Identification of a Global Repressor Gene, *rsmA*, of *Erwinia carotovora* subsp. *carotovora* That Controls Extracellular Enzymes, *N*-(3-Oxohexanoyl)-L-Homoserine Lactone, and Pathogenicity in Soft-Rotting *Erwinia* spp.†

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The production of extracellular enzymes such as pectate lyase (Pel), polygalacturonase (Peh), cellulase (Cel), and protease (Prt) is activated by the cell density (quorum)-sensing signal, *N*-(3-oxohexanoyl)-L-homoserine lactone (HSL); plant signals; and *aep* genes during postexponential growth of *Erwinia carotovora* subsp. *carotovora* 71. Studies with mutants of *E. carotovora* subsp. *carotovora* 71 derepressed in exoenzyme production led to the identification of a negative regulator gene, *rsmA* (*rsm*, repressor of secondary metabolites). Nucleotide sequencing, transcript assays, and protein analysis established that a 183-bp open reading frame encodes the 6.8-kDa RsmA. *rsmA* has extensive homology with the *csrA* gene of *Escherichia coli*, which specifies a negative regulator of carbon storage. Moreover, the suppression of glycogen synthesis in *E. coli* by *rsmA* indicates that the *Erwinia* gene is functionally similar to *csrA*. Southern hybridizations revealed the presence of *rsmA* homologs in soft-rotting and non-soft-rotting *Erwinia* spp. and in other enterobacteria such as *Enterobacter aerogenes*, *E. coli*, *Salmonella typhimurium*, *Shigella flexneri*, *Serratia marcescens*, and *Yersinia pseudotuberculosis*. *rsmA* suppresses production of Pel, Peh, Cel, and Prt, plant pathogenicity, and synthesis of HSL in *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *betavasculorum*, *E. carotovora* subsp. *carotovora*, and *E. chrysanthemi*. In the *E. carotovora* subsp. *carotovora* 71, *rsmA* reduces the levels of transcripts of *hslI*, a *luxI* homolog required for HSL biosynthesis. This specific effect and the previous finding that HSL is required for extracellular enzyme production and pathogenicity in soft-rotting *Erwinia* spp. support the hypothesis that *rsmA* controls these traits by modulating the levels of the cell density (quorum)-sensing signal.

The activation of extracellular pectate lyase (Pel), cellulase (Cel), polygalacturonase (Peh), and protease (Prt) production in *Erwinia carotovora* subsp. *carotovora* requires the cell density (quorum)-sensing signal, *N*-(3-oxohexanoyl)-L-homoserine lactone (HSL) (4, 20, 34); plant signals (9, 32); and the functions of activator genes, such as *aep* (24, 30, 32), *exp* (35), or *rex* (20). HSL and its analogs function as signals for the expression of traits responding to starvation, growth phase, or cell density (13, 19, and references cited therein). In *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *betavasculorum* extracellular enzyme production may also be controlled by plant signals, *aep* products, and HSL (11, 30, and this study).

To identify the regulatory components controlling extracellular enzyme production in *E. carotovora* subsp. *carotovora*, we have initiated mutant studies. In a previous report (4), we described a novel mutant capable of producing extracellular enzymes in the absence of HSL. Genetic evidence showed that inactivation of the *rsmA* locus (*rsm*, repressor of secondary metabolites) resulted in HSL independence. We present here the characteristics of *rsmA* of *E. carotovora* subsp. *carotovora* 71. Our data show that *rsmA* is a homolog of *csrA*, a gene previously found by Romeo et al. (38) to control glycogen accumulation, cell size, and cell surface properties in *Escherichia coli*. Our most remarkable findings with *rsmA* are (i) the suppression of extracellular enzyme production, HSL synthesis, and pathogenicity not only in *E. carotovora* subsp. *caroto-*

vora but also in other soft-rotting *Erwinia* spp. such as *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *betavasculorum* as well as *E. chrysanthemi* and (ii) the occurrence of *rsmA* homologs in every enterobacterial strain tested. In addition, we have noted repression of HSL production by *rsmA* in *Erwinia* spp., raising the possibility that *rsmA* may control gene expression by modulating the levels of the cell density (quorum)-sensing signal.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids are described in Table 1. The strains carrying drug markers were maintained on Luria-Bertani agar containing appropriate antibiotics. The wild-type strains of *Erwinia* were maintained on yeast extract-glucose-calcium carbonate (YGC) agar.

The compositions of lecithin medium, LB medium, minimal salts medium, nutrient gelatin (NG) agar, polygalacturonate-yeast extract agar (PYA), salts-yeast extract-glycerol (SYG) medium, SYG plus celery extract medium, and YGC agar have been described previously (2, 6, 7, 31). When required, the media were supplemented with the following antibiotics and drugs as indicated (each at 50 µg/ml): ampicillin, kanamycin, nalidixic acid, and spectinomycin. Media were solidified by the addition of 1.5% agar.

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

Preparation of samples for enzyme assays and assay conditions. Growth conditions, preparation of culture supernatants, and assay conditions for Pel, Peh, Prt, and Cel were previously described (4, 10, 32). β-Galactosidase activity was assayed according to the method of Miller (29).

Bioluminescence assay for HSL. The *E. coli*-based bioassay (4) was utilized for the estimation of HSL produced by *Erwinia* strains.

Glycogen production. Cells of *E. coli* B carrying pAKC877 or pBluescript KS⁺ were inoculated on enriched agar medium (14) supplemented with ampicillin and incubated at 28°C for 4 days. The *E. coli* colonies were then stained with iodine vapor as described previously (38).

Plant tissue maceration. The celery petiole assay was previously described (32). The extent of tissue maceration was visually estimated.

TABLE 1. Bacterial strains and plasmids

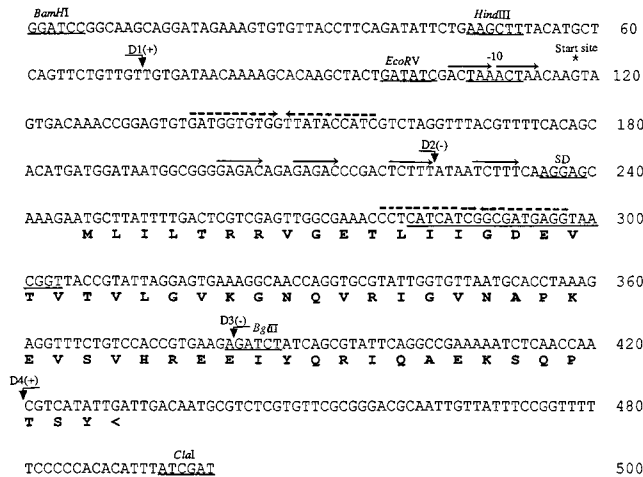| Strain and plasmid	Relevant characteristic(s)	Reference or source
Strains		
E. carotovora subsp. *carotovora*		
71	Wild type	45
193	Wild type	45
AC5006	Lac$^-$ mutant of 71	31
AC5092	Hsl$^-$ Km^r	*hslI-lacZ* derivative of AC5006 (laboratory collection)
AC5047	Nal^r derivative of AC5006	4
AC5070	RsmA$^-$ Km^r	4
AH2	Str^r Nal^r	18
SCC3193	Wild type	34
SCRI193	Wild type	39
E. carotovora subsp. *atroseptica*		
Ec	Wild type	21
Eca12	Wild type	45
E. carotovora subsp. *betavasculorum* Ecb11129	Wild type	J. E. Loper
E. chrysanthemi		
EC16	Wild type	8
EC183	Wild type	7
0706	Wild type	Laboratory collection
E. rhapontici Er1	Wild type	26
E. herbicola EH105	Wild type	Laboratory collection
E. amylovora		
E9	Wild type	36
EA246	Wild type	3
E. stewartii Es1	Wild type	Laboratory collection
E. coli		
DH5α	φ*80lacZ* Δ*M15* Δ(*lacZYA-argF*) *U169 hsdR17 recA1 endA1 thi-1*	Bethesda Research Laboratory
B (ICPB 2262)	Wild type	Laboratory collection
HB101	*proA1 lacY hsdS20* (r$_B^-$ m$_B^-$) *recA56 rpsL20*	45
K-12	Wild type	43
K38	F$^-$ HfrC *phoA4 pit-10 tonA22 ompF627 relA1* λ$^+$	25
VJS533	*ara* Δ(*lac-proAB*) *rpsL* φ*80lacZ* Δ*M15 recA56*	15
Salmonella typhimurium LT2	Wild type	Laboratory collection
Serratia marcescens Sm1	Wild type	Laboratory collection
Yersinia pseudotuberculosis Yp1(ICPB 3821)	Wild type	8
Shigella flexneri Sf1	Wild type	Laboratory collection
Enterobacter arogenes Ena1	Wild type	Laboratory collection
Plasmids		
pCL1920	Spc^r Sm^r	22
pBluescript KS$^+$	Ap^r	Stratagene
pHV200	8.8-kb *Sal*I fragment containing the *lux* opron, Ap^r	15
pHV200I	Frameshift mutant of *luxI* in pHV200, Ap^r	17
pGP1-2	Km^r	42
pAKC856	Ap^r	4
pAKC875	RsmA$^+$ Tc^r	4
pAKC877	RsmA$^+$ Ap^r	0.5-kb *Bam*HI-*Cla*I fragment of pAKC875 in pBluescript KS$^+$ (this study)
pAKC880	RsmA$^+$ Spc^r	0.5-kb *Bam*HI-*Cla*I fragment of pAKC875 in pCL1920 (this study)

BamHI   HindIII
GGATCCGGCAAGCAGGATAGAAAGTGTGTTACCTTCAGATATTCTGAAGCTTTACATGCT   60
                                                                    Start site
        D1(+)
CAGTTCTGTTGTTGTGATAACAAAAGCACAAGCTACTGATATCGACTAAAACTAACAAGTA   120
                                     EcoRV   -10   *

GTGACAAACCGGAGTGTGATGGTGTGGTTATACCATCGTCTAGGTTTACGTTTTCACAGC   180

                                          D2(-)
ACATGATGGATAATGGCGGGGGAGACAGAGAGACCCGACTCTTTATAATCTTTCAAGGAGC   240
                                                         SD

AAAGAATGCTTATTTTGACTCGTCGAGTTGGCGAAACCCTCATCATCGGCGATGAGGTAA   300
            M L I L T R R V G E T L I I G D E V

CGGTTACCGTATTAGGAGTGAAAGGCAACCAGGTGCGTATTGGTGTTAATGCACCTAAAG   360
T V T V L G V K G N Q V R I G V N A P K

          D3(-)   BglII
AGGTTTCTGTCCACCGTGAAGAGATCTATCAGCGTATTCAGGCCGAAAAATCTCAACCAA   420
E V S V H R E E I Y Q R I Q A E K S Q P
D4(+)
CGTCATATTGATTGACAATGCGTCTCGTGTTCGCGGGACGCAATTGTTATTTCCGGTTTT   480
T S Y <
                     ClaI
TCCCCCACACATTTATCGAT   500

FIG. 1. Nucleotide and deduced amino acid sequences of *rsmA*. The transcriptional start site, putative Shine-Dalgarno (SD) sequence, and the −10 region are shown. Tandem repeats are indicated by arrows. The potential hairpin loop structures are identified with dashed arrows. The nucleotide sequence used for the synthesis of complementary oligonucleotide for the primer extension assay is underlined. The deletion limit points, D1 and D2 from the 5′ end and D3 and D4 from the 3′ end, are indicated by right-angled arrows (⌐). The deletions D1 and D4 that suppress extracellular enzyme production (+) and D2 and D3 that do not suppress extracellular enzyme production (−) are also indicated. The numbers on the right refer to the positions of the nucleotides.

Recombinant DNA techniques. Standard procedures were used in the isolation of plasmid and chromosomal DNAs, transformation and electroporation, restriction digests, gel electrophoresis, electroelution of DNA fragments, and DNA ligation (40). Southern blot hybridizations were carried out according to the method of Murata et al. (31) except that hybridization and washes were conducted at 65°C instead of 63°C. Restriction and modifying enzymes were obtained from Promega Biotec (Madison, Wis.) and U.S. Biochemicals (Cleveland, Ohio). The random primer system from U.S. Biochemicals was used for labeling DNA.

Nucleotide sequence determination. Unidirectional 5′ to 3′ deletions within the 0.5-kb internal fragment of the RsmA+ plasmid pAKC877 were made by using the Erase-a-Base system (Promega Biotec). Overlapping deletions differing in size by approximately 200 bp were used for sequence analysis with the Sequenase system II (U.S. Biochemicals). The nucleotide sequence was analyzed with the PC/gene program (IntelliGenetics, Inc., Mountain View, Calif.).

Analysis of *rsmA* and *hslI* transcripts. Bacterial cultures were grown to a value of ca. 200 Klett units at 28°C in appropriate media. The procedures for RNA isolation and Northern blot (RNA) analysis have been described previously (5, 24). Total RNA samples from AC5070 carrying pCL1920 or pAKC880 were analyzed for *rsmA* mRNA by using the 282-bp *Eco*RV-*Bgl*II fragment of pAKC877 (Table 1) as the probe. For the assay of *hslI* mRNA, total RNA preparations from *E. carotovora* subsp. *carotovora* 71 carrying pCL1920 or pAKC880 were examined by using the 386-bp *Dra*I-*Eco*RI fragment of pAKC856 (Table 1) as the probe.

Identification of transcriptional start site. RNA samples from *E. carotovora* subsp. *carotovora* 71 and AC5070 were utilized in the primer extension assay (5, 24). The ^{32}P-labeled oligonucleotide primer (5′-ACCGTTACCTCATCGC CGATGATG-3′), complementary to the sequence shown in Fig. 1 (positions 304 to 281), was annealed to the RNA. The extension products were run in an 8% acrylamide–urea sequencing gel in parallel with a DNA ladder obtained by using the same primer and pAKC877 as the template DNA.

Overexpression of *rsmA* and identification of gene product. The plasmid pAKC877D2 carrying the promoterless *rsmA* behind the T7 promoter in pBluescript KS+ was transformed into *E. coli* K38 containing pGP1-2, which specifies the T7 RNA polymerase (42). Proteins were labeled with [^{35}S]methionine (42) and fractionated by (0.1%) sodium dodecyl sulfate-(16%) polyacrylamide gel electrophoresis, and the labeled bands were visualized by autoradiography with Kodak direct exposure film.

Nucleotide sequence accession number. DNA sequence has been deposited in the GenBank database under accession no. L40173.

RESULTS

Identification and nucleotide sequence of *rsmA*. We recently described the isolation of pAKC875 carrying the *rsmA*+ DNA

TABLE 2. Effect of *rsmA* on extracellular Pel production in soft-rotting *Erwinia* spp.

Bacterial strain[a]	Sp act with[b]:	
	pCL1920	pAKC880
E. carotovora subsp. *carotovora*		
AC5070	27.7	1.8
71	2.5	0.1
E. carotovora subsp. *atroseptica* Eca12	4.4	0.1
E. carotovora subsp. *betavasculorum* Ecb11129	2.9	0.1
E. chrysanthemi EC183	10.1	1.1

[a] *E. carotovora* constructs were grown in SYG supplemented with celery extract and spectinomycin to a Klett value of ca. 350, and *E. chrysanthemi* EC183 constructs were grown in polygalacturonate-yeast extract broth supplemented with spectinomycin to a Klett value of ca. 450. Culture supernatants were assayed for Pel activity.

[b] Pel-specific activity is expressed as units per A_{600} of culture.

of *E. carotovora* subsp *carotovora* 71 (4). The plasmid suppressed extracellular enzyme production in AC5070, the mini-Tn5 insertion RsmA− mutant, and in AC5047, its RsmA+ parent. A 0.5-kb *Bam*HI-*Cla*I fragment of pAKC875, which hybridized with the *rsmA* DNA flanking mini-Tn5, was cloned into pBluescript KS+ and pCL1920 to produce pAKC877 and pAKC880, respectively. To test the phenotypes conferred by this 0.5-kb DNA fragment, pAKC880 was transformed into AC5070, the bacterial constructs were grown in SYG plus celery extract medium, and culture supernatants were assayed for Pel activity (Table 2) and Peh, Cel, and Prt activities (Fig. 2). Since pAKC880 suppressed Pel, Peh, Cel, and Prt levels, we concluded that the 0.5-kb *Bam*HI-*Cla*I fragment contained the functional *rsmA* gene.

The DNA sequence of the 0.5-kb *Bam*HI-*Cla*I fragment disclosed a single 183-bp open reading frame which could encode a 6.8-kDa polypeptide of 61 amino acid residues (Fig. 1). A putative Shine-Dalgarno site (5′-AGGAG-3′) is located 6 bp upstream of the ATG start codon. In addition, there are three pairs of tandem repeats: one pair around the −10 region and the other two near the putative Shine-Dalgarno sequence (Fig. 1). Two potential hairpin loops (5′-GATGGTGTGG TTATACCATC-3′, −6.4 kcal [ca. −26.8 kJ], and 5′-CCTCAT CATCGGCGATGAGG-3′, −11.6 kcal [ca. −48.5 kJ]) are also positioned 20 to 39 and 160 to 179 bp downstream of the transcriptional start site. The significance of these structures in the expression of *rsmA* remains to be determined.

Several derivatives of pAKC877 carrying deletions from the 5′ end (D1 and D2) and the 3′ end (D3 and D4) were constructed and analyzed to determine the limits of *rsmA* required for repressor function (Fig. 1). Since *E. carotovora* subsp. *carotovora* 71 and its derivatives are naturally resistant to ampicillin, plasmids carrying these deletions were transformed into another strain, SCC3193, which, like strain 71, produces extracellular enzymes but is sensitive to ampicillin. SCC3193 carrying such plasmids or pBluescript KS+ was tested for the production of pectinases and protease on PYA or nutrient gelatin agar, respectively. With pAKC877D1, which contains 173 bp upstream of the start codon, extracellular enzyme production was suppressed. However, pAKC877D2, which retains only 22 bp upstream of the start codon, had lost the repressor function, most likely because of the deletion of the promoter region (see below) (Fig. 1). pAKC877D4, containing a deletion within the 3′ end which apparently removed three amino acids from the
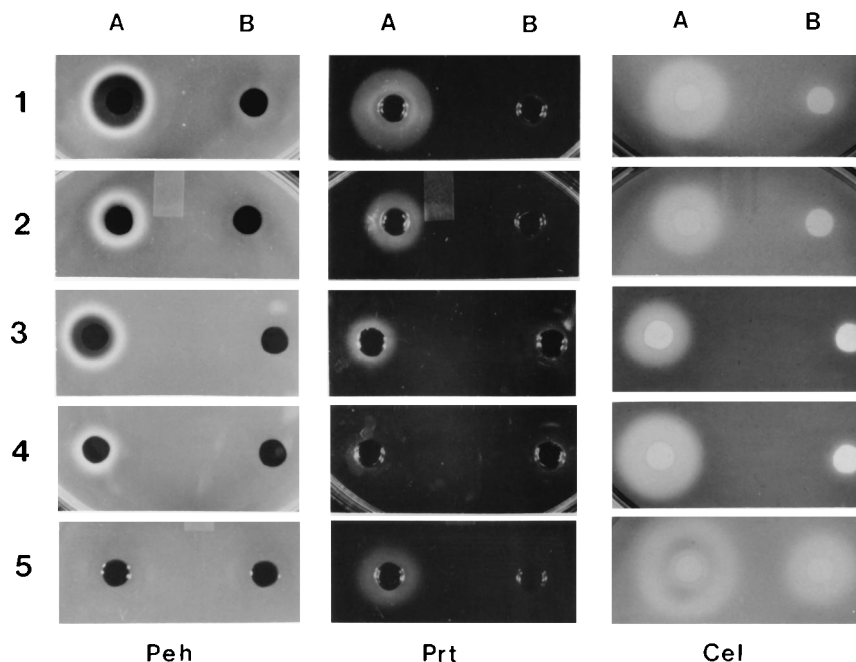
FIG. 2. Agarose plate assays for Peh, Prt, and Cel activities of *Erwinia* spp. carrying pAKC880 (columns B) or cloning vector pCL1920 (columns A). Bacteria were grown as described in Table 2, footnote *a*. Each well contained a mixture of 5 μl of culture supernatant and 5 μl of 10 mM Tris-HCl (pH 7.0). Rows: 1 and 2, *E. carotovora* subsp. *carotovora* AC5070 and 71, respectively; 3, *E. carotovora* subsp. *atroseptica* Eca12; 4, *E. carotovora* subsp. *betavasculorum* Ecb11129; 5, *E. chrysanthemi* EC183.

carboxyl end of RsmA, retained the repressor function. By contrast, the deletion in pAKC877D3 (Fig. 1), which presumably removed 16 amino acids from the carboxyl end, resulted in the loss of repressor function. These findings along with the analyses of transcriptional and translational products of *rsmA* demonstrate that the 183-bp open reading frame is responsible for the repressor function.

Homology between RsmA and *E. coli* regulatory protein CsrA. Homology search revealed 95% identity (Fig. 3) between the predicted products of *rsmA* and the *E. coli* gene *csrA* (38). As in CsrA (23), the predicted product of *rsmA* also contains a putative RNA binding domain (Fig. 3) that is similar to the KH (K protein homology) motif found only in proteins associated with RNA. To test whether *rsmA*, like *csrA*, affected glycogen synthesis, we constructed *E. coli* B carrying pAKC877 or pBluescript KS⁺. Cells of *E. coli* B carrying pBluescript KS⁺ became dark brown on exposure to iodine vapors. By contrast, the colony of *E. coli* B carrying pAKC877 remained light brown in color on exposure to iodine, indicating suppression of glycogen production by *rsmA* (data not shown).

RsmA of *E. carotovora* subsp. *carotovora* 71 has no discernible homology with the predicted products of the regulator

genes of *E. chrysanthemi*, i.e., *kdgR*, which controls the production of pectinase and pectate catabolic enzyme (33), and *pecS*, which controls the synthesis of pectinases and the blue pigment indigoidine (37).

Occurrence of *rsmA* homologs in *Erwinia* spp. and other enterobacteria. To determine the presence of *rsmA* homologs, Southern hybridizations were carried out under stringent conditions by using the 282-bp *Eco*RV-*Bgl*II *rsmA* fragment (Fig. 1) of pAKC877 as the probe. Hybridization signals were detected with *Eco*RI-digested chromosomal DNAs of *Erwinia* spp. and all other enterobacteria tested: *E. amylovora* E9 and EA246; *E. carotovora* subsp. *carotovora* 71, 193, SCRI193, SCC3193, and AH2; *E. carotovora* subsp. *betavasculorum* Ecb11129; *E. carotovora* subsp. *atroseptica* Ec and Eca12; *E. chrysanthemi* EC16, EC183, and 0706; *E. herbicola* EH105; *E. rhapontici* Er1; *E. stewartii* Es1; *Enterobacter aerogenes* Ena1; *E. coli* B and K-12; *Salmonella typhimurium* LT2; *Serratia marcescens* Sm1; *Shigella flexneri* Sf1; and *Yersinia pseudotuberculosis* Yp1 (Fig. 4). Single *Eco*RI fragments of these bacterial strains hybridized with the probe; however, the sizes of the hybridizing fragments varied depending on the bacterium. Despite these differences, the data indicate that *rsmA* sequences have been conserved in enterobacterial species that have adapted to different habitats.

Identification of *rsmA* transcripts and transcriptional start site. Northern blot analysis was carried out to identify *rsmA* transcripts. We detected a band of about 310 bases in the RNA preparation from the RsmA⁺ construct, i.e., AC5070 carrying pAKC880. By contrast, *rsmA* mRNA was not detected in the RNA sample from the RsmA⁻ construct, i.e., AC5070 carrying the cloning vector pCL1920.

The transcriptional start site was determined by primer extension (Fig. 5) to be at the guanine residue 128 bp upstream of the ATG start codon (Fig. 1). While there is a −10 consensus sequence (5′-TAAACT-3′) located 5 bp upstream of the

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RsmA  MLILTRRVGETLIIGDEVTVTVLGVKGNQVRIGVNAPKEVSVHREEIYQR  50
      *********** ************************************
CsrA  MLILTRRVGETLMIGDEVTVTVLGVKGNQVRIGVNAPKEVSVHREEIYQR  50

RsmA  IQAEKSQPTSY  61
      ******* **
CsrA  IQAEKSQQSSY  61
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FIG. 3. Alignment of deduced amino acid sequence of *rsmA* of *E. carotovora* subsp. *carotovora* 71 with that of *csrA* of *E. coli* (38). Asterisks denote identical amino acids. The putative RNA binding motifs of CsrA and RsmA that are similar to the consensus amino acid sequence of the KH (for K protein homology) motif are underlined. Numbers on the right indicate amino acid positions in each protein.

FIG. 4. Southern hybridization of *Eco*RI-digested chromosomal DNAs of *Erwinia* strains and other enterobacterial strains with *rsmA*. Lanes: 1 to 5, *E. carotovora* subsp. *carotovora* 71, 193, SCRI193, SCC3193 and AH2, respectively; 6, *E. carotovora* subsp. *betavasculorum* Ecb11129; 7 and 8, *E. carotovora* subsp. *atroseptica* Eca12 and Ec, respectively; 9 to 11, *E. chrysanthemi* EC16, EC183, and 0706, respectively; 12, *E. rhapontici* Er1; 13 and 14, *E. amylovora* E9 and EA246, respectively; 15, *E. herbicola* EH105; 16, *E. stewartii* Es1; 17 and 18, *E. coli* B and K-12, respectively; 19, *Y. pseudotuberculosis* Yp1; 20, *Salmonella typhimurium* LT2; 21, *Serratia marcescens* Sm1, 22, *Shigella flexneri* Sf1; 23, *Enterobacter aerogenes* Ena1. The lambda *Hin*dIII marker sizes are shown on the left.

transcriptional start site, we did not detect a −35 consensus sequence. On examining the sequences upstream of *csrA* (38), we also detected several putative −10 regions but no corresponding −35 regions. Since the transcriptional start site in *csrA* has not been reported, we cannot, at this juncture, predict which of these −10 regions is actually involved in the initiation of transcription. However, the presence of −10 regions and the absence of a recognizable −35 region in both *rsmA* and *csrA* raise the possibility that these genes may also be similarly regulated.

Identification of *rsmA* product. The *E. coli* T7 expression system was utilized to analyze the proteins encoded by pAKC877D2, i.e., pBluescript KS$^+$ carrying promoterless *rsmA* behind the T7 promoter. The results (Fig. 6) show that *E. coli* carrying pAKC877D2 produced a 6.8-kDa polypeptide that was not present in the lysate of *E. coli* carrying pBluescript KS$^+$. Since this overexpressed polypeptide has a molecular mass identical to the predicted *rsmA* product, we concluded that the 6.8-kDa polypeptide is RsmA.

Effect of *rsmA* on extracellular enzyme production in soft-rotting *Erwinia* spp. In order to quantify the effects of extrachromosomal copies of *rsmA* on Pel, Peh, Cel, and Prt production, pAKC880 and pCL1920 were transformed into *E. carotovora* subsp. *carotovora* 71 and AC5070, *E. carotovora* subsp. *atroseptica* Eca12, *E. carotovora* subsp. *betavasculorum*
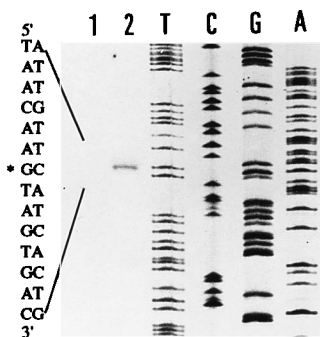

FIG. 6. Identification of *rsmA* product by T7 expression system. Lanes: 1, 5 μl of lysate of *E. coli* K38 carrying pGP1-2 and pBluescript KS$^+$; 2, 5 μl of lysate of K38 carrying pGP1-2 and pAKC877D2 (See Fig. 1 for the nucleotide sequence of the insert DNA). RsmA polypeptide with a size of ca. 6.8 kDa is indicated by the arrow.

Ecb11129, and *E. chrysanthemi* EC183. The culture supernatants of these bacteria carrying the cloning vector pCL1920 contained substantial levels of the enzymes (Fig. 2; Table 2). It should be noted that EC183 does not produce endo-Peh as shown in the Peh assay plate (Fig. 2). The levels of Pel, Peh, Cel, and Prt were either undetectable or barely detectable in culture supernatants of *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *betavasculorum*, and *E. carotovora* subsp. *carotovora* strains carrying the *rsmA$^+$* plasmid, pAKC880 (Table 2; Fig. 2). By contrast, in *E. chrysanthemi* EC183 carrying pAKC880, the level of repression of Pel and Cel was not as high as that in the *E. carotovora* subspecies. However, Prt (Fig. 2), phospholipase C (lecithinase), and exo-Peh (12) activities were totally suppressed by *rsmA* in EC183.

Suppression of plant tissue maceration by *rsmA*. Our previous work (4) had revealed that the RsmA$^−$ mutant AC5070 caused more-extensive maceration of plant tissue than the parent RsmA$^+$ strain, AC5047. To determine the effects of multiple copies of the *rsmA$^+$* allele, we transferred pAKC880 or pCL1920 into AC5070, AC5047, and the wild-type strain 71. Bacterial cells carrying these plasmids were inoculated into celery petioles. Figure 7 shows that *rsmA* prevented tissue maceration by these strains.

We then determined whether *rsmA* suppressed plant tissue macerating ability in other soft-rotting *Erwinia* spp. *E. carotovora* subsp. *batavasculorum* Ecb11129, *E. carotovora* subsp. *atroseptica* Eca12, and *E. chrysanthemi* EC183 carrying pAKC880 or pCL1920 were inoculated into celery petioles. The results (Fig. 7) demonstrate that these bacteria carrying the vector (pCL1920), but not those carrying the RsmA$^+$ plasmid, macerated celery petioles.

Effect of *rsmA* on HSL production. Since previous studies have shown that HSL production is growth-phase dependent (28, 34, 41, 44), we examined the effect of *rsmA* on HSL production during various growth stages of *E. carotovora* subsp. *carotovora* 71. Bacterial strains were grown in SYG plus celery extract supplemented with spectinomycin, and culture supernatants were tested for their ability to elicit light production in the *E. coli* Lux assay system. In this assay, the degree of bioluminescence reflects the levels of HSL in spent cultures. The data (Fig. 8) show that in strain 71 carrying pCL1920, as in other *Erwinia* spp. (44), HSL production is stimulated during later growth stages when the culture has attained high cell density. It is also apparent that while *rsmA* suppressed HSL levels during the entire growth cycle (Fig. 8), repression was more pronounced during log phase than in the stationary phase.

FIG. 5. Primer extension analysis of *rsmA* mRNA. Lanes: 1 and 2, RNA samples from RsmA$^−$ mutant AC5070 and RsmA$^+$ wild-type *E. carotovora* subsp. *carotovora* 71, respectively. The nucleotides on the left refer to the nucleotide sequence beyond the transcriptional start site. The asterisk denotes the guanine residue at which transcription was initiated.
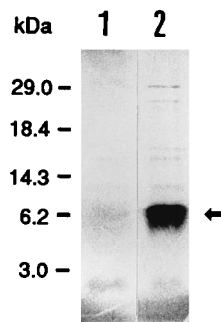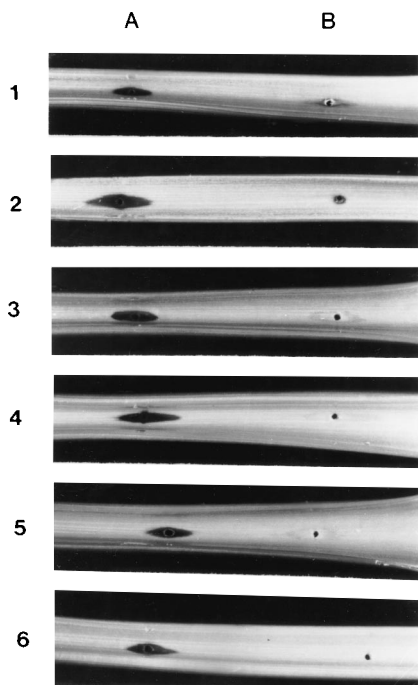
FIG. 7. Plant tissue maceration by soft-rotting *Erwinia* spp. carrying pCL1920 (columns A) or pAKC880 (columns B). About 2×10^8 cells were injected into celery petioles at each inoculation site. Inoculated celery petioles were incubated in a moist chamber at 25°C for 24 h. Rows: 1, 2, and 3: *E. carotovora* subsp. *carotovora* AC5047, AC5070, and 71, respectively; 4, *E. carotovora* subsp. *atroseptica* Eca12; 5, *E. carotovora* subsp. *betavasculorum* Ecb11129; 6, *E. chrysanthemi* EC183.
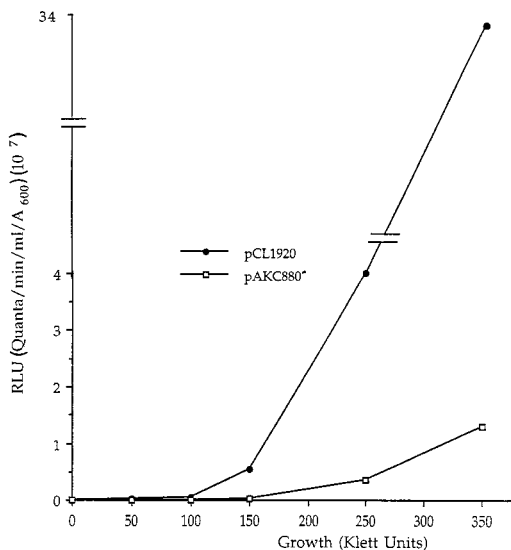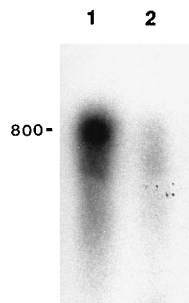

FIG. 9. Northern blot analysis of *hslI* mRNA of *E. carotovora* subsp. *carotovora* 71 carrying pCL1920 (lane 1) or pAKC880 (lane 2). Bacteria were grown in SYG supplemented with spectinomycin to a Klett value of ca. 200 at 28°C. Each lane contained 40 μg of total RNA. The position of the 800-base marker is indicated.

The suppression of HSL levels by pAKC880 raised the possibility that *rsmA* interfered with the expression of the HSL biosynthetic genes, for example, *hslI* (4). To test this possibility, we transferred the *rsmA*⁺ plasmid pAKC880 or the cloning vector pCL1920 into AC5092, which carries a copy of the *hslI-lacZ* operon fusion in the chromosome (Table 1). On McConkey lactose agar medium plus spectinomycin, AC5092 carrying pCL1920 produced pink colonies whereas AC5092 carrying pAKC880 produced white colonies. The results of quantitative assays revealed that the β-galactosidase activity in AC5092 carrying pCL1920 was about ninefold higher than the level in AC5092 carrying pAKC880 (12). Likewise, the findings of Northern blot analysis (Fig. 9) also demonstrated that the levels of *hslI* transcripts were substantially reduced in the wild-type strain 71 carrying the *rsmA*⁺ plasmid, pAKC880.

We subsequently examined the effect of *rsmA* in the other *Erwinia* strains that we had previously found to produce HSL (4). Figure 10 shows the data for the induction of bioluminescence by culture filtrate of *E. carotovora* subsp. *atroseptica* Eca12, *E. carotovora* subsp. *betavasculorum* Ecb11129, and *E. chrysanthemi* EC183 carrying pAKC880 or pCL1920. The degree of bioluminescence was consistently higher in bacteria
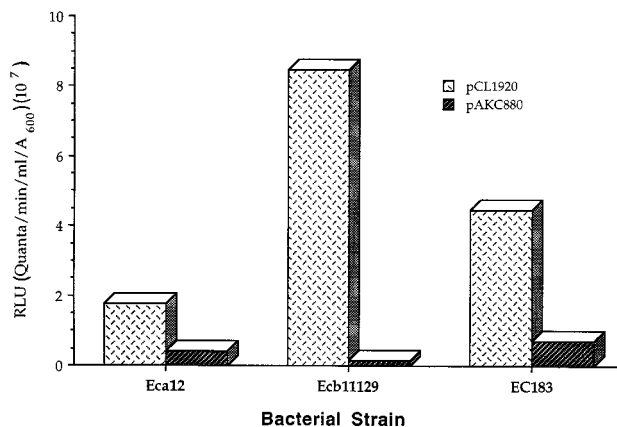

FIG. 8. Effect of spent cultures of *E. carotovora* subsp. *carotovora* 71 carrying pAKC880 and pCL1920 on light production by *E. coli* VJS533 harboring the LuxI⁻ plasmid, pHV200I. The *Erwinia* constructs were grown at 28°C in SYG supplemented with celery extract and ampicillin. Culture samples were removed at different growth stages. Filtered culture supernatants were mixed with LB plus spectinomycin to yield a final concentration of 0.5% (vol/vol). The *E. coli* cells were inoculated to produce a value of ca. 35 Klett units and incubated at 28°C. Cell density and bioluminescence were measured after 5 h of incubation. Relative light units (RLU) are expressed as counts per minute per milliliter of culture normalized for culture turbidity.

FIG. 10. Effect of spent cultures of *Erwinia* strains carrying pAKC880 and pCL1920 on light production by *E. coli* VJS533 carrying pHV200I. *E. carotovora* subsp. *atroseptica* (Eca 12) and *E. carotovora* subsp. *betavasculorum* (Ecb11129) constructs were grown in SYG supplemented with celery extract and spectinomycin to a Klett value of ca. 350, and *E. chrysanthemi* (EC183) constructs grown in polygalacturonate-yeast extract supplemented with spectinomycin to a Klett value of ca. 450. Assay conditions were the same as those described in the legend to Fig. 8.

carrying pCL1920 than in bacteria carrying the RsmA$^+$ plasmid. The data also show that the level of suppression by RsmA was higher in *E. carotovora* subsp. *betavasculorum* and *E. carotovora* subsp. *carotovora* than in *E. carotovora* subsp. *atroseptica* and *E. chrysanthemi* (Fig. 8 and 10).

DISCUSSION

In a previous communication we reported the isolation of the negative regulator gene, *rsmA*, of *E. carotovora* subsp. *carotovora* 71 (4). The data presented here establish that *rsmA* is a homolog of *csrA* of *E. coli* (38). The predicted products of these genes share a very high proportion (95%) of identical amino acids (Fig. 3). Moreover, the *Erwinia* gene suppressed glycogen synthesis in *E. coli*, and thus *rsmA* and *csrA* are functionally similar as well.

One key characteristic of the RsmA$^-$ mutant is its ability to produce extracellular enzymes in the absence of HSL (4). This observation led us to postulate that HSL interacts with RsmA in some manner to control the expression of the genes that are growth-phase dependent. As a first step in analyzing the relationship between RsmA and HSL, we tested the effect of multiple copies of *rsmA* on HSL production. Our results (Fig. 8 and 10) revealed that *rsmA* suppressed the levels of HSL in *Erwinia* strains. Moreover, in *E. carotovora* subsp. *carotovora* 71, *rsmA* reduced the levels of transcripts of *hslI*, whose product is involved in the production of HSL. Whether RsmA elicits this response by regulating *carR* (27) and *expR* (34), the *luxR* (16) homologs, has yet to be determined. However, the suppression of HSL levels by *rsmA* and the concomitant repression of extracellular enzyme production as documented here support the notion that in the presence of RsmA, HSL levels are not high enough to activate gene expression. This hypothesis is supported by the observation that the levels of HSL or its analogs must reach a high threshold level in the culture medium or the extracellular milieu in order to activate gene expression (1, 13, 20, 28, 34, 44).

Our findings strongly support the hypothesis that *rsmA* functions as a global regulator. Inhibition of extracellular Pel, Peh, Cel, and Prt production by *rsmA* occurred not only with *E. carotovora* subsp. *carotovora* strains but also with *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *betavasculorum*, and *E. chrysanthemi* strains (Table 2; Fig. 2). These bacteria carrying the *rsmA* plasmid were unable to macerate plant tissues (Fig. 7) as would be expected, since the production of extracellular pectinases was severely repressed (Fig. 2; Table 2). Southern hybridization (Fig. 4) also documented that *rsmA* or *rsmA*-like genes occur in various enterobacteria. Moreover, multiple copies of *E. carotovora rsmA* affect diverse phenotypes in enterobacteria, including the production of extracellular proteins, polysaccharides, antibiotics and pigments; motility; flagellum formation; and factors controlling host interaction (12). It is noteworthy that *csrA*, which shares extensive homology with *rsmA*, was also found to control the expression of the genes for glycogen synthesis, cell size, and cell surface properties in *E. coli* (38). In addition, Liu et al. (23) recently documented that CsrA controls glycogen production in *E. coli* by affecting mRNA stability of one of the glycogen biosynthetic genes, *glgC*, which encodes ADP-glucose pyrophosphorylase. Studies have been initiated to determine if RsmA regulates gene expression by a similar mechanism in *Erwinia* spp.

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