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Identification and Characterization of the *Erwinia amylovora rpoS* Gene: RpoS Is Not Involved in Induction of Fireblight Disease Symptoms

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The *Erwinia amylovora rpoS* gene, encoding the alternative sigma factor RpoS, has been cloned and characterized. Though highly sensitive to a number of environmental stresses, an *E. amylovora rpoS* mutant was not compromised in its ability to grow or cause disease symptoms within apple seedlings or in an overwintering model.

Erwinia amylovora is the causative organism of the disease fireblight, which affects members of the family Pomoideae (7). Studies on nonpathogenic mutants of *E. amylovora* have identified a number of virulence factors, including the extracellular polysaccharide (EPS) and the products of the *hrp* and *dsp* gene clusters (29). Little is known concerning how pathogens sense entry into the host; however, it has been proposed that bacteria respond to a generalized nutrient limitation which activates expression of the alternative sigma factor RpoS, promoting transcription of genes required for in vivo survival (12). The alternative sigma factor RpoS regulates a panel of genes which serve to maintain viability during periods of starvation and environmental stress (15). In addition, a direct role for RpoS in regulation of virulence factor expression has been established for a number of pathogens (11, 13). We describe the cloning and characterization of the *E. amylovora rpoS* gene and the effect of insertional inactivation of the chromosomal copy on *E. amylovora* virulence.

Cloning and expression of the *E. amylovora rpoS* gene. An *E. amylovora* cosmid library (2) was introduced into strain ZK918 (R. Kolter, Harvard Medical School, Boston, Mass.), which has a β-galactosidase reporter fusion in the *bolZ* gene and an insertional mutation in the chromosomal copy of *rpoS*. Transcription of *bolZ* is dependent on the *rpoS* gene product, and complementation of the mutation by *E. amylovora rpoS* was visualized as β-galactosidase activity (30). Of 1,200 *E. amylovora* cosmid clones, two, pPCL911 and pPCL420, showed high levels of β-galactosidase expression during stationary-phase growth (Fig. 1). Southern blot analysis of pPCL911 and pPCL420, using the *Escherichia coli rpoS* gene (24) as a probe, confirmed that both cosmids carried a homologue of *E. coli rpoS* (data not shown). A single 4-kb *Eco*RV fragment of pPCL911 that hybridized with the *E. coli rpoS* probe was subcloned into pBluescript SK+ (Stratagene) to generate plasmid pMAEV9, which retained the ability to complement ZK918. The *rpoS* gene was further localized to a 3.2-kb *Eco*RV/*Kpn*I fragment on plasmid pMAKI (Fig. 1).

Nucleotide sequence analysis of the *E. amylovora rpoS* gene. The nucleotide sequence of the *E. amylovora rpoS* gene was determined by cycle sequencing (Applied Biosystems). Sequence analysis (1, 8) identified a single open reading frame (ORF) of 990 bp that was 81% homologous to the *rpoS* genes of *E. coli* and *Salmonella* species. Six bases upstream of the predicted start site is a putative Shine-Dalgarno sequence, AGGAG. The *E. amylovora rpoS* gene encoded a 330-amino-acid protein, the predicted sequence of which was 97% identical to the RpoS sequences of *E. coli* and *Salmonella* species. Fifty bases 5′ to the start site of the *E. amylovora rpoS* gene is an ORF with 64% identity to the *E. coli nlpD* gene (20). Further sequence analysis identified an incomplete ORF, 3′ to the *E. amylovora rpoS* gene, which was 76% identical to *E. coli mutS*. Therefore, the gene organization of *nlpD*, *rpoS*, and *mutS* would appear to be conserved in both *E. amylovora* and *Erwinia carotovora*.

Transcript mapping of the *rpoS* promoter region. Transcript mapping of the *E. amylovora rpoS* gene (with the Promega RT primer extension system) identified a single transcriptional start site, within the *nlpD* gene, 536 bases 5′ of the start of the *rpoS* coding sequence (Fig. 2A). Putative −10 and −35 consensus sequences were identified 5′ of the transcriptional start. In *E. coli* the transcription start site for *rpoS* (P2) is also within the *nlpD* gene and is approximately 21 bp 5′ to that of *E. amylovora* (18, 32). No additional transcriptional start sites were detected.

Analysis of the secondary structure of the region containing the *rpoS* ribosome binding site demonstrated extensive folding (Fig. 2B), which could facilitate formation of a stem structure comparable to that seen in *Salmonella typhimurium* (3). Though only 66% identity is seen with the intergenic region of *S. typhimurium nlpD* and *rpoS*, the nucleotides postulated to be required for formation of the stem structure are conserved. This similarity in folding at the ribosome binding site suggests that the regulation of RpoS expression at the translational level may be comparable to that in other gram-negative bacteria (3, 19).

Characterization of an *E. amylovora rpoS* mutant. The *E. amylovora rpoS* gene was mutated by insertional inactivation with a streptomycin resistance cassette. The *Eco*RV/*Pst*I fragment from pMAKI was cloned into pRDH20 (9), and the streptomycin cassette from pHP45Ω (38) was ligated into the unique *Bam*HI site in the *rpoS* gene. The resulting suicide vector, pMA16, was introduced into *E. amylovora* OT1 by filter mating (6), and the second crossover event was selected by using a sucrose-resistant phenotype. The *E. amylovora rpoS* mutant was termed OT1.Cm5.

RpoS has been shown to be important for the virulence of a

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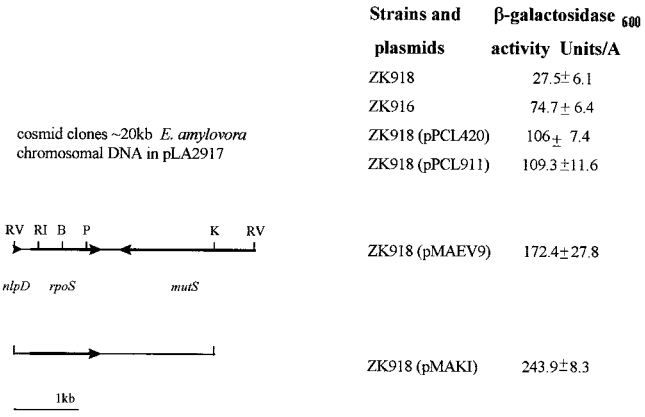

Strains and plasmids	β-galactosidase activity Units/A$_{600}$
ZK918	27.5± 6.1
ZK916	74.7± 6.4
ZK918 (pPCL420)	106± 7.4
ZK918 (pPCL911)	109.3 ±11.6
ZK918 (pMAEV9)	172.4±27.8
ZK918 (pMAKI)	243.9±8.3

FIG. 1. Restriction map of plasmid clones containing the *E. amylovora rpoS* gene and β-galactosidase activities of the plasmids in the reporter strain ZK918. ZK916 is the parent strain, with an intact chromosomal copy of *rpoS*.

number of animal pathogens. In *Salmonella* species, *rpoS* mutants are attenuated for virulence (28, 35) and RpoS protein is required for colonization and destruction of GALT tissue (25), persistence in the liver and spleen (16, 35), and survival in phagocytic cells (5). The ability of OT1.Cm5 to cause disease in the apple seedling assay (23) was determined. The virulence of OT1.Cm5 was not compromised: 61% of the plants developed symptoms compared to 57% of the plants inoculated with OT1 (Fig. 3A). In addition, the times required for the onset of disease symptoms caused by OT1 and OT1.Cm5 were comparable, with necrotic lesions becoming apparent 5 days after inoculation in both cases.

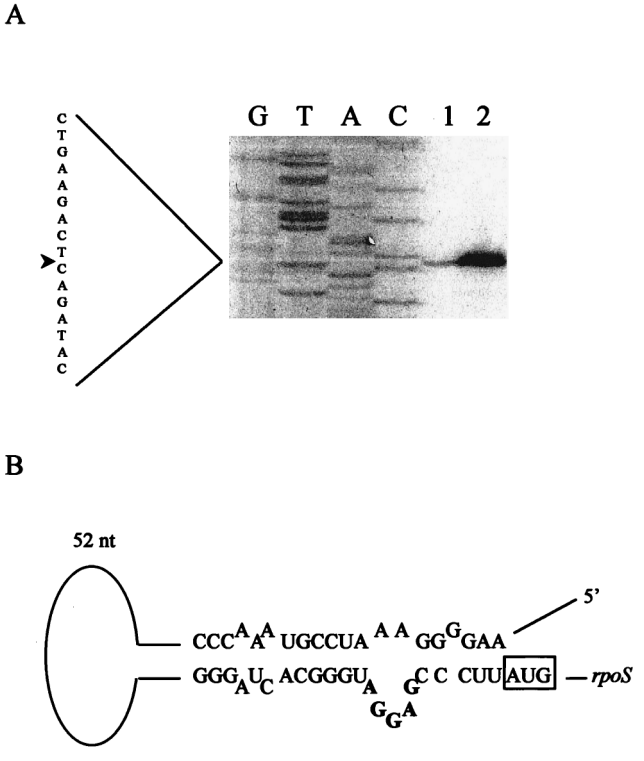

FIG. 2. (A) Promoter mapping of the *E. amylovora rpoS* gene. The first four lanes show the nucleotide sequence analysis of the promoter region. Lanes 1 and 2, reverse transcriptase primer extension of RNA from stationary-phase JM101 (pMAKI). The arrow indicates the transcriptional start site. (B) Model of the secondary structure of *rpoS* promoter region. The Shine-Dalgarno sequence is indicated in bold. nt, nucleotides.

The *E. amylovora* mutant showed in planta growth characteristics comparable to those of the wild type, with a maximal population of approximately 5×10^6 CFU per plant at day 7 (Fig. 3B). The ability of OT1.Cm5 to grow in planta indicates the absence of nutritional stress. The level of glucose in the apoplast has been estimated at 3 to 6 mM, a level likely to be sufficient to permit bacterial growth (10) and in excess of the 10^{-4} mM glucose concentration thought to induce the onset of stationary phase (26). As such, it is unclear how nutritionally limited the plant environment is to a pathogen and whether expression of the RpoS regulon would be activated. In our model the situation may be complicated by the release of cellular nutrients due to localized tissue damage at the inoculation site.

The entry of a pathogen into plant tissue evokes a defense response in the form of the generation of active oxygen species. Elevated levels of hydrogen peroxide in transgenic plants results in increased disease resistance (37). It has been proposed that for successful induction of disease symptoms plant pathogens require catalase activity to degrade hydrogen peroxide formed in response to infection (8, 17). Bacterial mechanisms for surviving oxidative stress have been well characterized; in *E. coli* a panel of protective genes, including *katE* and *katG*, are induced in an RpoS-dependent manner (4, 22). The ability of OT1.Cm5 to resist oxidative stress was determined. Strains OT1 and OT1.Cm5 were exposed to 25 mM H_2O_2 and assessed for viability at 30-s intervals over a 2-min period. Strain OT1 retained over 90% viability after 2 min of exposure, whereas OT1.Cm5 was highly sensitive, with no viable bacteria being recovered after as little as 30 s of exposure (Fig. 3C). Given the postulated role of hydrogen peroxide as a plant defense mechanism, it is surprising to find that, though sensitive to oxidative stress, OT1.Cm5 retains virulence. A likely explanation is that when infecting host plants *E. amylovora* evades recognition and does not trigger defense mechanisms (21), so levels of hydrogen peroxide in plant tissues remain low and the survival of the mutant strain is not compromised.

Although our results do not identify a role for RpoS in induction of disease symptoms in the apple seedling assay, we can readily propose a function for RpoS in the complex *E. amylovora* disease cycle. The most common route of fireblight infection follows colonization of the stigma and subsequent invasion via the nectaries (36). To colonize these sites, *E. amylovora* needs to resist the osmotic stress imposed as a consequence of the high sugar concentration in nectar and stigmal secretions (33). In an in vitro assay, OT1.Cm5 was more sensitive to osmotic shock than wild-type *E. amylovora*. Strain OT1 retained 70 to 80% viability after 2 min of exposure to 0.5 M NaCl, whereas OT1.Cm5 showed a rapid reduction over the first minute and exhibited only 45% survival (Fig. 3D). The sensitivity of OT1.Cm5 to osmotic stress suggests that RpoS may be essential for successful colonization, allowing the organism to survive the osmotic stress encountered during blossom infection.

During systemic infection the host may attempt to limit *E. amylovora* spread by walling off diseased tissue to form a canker. Cankers act as a reservoir in which *E. amylovora* overwinters (34). Canker formation may impose a nutritional stress on the infecting bacteria, inducing *rpoS* expression and promoting survival under the harsh environmental conditions associated with overwintering. To assess the role of *rpoS* in the starvation survival of *E. amylovora*, OT1 and OT1.Cm5 were grown to stationary phase and viable counts were determined every 24 h

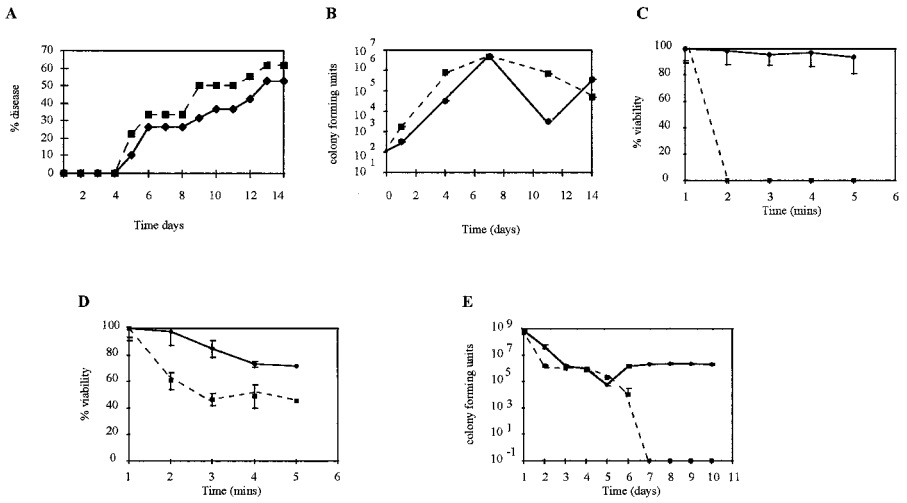

FIG. 3. Phenotypic analysis of the *E. amylovora rpoS* mutant. (A) Disease incidence in apple seedlings inoculated with OT1 (solid line) and OT1.Cm5 (dashed line); (B) growth of *E. amylovora* in apple seedlings; (C) survival of exposure to 25 mM H_2O_2; (D) survival of exposure to 0.5 M NaCl; (E) survival post-stationary phase. All results are presented as means ± standard errors of the means.

over a 10-day period. During the first 5 days there was a rapid loss of viability of both OT1 and OT1.Cm5 (Fig. 3E). However, after day 5 there was a slight increase in the numbers of viable OT1, with levels remaining constant for the rest of the experiment. In contrast, OT1.Cm5 continued to lose viability, until by day 7 no bacteria were recovered.

To determine whether this starvation sensitivity affects in planta survival of *E. amylovora* in mature tissue, OT1 and OT1.Cm5 (1,000 CFU/plant) were inoculated into apple bud sticks and incubated at 1°C for 10 weeks. The *rpoS* mutation had no effect on survival, with similar bacterial populations being recovered from both sets of bud sticks following overwintering (data not shown). In addition, the ability of OT1.Cm5 to replicate in mature tissue and induce water-soaking symptoms following temperature increase (1°C per day up to 25°C) was also unaffected (data not shown). These results suggest that RpoS plays no role in the survival of *E. amylovora* during overwintering in mature tissue. This result is surprising, since it is known that there is increased expression of RpoS at reduced temperatures during exponential growth, which leads to activation of a subset of RpoS-dependent genes (31). It has been suggested that under low-temperature conditions RpoS may act as a housekeeping sigma factor and that increased expression of members of the RpoS regulon facilitates efficient growth. The mechanisms by which *E. amylovora* is able to survive overwintering are currently being investigated in our laboratory.

These results identify an *rpoS* homologue in the plant pathogen *E. amylovora* and demonstrate a role for the gene in survival of stationary-phase, oxidative and osmotic stresses. In contrast to the situation seen with a number of animal pathogens, an *rpoS* mutation does not compromise the in planta growth or virulence of *E. amylovora* at different stages of the disease cycle. This implies that major differences exist in the natures of the bacterium-host interactions between plant and animal pathogens.

Nucleotide sequence accession number. The GenBank accession number of the nucleotide sequence of the *E. amylovora rpoS* gene is AJ222716.

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