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RpoS (Sigma-S) Controls Expression of *rsmA*, a Global Regulator of Secondary Metabolites, Harpin, and Extracellular Proteins in *Erwinia carotovora*†

ASITA MUKHERJEE,[1] YAYA CUI,[1] WEILEI MA,[1] YANG LIU,[1] AKIRA ISHIHAMA,[2] ABRAHAM EISENSTARK,[3] AND ARUN K. CHATTERJEE[1]*

Department of Plant Pathology, University of Missouri, Columbia, Missouri 65211[1]; Cancer Research Center, Columbia, Missouri 65201[3]; and Department of Molecular Genetics, National Institute of Genetics, Mishima, Shizuoka 411, Japan[2]

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RpoS (sigma-S or sigma-38) controls a large array of genes that are expressed during stationary phase and under various stress conditions in *Escherichia coli* and other bacteria. We document here that plant pathogenic and epiphytic *Erwinia* species, such as *E. amylovora*; *E. carotovora* subsp. *atroseptica*, *betavasculorum*, and *carotovora*; *E. chrysanthemi*; *E. herbicola*; *E. rhapontici*; and *E. stewartii*, possess *rpoS* genes and produce the alternate sigma factor. We show that *rpoS* transcription in *E. carotovora* subsp. *carotovora* is driven from a major promoter which resides within the *nlpD* gene located upstream of *rpoS* as in *E. coli*. RpoS$^-$ *E. carotovora* subsp. *carotovoa* strain AC5061, constructed by marker exchange, is more sensitive to hydrogen peroxide, carbon starvation, and acidic pH than its RpoS$^+$ parent strain, AC5006. The basal levels of extracellular pectate lyase, polygalacturonase, and cellulase as well as those of transcripts of *E. carotovora* subsp. *carotovora hrpN* ($hrpN_{Ecc}$), the gene for the elicitor of the hypersensitive reaction, are higher in the RpoS$^-$ strain than in the RpoS$^+$ parent. Likewise, compared to AC5006, AC5061 causes more extensive maceration of celery petioles. Our findings with the RpoS$^-$ mutant and strains carrying multiple copies *rpoS*$^+$ DNA reveal that *rpoS* positively controls *rsmA* expression. We also present evidence that supports the hypothesis that the RpoS effect on extracellular enzyme levels, $hrpN_{Ecc}$ expression, and virulence manifests itself by the modulation of *rsmA* expression.

Bacteria have the remarkable capacity to quickly adapt to various stresses including nutrient limitations, temperature extremes, acidic conditions, osmolarity extremes, and toxic chemicals. Under such conditions bacteria generally deploy sigma factors to activate specific sets of genes. In *Escherichia coli* and various other bacteria, several alternate sigma factors such as sigma-24, sigma-28, sigma-32, sigma-38, and sigma-54 have been identified (reference 14 and references cited therein). Of these, sigma-38 (also called KatF, RpoS, and sigma-S) is involved in the expression of a number of genes that are activated during postexponential growth or upon nutrient limitation or exposure to acidic pH (12, 36). In addition, sigma-S contributes to bacterial resistance to the toxic effects of hydrogen peroxide (1, 6), to the production of certain secondary metabolites (31), and in some instances to the virulence of animal pathogens, for example in *Salmonella typhimurium* (8, 9). Several studies with soft-rotting *Erwinia* spp. have disclosed that virulence factors are expressed during postexponential growth and are subject to global regulation (2, 4, 5, 11, 13, 15, 25, 28, 34, 35). These features prompted the notion that such virulence factors may also be controlled by sigma-S. To test this idea we have undertaken studies with *rpoS* genes of *Erwinia* species. Here we (i) document the occurrence of *rpoS* homologs and the production of the alternate sigma factor; (ii) show that transcription of *rpoS* is mainly driven by a promoter located within the *nlpD* gene upstream of *rpoS*; (iii) report that RpoS of *Erwinia carotovora* is required for the bacterium to cope with carbon starvation, hydrogen peroxide toxicity, and acidic pH; and (iv) document that RpoS controls the expression of *rsmA*, a global negative regulator gene. We also present data that demonstrate that RpoS affects extracellular enzyme production, *E. carotovora* subsp. *carotovora hrpN* ($hrpN_{Ecc}$) expression, and virulence by modulating *rsmA* expression.

* Corresponding author. Mailing address: Department of Plant Pathology, College of Agriculture, Food and Natural Resources, University of Missouri-Columbia, 108 Waters Hall, Columbia, Mo. 65211. Phone: (573) 882-2643. Fax: (573) 882-0588. E-mail: achatterjee@psu.missouri.edu.

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MATERIALS AND METHODS

Bacterial strains, plasmids and media. Bacterial strains and plasmids are described in Table 1. The strains carrying antibiotic markers were maintained on Luria-Bertani (LB) agar containing appropriate antibiotics. The wild-type *Erwinia* and other enterobacterial strains, described in our previous report (5), were maintained on LB agar.

The compositions of King's B (KB) medium, LB medium, minimal salts medium, nutrient gelatin agar, polygalacturonate-yeast extract agar, and M9 medium have been described previously (23, 27, 30). To test the effects of osmolarity on *rpoS* expression, bacteria were grown in TY medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract) either without NaCl or with 0.1 or 0.25 M NaCl. When required, antibiotics and drugs were supplemented as follows (concentrations are in micrograms per milliliter): ampicillin, 100; kanamycin, 50; nalidixic acid, 50; spectinomycin, 50; and tetracycline (TC), 10. Media were solidified by the addition of 1.5% agar.

The compositions of agarose media for semiquantitative assays of enzymatic activities were described previously by Chatterjee et al. (4).

Enzyme assays. The preparation of enzyme samples for pectate lyase (Pel), polygalacturonase (Peh), cellulase (Cel), and protease (Prt) assays and the assay procedures were described previously (4, 27).

DNA techniques. Standard procedures were used in the isolation of plasmid and chromosomal DNAs, transformation, restriction endonuclease digests, gel electrophoresis, and DNA ligation (30). Southern hybridizations were carried out as described by Cui et al. (5). Restriction and modifying enzymes were obtained from Promega Biotec (Madison, Wis.). The Prime-a-Gene DNA labeling system of Promega Biotec was used for labeling DNA.

TABLE 1. *Erwinia carotovora* subsp. *carotovora* strains and plasmids

Bacterial strain or plasmid	Relevant characteristics	Reference or source
Strains		
71	Wild type	35
AC5006	Lac$^-$ mutant of 71	25
AC5047	Nal^r derivative of AC5006	4
AC5061	RpoS$^-$ derivative of AC5006 by marker exchange with pAKC941; Spc^r	This study
AC5070	RsmA$^-$ mini-Tn*5*-Km mutant of AC5047; Km^r	4
AC5072	RpoS$^-$ derivative of AC5070 by marker exchange with pAKC941; Km^r Spc^r	This study
Plasmids		
pRK415	Tc^r	16
pTB2	RpoS$^+$; 4.3-kb *Eco*RI-*Pst*I fragment in pGem3Z; Ap^r	3
pAKC940	RpoS$^+$; 4.3-kb *Eco*RI-*Pst*I fragment from pTB2 in pRK415; Tc^r	This study
pAKC941	RpoS::Ω_{spc}; pAKC940 inactivated with Ω-Spc^r; Spc^r Tc^r	This study
pAKC943	*rpoS-lacZ*; 0.76-kb *Ssp*I-*Hpa*I fragment of pTB2 in pMP220; Tc^r	This study
pAKC944	*rpoS-lacZ*; 0.3-kb *Ssp*I-*Pvu*II fragment of pTB2 in pMP220; Tc^r	This study
pAKC945	*rpoS-lacZ*; 0.45-kb *Pvu*II-*Hpa*I fragment of pTB2 in pMP220; Tc^r	This study

RNA isolation and Northern blot analyses. Bacterial cultures were grown at 28°C in different media and harvested for total RNA extraction at the Klett values indicated in the figure legends. The procedures for RNA isolation and Northern blot analysis have been described previously (21). The 875-bp *Hpa*I-*Mlu*I fragment from pTB2 (Table 1) was used as the *rpoS* probe, the 779-bp *Eco*RV-*Sma*I fragment of pAKC924 was used as the $hrpN_{Ecc}$ probe (25), and the 183-bp *Nde*I-*Sal*I fragment of pAKC882 was used as the *rsmA* probe (24).

S1 nuclease protection assay. Ten picomoles of primer rpoS1 (5′-TGCCGA CAGCAGCTGTATTGCTG-3′; complementary to the base positions −417 to −440 from the translational start codon) was end labeled by polynucleotide kinase (Promega Biotec) and [γ-^{32}P]ATP (New England Nuclear Life Science Products, Boston, Mass.). The end-labeled probe was amplified by PCR using end-labeled primer rpoS1, the opposing unlabeled primer rpoS2 (5′-TGGCAA CAACCGATGCCACACGCG-3′; corresponding to the base positions −633 to −610 from the translational start codon), and pAKC942 as the template DNA. The conditions of PCR, hybridization, S1 nuclease digestion, and analysis of products were as described previously (19).

Western blot analysis. Bacterial strains were grown at 28°C in LB medium to a Klett value of ca. 180. Cells were collected by centrifugation (4,000 × *g*, 10 min, 4°C), resuspended in TESP buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 100 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride), and sonicated with a Braunsonic 1510 sonicator (B. Braun Biotech Inc., Allentown, Pa.) at a 100-W output. The samples were centrifuged at 15,000 × *g* for 15 min at 4°C, and the supernatants were collected. The protein concentration of the cell lysates was determined by using the bicinchoninic acid (Pierce Corp., Rockford, Ill.) method with bovine serum albumin as a standard. Double-strength sodium dodecyl sulfate (SDS) loading buffer (100 mM Tris-HCl [pH 6.8], 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 2% glycerol) was added, and the samples were boiled for 5 min. Proteins were fractionated by 0.1% SDS–12% polyacrylamide gel electrophoresis and transferred to a NitroBind nitrocellulose membrane (Micron Separations, Inc., Westboro, Mass.). The blots were probed with antibodies raised against *E. coli* sigma-38 (14) and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase (Promega Biotec).

Construction of RpoS$^-$ strains by marker exchange. The plasmid pAKC941 (Table 1), wherein *rpoS* was inactivated by inserting an Ω-Spc cassette (29) at the *Hpa*I site (Fig. 1), was transferred into AC5006 and AC5070 by using helper plasmid pRK2013 (10). Transconjugants were selected on minimal salts agar containing sucrose and supplemented with spectinomycin. Isolates that were Spc^r and Tc^s were selected for further studies. The marker exchange was confirmed by Southern blot hybridization as well as Northern and Western blot analyses.

Construction of *rpoS-lacZ* fusions and β-galactosidase assay. A series of *rpoS-lacZ* transcriptional fusions were constructed by cloning sequences from nucleotides (nt) −736 to +24, from nt −736 to −429, and from nt −429 to +24 into promoter probe vector pMP220 (32) to produce pAKC943, pAKC944, and pAKC945, respectively. The orientation of the cloned fragments in these constructs was determined by restriction mapping. β-Galactosidase assays were carried out as described by Miller (23), and the units of activity are expressed as A_{420} units per min per A_{600} unit.

Sensitivity to environmental stress. The RpoS$^-$ strain and its parent were grown overnight in M9 medium at 28°C in a shaker incubator. Stationary-phase cells were collected by centrifugation and resuspended in the desired media (A_{600} of 1.0) and exposed to the indicated stress. For H_2O_2 treatment cells were washed and resuspended in 0.9% NaCl to an A_{600} of 1.0. H_2O_2 was added to a final concentration of 5 mM, and the mixture was incubated for 10 min (1, 18). To find out the effects of starvation, cells were washed and resuspended in M9 medium containing 0.025% glucose and incubated for 100 h. For acid tolerance determination cells were grown overnight in LB broth and resuspended in LB medium buffered with MES (morpholineethanesulfonic acid; final concentration, 5 mM; pH 3.0) and incubated for 3 min (1). After exposure to the respective stresses in the incubation media at 28°C in a shaker incubator, the bacterial suspensions were diluted and appropriate dilutions were plated on LB agar.

Plant tissue maceration. The celery petiole assay has been previously described (28). The extent of tissue maceration was visually estimated.

RESULTS AND DISCUSSION

Evidence for the occurrence of *rpoS* homologs. Calcutt et al. (3) determined the nucleotide sequence of *rpoS* and DNA flanking this gene for *E. carotovora* subsp. *carotovora* strain 71 (hereafter strain 71). Their data showed that (i) the nucleotide sequence of the *rpoS* structural gene is 81% identical to that of the corresponding *E. coli* gene; (ii) the *Erwinia* gene could encode a protein of 331 amino acid residues that is 89 to 91% identical to RpoS of *E. coli*; and (iii) the putative *nlpD* gene is located upstream of *rpoS*, as in *E. coli*. To ascertain if homologs of strain 71 *rpoS* occur in other *Erwinia* species, we conducted Southern blot hybridizations using an internal fragment of strain 71 *rpoS* (the 875-bp *Hpa*I-*Mlu*I fragment; Fig. 1) as the probe. The data in Fig. 2 show that strain 71 *rpoS* DNA hybridized with genomic fragments of all tested strains of *Erwinia* species as well as *E. coli*, *Yersinia enterocolitica*, and *S. typhimurium*.

To detect if *rpoS* genes are expressed in these *Erwinia* species, cell lysates of bacteria were subjected to Western blot analysis. All the tested *Erwinia* strains produced protein species of about 38 kDa that cross-reacted with polyclonal antibodies raised against *E. coli* RpoS (data not shown). Since this cross-reacting material was absent in the extract of an RpoS$^-$ *E. carotovora* subsp. *carotovora* strain, we concluded that the 38-kDa protein represents the RpoS species. These data, taken along with the results of Southern blot analysis (Fig. 2), establish the occurrence of active *rpoS* alleles in these plant-pathogenic and plant-associated bacteria.

The 5′ end of the *rpoS* transcript of strain 71 was localized by an S1 nuclease protection assay at the A residue at base −525 relative to the translational start site (Fig. 3; also see Fig. 1). The calculated sizes of the *rpoS* transcripts presumed to be initiated from this start site matched well to the 1,600-base size determined by Northern blot assays (Fig. 4A). Upstream of the putative *rpoS* transcriptional start site, there are a −10 (TAT TCT) element and a −35 (TTGATT) element, which are highly similar to the consensus *E. coli* sigma-70 promoter. The results of the S1 nuclease protection assay also indicated that this putative promoter is located within the coding region of *nlpD* in strain 71, as is the major *rpoSp1* promoter of *E. coli* (17). To confirm that this promoter is actually functional in *E. carotovora* subsp. *carotovora*, we made the following transcriptional *rpoS-lacZ* fusions: an *Ssp*I-*Hpa*I fragment corresponding to nt −736 to +24 in pAKC943; the upstream region, i.e., the *Ssp*I-*Pvu*II fragment from nt −736 to −429 in pAKC944; and the downstream region, i.e., the *Pvu*II-*Hpa*I fragment from nt −429 to +24 in pAKC945 (Fig. 1 and Table 1). *E. carotovora* subsp. *carotovora* AC5006 carrying each of these plasmids or the promoter probe vector, pMP220, was grown in LB broth to a Klett value of ca. 180, and β-galacto-

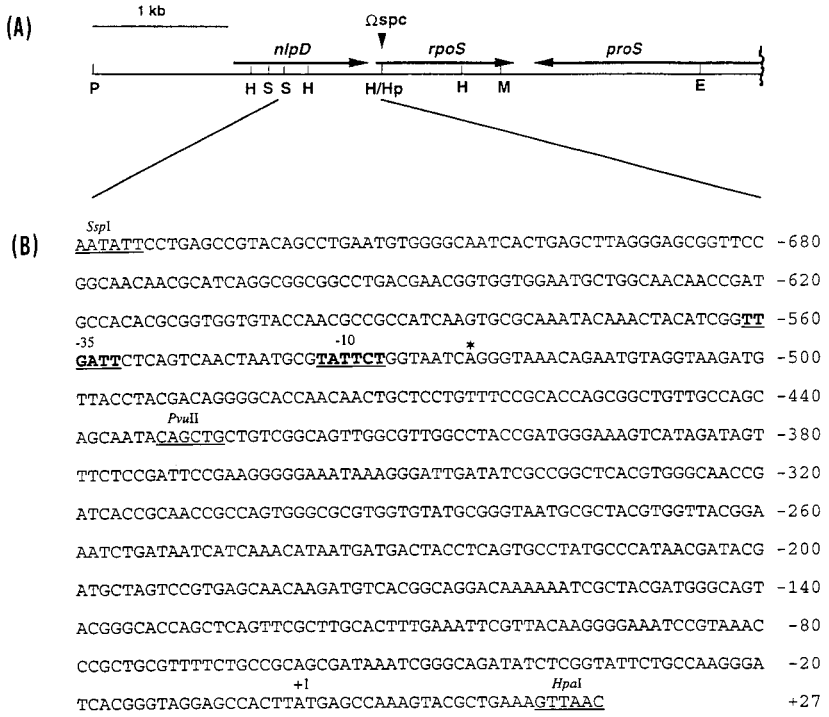

FIG. 1. (A) Restriction map of the 4.5-kb DNA segment of *E. carotovora* subsp. *carotovora* 71 containing *rpoS*, *nlpD*, and *proS* genes. This genetic organization is deduced from the nucleotide sequence data of Calcutt et al. (3). The locations and directions of the genes are indicated by arrows. The site of Ω-Spc insertion in the *Hpa*I site, resulting in the inactivation of *rpoS*, is indicated. The 875-bp *Hpa*I-*Mlu*I fragment was used as the probe in Northern and Southern blot hybridizations. E, *Eco*RI; H, *Hin*cII; Hp, *Hpa*I; M, *Mlu*I; P, *Pst*I; S, *Ssp*I. (B) Nucleotide sequence of the *Ssp*I-*Hpa*I fragment containing parts of *nlpD* and *rpoS* genes of strain 71. The asterisk and +1 indicate transcriptional and putative translational start sites, respectively. The putative −10 and −35 regions and some restriction enzyme sites are shown. The numbers on the right refer to the positions of the nucleotides.

sidase activity was assayed. AC5006 carrying pAKC943 produced 3,694 Miller units of β-galactosidase activity, and AC5006 carrying pAKC944 produced 5,568 Miller units. By contrast, AC5006 carrying vector pMP220 produced 155 Miller units of β-galactosidase activity. The high expression levels of the *rpoS-lacZ* fusions in these constructs and the location of the consensus sigma-70 promoter strongly suggest that the major *rpoS* promoter (rpoSpM) is present within nt −561 and −525 (Fig. 1). The transcription of *rpoS* from this promoter was stimulated during postexponential growth and by medium osmolarity (data not shown), as in *E. coli* (12, 22).

AC5006 carrying pAKC945 produced 887 Miller units of β-galactosidase activity, which is about sixfold higher than the levels produced by bacteria carrying promoter probe vector pMP220 but much lower than the levels produced by bacteria carrying either pAKC943 or pAKC944. These data suggest that there may be another weak promoter (rpoSpW) behind rpoSpM. This conclusion is supported by the S1 nuclease protection assay, which revealed the presence of weakly protected bands within nt −166 and +19 (data not shown). It is noteworthy that in *E. coli*, *rpoS* transcription is mainly driven by a single major promoter, rpoSp1, which is homologous to the strain 71 rpoSpM promoter. Although there is a second putative promoter about 251 bp downstream of rpoSp1 (17), this promoter apparently does not play a significant role in the expression of *rpoS* in *E. coli*. A very similar situation probably occurs in strain 71 since by Northern blot analysis we did not detect an *rpoS* transcript smaller than 1,600 bases (Fig. 4A).

Effects of RpoS on the production of extracellular enzymes and expression of $hrpN_{Ecc}$. To analyze the regulatory role of RpoS in *E. carotovora* subsp. *carotovora*, we constructed an RpoS-deficient mutant, AC5061, by a marker exchange procedure. The exchange of the wild-type *rpoS* gene by inactivated *rpoS*::Ω fragment was confirmed by Northern blot hybridizations (Fig. 4A) as well as Western blot assay and Southern blot

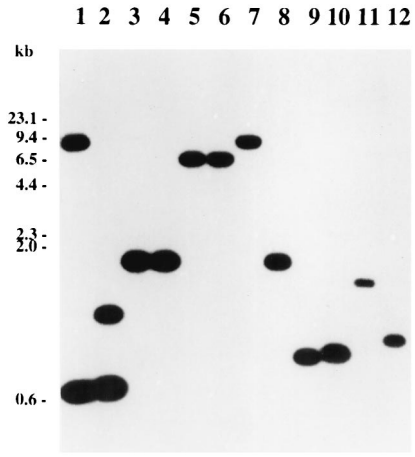

FIG. 2. Southern blot hybridization of *Hin*cII-digested chromosomal DNAs of wild-type strains of *Erwinia*, *Salmonella*, *Yersinia*, and *E. coli* with *rpoS* of *E. carotovora* subsp. *carotovora* 71. Lane 1, *E. carotovora* subsp. *carotovora* 71; lane 2, *E. carotovora* subsp. *atroseptica* Eca12; lane 3, *E. carotovora* subsp. *betavasculorum* Ecb11129; lane 4, *Erwinia chrysanthemi* EC16; lanes 5 and 6, *Erwinia amylovora* E9 and Ea321; lane 7, *Erwinia rhapontici* Er1; lane 8, *Erwinia herbicola* EH105; lane 9, *Erwinia stewartii* Es1; lane 10, *S. typhimurium* LT2; lane 11, *Y. enterocolitica* 8081v; and lane 12, *E. coli* AE908.

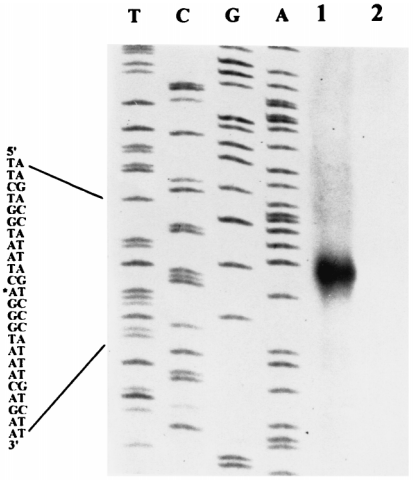

FIG. 3. S1 nuclease mapping of the putative transcriptional start site of *rpoS*. Strain 71 was grown in LB medium to a Klett value of ca. 200 for RNA isolation. Lane 1, 100,000 cpm of end-labeled DNA probe with 20 μg of total RNA; lane 2, 100,000 cpm of end-labeled DNA probe without RNA. The nucleotides on the left refer to the nucleotide sequence beyond the 5′ end. The asterisk denotes the A residue at which transcription was presumed to be initiated.

hybridization (data not shown). The RpoS$^-$ mutant strain was also tested for various characteristics. The inactivation of *rpoS* resulted in enhanced sensitivity to carbon starvation, acidic pH, and hydrogen peroxide (Table 2). These characteristics are typical of RpoS$^-$ bacteria (22).

In the course of characterization of RpoS$^-$ strain AC5006, we noted that the levels of extracellular Pel, Peh, and Cel were higher in RpoS$^-$ bacteria than in the RpoS$^+$ parent strain. The results of quantitative assays (Table 3) show that Pel-specific activity was about twofold higher in AC5061 than in the RpoS$^+$ parent, AC5006. Similarly, the levels of $hrpN_{Ecc}$ transcripts were higher in RpoS$^-$ bacteria than in the RpoS$^+$ parent strain (Fig. 4B). The RpoS$^-$ strain was more virulent (Fig. 5) than the RpoS$^+$ parent as would be expected from the production of higher levels of extracellular enzymes, specially the pectinases. These observations were unexpected since RpoS is generally known to activate gene expression in the stationary-growth phase (12). This pleiotropic effect of RpoS deficiency was somewhat reminiscent of the phenotype of the RsmA$^-$ strains of *E. carotovora* subsp. *carotovora* (4, 5). We therefore argued that RpoS positively regulates *rsmA* expression and that the reduced pool of RsmA in RpoS$^-$ bacteria accounts for the elevated levels of extracellular enzymes, the higher level of expression of $hrpN_{Ecc}$, and the greater plant tissue maceration. The data presented below substantiate this hypothesis.

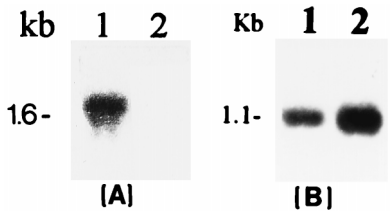

FIG. 4. Northern blot analysis of *rpoS* (A) and $hrpN_{Ecc}$ (B) mRNA in *E. carotovora* subsp. *carotovora* AC5006 (lane 1) and its RpoS$^-$ derivative, AC5061 (lane 2). Total RNA was extracted from bacteria grown in minimal salts medium supplemented with sucrose (0.5% [wt/vol]) to a Klett value of 200. Each lane contained 10 μg of total RNA.

TABLE 2. Survival of *E. carotovora* subsp. *carotovora* AC5006 and its RpoS$^-$ mutant (AC5061) upon exposure to environmental stresses

Stress factor	Strain	% Survival[a]
Hydrogen peroxide	AC5006	35
	AC5061	2.7
Low pH	AC5006	29
	AC5061	2.5
Carbon starvation	AC5006	150
	AC5061	36

[a] These values were obtained by dividing bacterial CFU after exposure to stresses by CFU at zero time, i.e., immediately prior to exposure to stresses, and multiplying by 100.

RpoS causes accumulation of *rsmA* transcripts. The data shown in Fig. 6 demonstrate that the levels of *rsmA* transcripts were much lower in RpoS$^-$ strain AC5061 than in RpoS$^+$ strain AC5006. To assess the effect of *rpoS* gene dosage, the levels of *rpoS* and *rsmA* transcripts in the RpoS$^-$ strain, the parent strain carrying a chromosomal copy of *rpoS*$^+$, and the RpoS$^-$ strain carrying low-copy-number RpoS$^+$ plasmid pAKC940 were determined. Bacteria were grown in KB medium containing TC. Total RNA was extracted from cells and subjected to Northern blot analysis. The results were as follows. (i) The level of *rpoS* mRNA was higher in the strain carrying multiple copies of *rpoS*$^+$ than in the strain carrying a single copy of *rpoS*$^+$ (data not shown). As expected, no *rpoS* mRNA was detected in RpoS$^-$ mutant AC5061. (ii) The highest level of *rsmA* transcripts was observed in bacteria producing the most *rpoS* mRNA (i.e., the strain carrying *rpoS*$^+$ plasmid pAKC940) (Table 4).

To determine if there was a correlation between the dosage of *rpoS*$^+$ DNA and the levels of extracellular enzymes, those bacterial constructs were grown in minimal salts medium containing sucrose and TC, and culture supernatants were assayed for enzymatic activities. The level of Pel activity in AC5061 carrying multiple copies of *rpoS*$^+$ DNA was 6% of the activity found in AC5061 carrying the vector (Table 3). A similar effect of *rpoS*$^+$ copies occurred with Peh, Cel, and Prt activities (data not shown). We attribute this effect to overexpression of *rsmA*

TABLE 3. The effect of *rpoS* on Pel production in *E. carotovora* subsp. *carotovora*

Bacterial construct[a]	Relevant phenotype	Pel activity[b]
AC5006*	RsmA$^+$ RpoS$^+$	0.35 ± 0.005
AC5061*	RsmA$^+$ RpoS$^-$	0.58 ± 0.004
AC5070*	RsmA$^-$ RpoS$^+$	9.6 ± 0.22
AC5072*	RsmA$^-$ RpoS$^-$	10.1 ± 0.35
AC5006/pRK415†	RsmA$^+$ RpoS$^+$/RpoS$^-$	0.059 ± 0.006
AC5061/pRK415†	RsmA$^+$ RpoS$^-$/RpoS$^-$	0.24 ± 0.016
AC5061/pAKC940†	RsmA$^+$ RpoS$^-$/RpoS$^+$	0.015 ± 0.0016
AC5070/pRK415†	RsmA$^-$ RpoS$^+$/RpoS$^-$	2.0 ± 0.036
AC5072/pRK415†	RsmA$^-$ RpoS$^-$/RpoS$^-$	2.1 ± 0.097
AC5072/pAKC940†	RsmA$^-$ RpoS$^-$/RpoS$^+$	2.0 ± 0.063

[a] Bacteria were grown in minimal salts plus sucrose (*) or minimal salts plus sucrose plus TC (†) to a Klett value of ca. 200. Cultural supernatants were assayed for Pel activity.

[b] Expressed as units per milliliter per A_{600} unit.

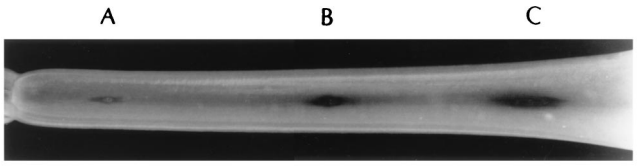

FIG. 5. Plant tissue maceration induced by *E. carotovora* subsp. *carotovora* AC5006 and its RpoS$^-$ mutant, AC5061. About 2×10^8 cells were injected into the celery petiole at each inoculation site. The inoculated celery petiole was incubated in a moist chamber at 25°C for 24 h. (A) water injection; (B) AC5006 injection; (C) AC5061 injection.

by *rpoS*$^+$ copies, followed by RsmA-promoted decay of the cognate transcripts of the extracellular enzyme genes.

We performed the following experiments to establish that the RpoS effect was mediated via RsmA. We constructed an RpoS$^-$ RsmA$^-$ double mutant, and determined that the levels of Pel were similar in the mutant, AC5072, and its RpoS$^+$ counterpart, AC5070 (Table 3). We then transferred RpoS$^+$ plasmid pAKC940 or cloning vector pRK415 into the RpoS$^-$ RsmA$^-$ double mutant, AC5072. The RpoS$^+$ RsmA$^-$ strain, AC5070, carrying pRK415 was used as a control. These constructs were grown in minimal salts medium containing sucrose and TC, and culture supernatants were assayed for enzymatic activities. The levels of Pel activity were very similar in these constructs (Table 3). Similar results were obtained with Peh, Cel, and Prt activities (data not shown). These observations demonstrate that in the absence of a functional *rsmA* allele RpoS does not have a significant effect on extracellular enzyme production. This clearly contrasts with the suppression of enzyme levels by RpoS in the RsmA$^+$ strain (see above). A straightforward interpretation of these observations is that the RpoS effect manifests itself primarily by regulating *rsmA* expression.

To determine if multiple copies of the strain 71 *rpoS*$^+$ gene have a generalized effect on *rsmA* expression in different bacteria, we introduced pAKC940 into *E. carotovora* subsp. *carotovora* SCRI193, *E. carotovora* subsp. *atroseptica* Eca12, and *E. carotovora* subsp. *betavasculorum* Ecb11129. Strain 71 carrying pAKC940 served as the positive control. The plasmid-carrying strains were grown in LB containing TC, and total RNA was extracted from cells grown to a Klett value of 100. The data in Table 4 show that *rsmA* transcripts were consistently higher in strains carrying the strain 71 *rpoS* allele than in cells carrying cloning vector pRK415.

Since high levels of *rsmA* transcripts are only detected in the presence of RpoS, we do not consider it merely coincidental that the *rsmA* promoter consists of a −10 region comprising CTAAACT and no consensus −35 region (5). This −10 sequence is typical of sigma-S-dependent promoters (see, for example, reference 7). These observations and the finding that the level of *rsmA* expression is higher in the postexponential than in the exponential growth stage (20) provide strong support for the hypothesis that *rsmA* expression is positively affected by RpoS in *E. carotovora* subsp. *carotovora*. In fact, we have found that the levels of *rsmA* (*csrA*) transcripts in *S. typhimurium* are reduced by RpoS deficiency (data not shown), raising the possibility that expression of *rsmA* may be under the control of this alternate sigma factor in other enterobacteria as well. We have initiated studies in collaboration with Tony Romeo to determine if *E. coli csrA*, the *rsmA* homolog, is also controlled by this alternate sigma factor. We should note that expression of *rsmA* occurs to some extent in RpoS-deficient *E. carotovora* subsp. *carotovora* strains. Thus, the gene is controlled by sigma-70 as well as sigma-S. In fact, this prediction is consistent with the observation that many sigma-S-controlled genes in *E. coli* are also activated by sigma-70 (33).

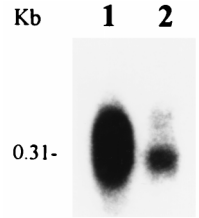

FIG. 6. Northern blot analysis of *rsmA* mRNA in *E. carotovora* subsp. *carotovora* AC5006 (lane 1) and its RpoS$^-$ derivative, AC5061 (lane 2). Each lane contained 20 μg of total RNA isolated from bacteria grown in LB medium to a Klett value of ca. 200.

TABLE 4. Effect of *rpoS*$^+$ copies on the levels of *rsmA* transcripts[a]

Bacterial construct	Relative value of contour (OD × mm^2)[b]
E. carotovora subsp. *carotovora*	
AC5006/pRK415*	100
AC5061/pRK415*	ND
AC5061/pAKC940*	249
71/pRK415†	100
71/pAKC940†	336
SCRI193/pRK415†	100
SCRI193/pAKC940†	258
E. carotovora subsp. *atroseptica*	
Eca12/pRK415†	100
Eca12/pAKC940†	207
E. carotovora subsp. *betavasculorum*	
Ecb11129/pRK415†	100
Ecb11129/pAKC940†	294

[a] Total RNA was isolated from bacteria grown in KB plus TC (*) to an A_{600} of 4.0 or in LB plus TC (†) to a Klett value of ca. 100. The RNA samples (10 μg of total RNA for each strain) were subjected to Northern blot analysis. The densities of the hybridization bands were quantified by using the QS30 optically enhanced densitometry system (Fisher Scientific, Pittsburgh, Pa.).

[b] Data are presented as relative values by defining the value for the wild-type strains carrying cloning vector pRK415 as 100. pAKC940 carries the *Erwinia rpoS* DNA cloned into pRK415 (see Table 1). ND, a band corresponding to *rsmA* mRNA was not detected; the relative value of the contour is <20. OD, optical density.

In summary, we have shown that some of the structural characteristics of *E. carotovora rpoS* and its functions are generally similar to those in *E. coli*. These similarities notwithstanding, our data reveal several novel features as well. For example, the levels of extracellular enzymes and $hrpN_{Ecc}$ transcripts and the degree of plant virulence are higher in RpoS-deficient bacteria than in the RpoS$^+$ parent. We have established that this effect manifests itself through the reduction in *rsmA* expression. The rationale for a dual control of *rsmA* expression by sigma-70 and sigma-S can perhaps be appreciated by invoking an important housekeeping role of RsmA as well as its function as a regulator of secondary metabolites.

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