to estimate the background levels. ND, Not detectable.

Strain	Peh	Pel	Prt
SCC3193	0.77 \pm 0.21	1.1 \pm 0.32	0.16 \pm 0.057
SCC8002	1.7 \pm 0.44	1.2 \pm 0.28	1.1 \pm 0.50
Uninfected	0.35	0.028	ND

We were not able to detect any significant difference in the growth of SCC3193 and SCC8002 on the SA-treated plants (data not shown). Taken together, our results suggest that SCC8002 is more sensitive to reactive oxygen species not only *in vitro* but also *in planta*. The results also suggest that the *rpoS* mutant is not affected in its ability to infect wild-type tobacco. On the contrary, it causes more severe symptoms than SCC3193 on wild-type tobacco plants. This suggests that the *rpoS* mutant produces more extracellular enzymes *in planta*. To investigate this, we performed enzyme assays by using macerated plant tissue to determine the *in planta* levels of Peh, Pel and Prt (Table 3). We found that the level of Peh and Prt was significantly higher (twofold for Peh and sevenfold for Prt) in tobacco plants infected with the *rpoS* mutant as compared to plants infected with the wild-type strain, whilst the production of Pel did not differ between the two strains. It is therefore likely that the increased symptoms caused by the *rpoS* mutant strain are due to increased extracellular enzyme production *in planta* and not to higher cell density. This is in contrast with the *in vitro* results, as we could not find any difference in the levels of extracellular enzymes *in vitro* when the cells were grown in L medium with or without PGA. Whilst our work indicates that RpoS is a positive regulator of *rsmA* in SCC3193, it appears that the level of *rsmA* is more important *in planta* than during growth in L medium. This is supported by our previous results showing that the *expM* mutant strain SCC3032, which overproduces RpoS and thereby shows enhanced *rsmA* expression, has significantly lower virulence on tobacco than the wild-type strain (Andersson *et al.*, 1999). Introduction of an *rpoS* mutation in the *expM* mutant background caused an *rsmA* expression similar to that seen in an *rpoS* mutant and the *expM rpoS* double mutant was found to have almost fully restored virulence (Andersson *et al.*, 1999). In addition, the *expM* mutant is also affected in production and secretion of the extracellular enzymes *in vitro* (L medium plus PGA), but this phenotype seems not to be *rsmA*-dependent as the *expM rpoS* double

mutant is similar to the *expM* mutant in this respect (Andersson *et al.*, 1999).

The *rpoS* mutant is unable to compete with the wild-type strain *in vitro* and *in planta*

It has previously been reported that an *Es. coli* strain with a null mutation in *rpoS* is unable to compete with an *rpoS*⁺ strain when grown *in vitro* (Zambrano *et al.*, 1993). Similarly, we found that the *Er. carotovora* subsp. *carotovora* *rpoS* mutant SCC8002 was unable to compete with SCC3193 when grown for 48 h in L medium. In these experiments, about 3×10^6 c.f.u. of the mutant and the wild-type strains were inoculated either together or separately. After incubation, only 0.3–1.0% of the total number of bacteria (about 1.5×10^9 c.f.u. ml⁻¹) were SCC8002. When the strains were grown separately under the same conditions the *rpoS* mutant and the wild-type strain grew to about 8.0×10^8 c.f.u. ml⁻¹ and 1.5×10^9 c.f.u. ml⁻¹, respectively. This indicates that the growth of the wild-type is not affected by the presence of the *rpoS* mutant, whilst the mutant is severely affected in growth in the presence of the wild-type strain. The competition experiments *in vitro* prompted us to test if the *rpoS* mutant is able to compete with the wild-type strain *in planta*. We infected tobacco plants with a mixture of equal numbers of bacteria of both strains (total c.f.u. 2.1×10^6 per plant) and incubated the plants for 48 h. We then extracted the bacteria and plated serial dilutions on L medium with and without Km. In plants infected by the mixture, approximately 25% of the cells (total c.f.u. 2.4×10^8 per plant) were SCC8002, whilst both strains grew similarly in control plants infected with SCC3193 and SCC8002 separately (means of 2.6×10^8 and 2.9×10^8 c.f.u. per plant, respectively). This indicates that the *rpoS* mutant is not able to compete with the wild-type strain SCC3193 *in planta*. When we examined the symptom levels, we found that the plants infected by the mixture showed symptoms very similar to the plants infected by the wild-type strain alone (mean symptom levels of 1.9 for the mixture and 2.1 for SCC3193), whilst plants infected by the *rpoS* mutant alone showed more severe symptoms (mean symptom level 3.3). This indicates that the *rpoS* mutant does not contribute to the development of the symptoms when a mixture of the two strains is used to infect tobacco. Taken together, our results indicate that a functional *rpoS* gene is needed mainly for survival in a competitive environment and in the long term. This is supported by the fact that Mukherjee *et al.* (1998) found that an *Er. carotovora* subsp. *carotovora* *rpoS* mutant of strain 71 is sensitive to carbon starvation.

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