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rsmC of the Soft-Rotting Bacterium *Erwinia carotovora* subsp. *carotovora* Negatively Controls Extracellular Enzyme and Harpin$_{Ecc}$ Production and Virulence by Modulating Levels of Regulatory RNA (*rsmB*) and RNA-Binding Protein (RsmA)†

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Previous studies have shown that the production of extracellular enzymes (pectate lyase [Pel], polygalacturonase [Peh], cellulase [Cel], and protease [Prt]) and harpin$_{Ecc}$ (the elicitor of hypersensitive reaction) in *Erwinia carotovora* subsp. *carotovora* is regulated by RsmA, an RNA-binding protein, and *rsmB*, a regulatory RNA (Rsm stands for regulator of secondary metabolites) (Y. Liu et al., Mol. Microbiol. 29:219–234, 1998). We have cloned and characterized a novel regulatory gene, *rsmC*, that activates RsmA production and represses extracellular enzyme and harpin$_{Ecc}$ production, *rsmB* transcription, and virulence in *E. carotovora* subsp. *carotovora*. In an *rsmC* knockout mutant of *E. carotovora* subsp. *carotovora* Ecc71 carrying the chromosomal copy of the wild-type *rsmA*$^+$ allele, the basal levels of Pel, Peh, Cel, Prt, and harpin$_{Ecc}$ as well as the amounts of *rsmB*, *pel-1*, *peh-1*, *celV*, and *hrpN*$_{Ecc}$ transcripts are high, whereas the levels of *rsmA* transcripts and RsmA protein are low. Furthermore, the expression of an *rsmA-lacZ* gene fusion is lower in the RsmC$^-$ mutant than in the RsmC$^+$ parent. Conversely, the expression of an *rsmB-lacZ* operon fusion is higher in the RsmC$^-$ mutant than in the RsmC$^+$ parent. These observations establish that RsmC negatively regulates *rsmB* transcription but positively affects RsmA production. Indeed, comparative studies with an RsmC$^-$ mutant, an RsmA$^-$ mutant, and an RsmA$^-$ RsmC$^-$ double mutant have revealed that the negative effects on exoprotein production and virulence are due to the cumulative regulatory effects of RsmC on *rsmA* and *rsmB*. Exoprotein production by the RsmC$^-$ mutant is partially dependent on the quorum sensing signal, *N*-(3-oxohexanoyl)-L-homoserine lactone. Southern blot data and analysis of PCR products disclosed the presence of *rsmC* sequences in *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *betavasculorum*, and *E. carotovora* subsp. *carotovora*. These findings collectively support the idea that *rsmA* and *rsmB* expression in these plant pathogenic *Erwinia* species is controlled by RsmC or a functional homolog of RsmC.

Erwinia species produce extracellular enzymes and proteins, polysaccharides, pigments, and small diffusible metabolites (2, 5, 43). The production of such substances is markedly stimulated during late exponential and early stationary growth phases when bacteria reach high cell density and experience nutrient limitation and other forms of stress (14, 31, 36, 39, 44). How bacteria perceive these conditions and then activate gene expression are issues that have lately attracted considerable attention. Consequently, at least four regulatory parameters are now recognized to be of critical importance in the activation of growth phase-dependent (secondary) metabolite production. One entails the production of RpoS, an alternate sigma factor responsible for the activation of many genes expressed mainly during the stationary phase (14, 44). The other factor is the cell density (quorum) sensing signal, N-acylated derivatives of homoserine lactone, that apparently accumulate during these growth conditions and activate the expression of an array of genes many of which are expressed during postexponential growth (11, 12, 36, 41). The other two parameters, RsmA and *rsmB* RNA, control the production of extracellular enzymes, phytohormones, antibiotics, pigments, and polysaccharides, the synthesis of flagella, and levels of the quorum sensing signal *N*-(3-oxohexanoyl)-L-homoserine lactone (OHL) in various *Erwinia* species; they also affect virulence and the production of *Erwinia carotovora* subsp. *carotovora* harpin (harpin$_{Ecc}$), the elicitor of the hypersensitive reaction (4, 6, 19, 26).

Recent studies have disclosed that RsmA is an RNA-binding protein and that it promotes message decay (6), although how this is brought about awaits clarification. *rsmB* (previously *aepH* [28]), on the other hand, specifies a unique RNA regulator that apparently neutralizes RsmA action by forming an inactive ribonucleoprotein complex (19). The current model postulates that RsmA and *rsmB* act antagonistically to modulate the expression of many genes, particularly those that are expressed in a growth phase-dependent manner. Romeo and associates have characterized a very similar system comprising CsrA (a RsmA homolog) and *csrB* (a *rsmB* homolog), which controls glycogen accumulation, cell surface properties, and cell size in *Escherichia coli* (reference 34 and references cited therein).

There is growing evidence that RsmA levels and RsmA activity are rigorously controlled by bacteria to prevent extensive decay of transcripts of genes for essential functions. Several lines of evidence support this view. First, overexpression of *rsmA* from high-copy-number plasmids or artificial strong promoters is generally detrimental to cell physiology and in certain hosts is even lethal (25). Second, *rsmA* expression in *Erwinia* and other enterobacteria (i.e., *Salmonella typhimurium*) is con-

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trolled by sigma-S as well as sigma-70 (27). Moreover, *E. carotovora* subsp. *carotovora* uses a novel regulatory mechanism involving KdgR, a global negative regulator of IclR family, to modulate the levels of *rsmB* RNA (20), which in turn controls RsmA action.

In the course of our search for regulatory mutants of *E. carotovora* subsp. *carotovora*, we discovered a class of transposon insertion mutants that produced very high basal levels of extracellular enzymes, as previously noted with RsmA$^-$ mutants (4, 6). Subsequent physical evidence, however, revealed that the phenotypes of the new mutants resulted from disruption of a previously unidentified locus, which we have designated, *rsmC* (for regulator of secondary metabolism). We describe here the structure and function of *rsmC*. Our data for the first time show that RsmC controls the production of RsmA and *rsmB* RNA and that the phenotypic changes in RsmC$^-$ mutants are due to these regulatory effects of RsmC. Physical evidence shows that homologs of *E. carotovora* subsp. *carotovora* Ecc71 *rsmC* occur in other *E. carotovora* subspecies, i.e., *atroseptica* and *betavasculorum*. Based on the data presented here and previously reported (4, 6, 19), we conclude that RsmA, *rsmB*, and RsmC are the major components of a global regulatory system that controls gene expression in several plant pathogenic enterobacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. The compositions of Luria-Bertani (LB) medium, minimal salts medium, minimal salts medium plus celery extract, nutrient gelatin agar have been previously described (4, 29). When required, antibiotics were added as follows: ampicillin, 100 μg/ml; gentamicin, 10 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 50 μg/ml; spectinomycin, 50 μg/ml; and tetracycline, 10 μg/ml. Media were solidified by the addition of 1.5% (wt/vol) agar. The compositions of agarose media for semiquantitative plate assay for extracellular pectate lyase (Pel), polygalacturonase (Peh), cellulase (Cel), and protease (Prt) have been described by Chatterjee et al. (4).

Preparation of samples for enzyme assays and assay conditions. The preparation of enzyme samples for extracellular Pel, Peh, Prt, and Cel, and the assay procedures were carried out according to Murata et al. (29). The semiquantitative agarose plate assays for extracellular Pel, Peh, Prt, and Cel were performed as described by Chatterjee et al. (4).

Isolation of RsmC$^-$ mutant by mini-Tn*5* mutagenesis. The Nal^r strain AC5047 (Table 1) was mutagenized with mini-Tn*5*-Km as described by Chatterjee et al. (4). Transconjugants were selected on nutrient gelatin agar medium containing kanamycin and nalidixic acid. Protease-overproducing mutants, identified by the size of halo around the colony, were tested for a pleiotropic phenotype by semiquantitative agarose plate assays for Pel, Peh, and Cel.

DNA techniques. Standard procedures were used in the isolation of plasmid and chromosomal DNAs, transformation, restriction endonuclease digests, gel electrophoresis, DNA ligation, and colony in situ hybridization (38). Southern blot hybridizations were carried out as described by Cui et al. (6). PCR was performed as described by Liu et al. (19). Restriction and modifying enzymes were obtained from Promega Biotec (Madison, Wis.).

Nucleotide sequence analysis of *rsmC*. For nucleotide sequence analysis, the *rsmC* fragments flanking the mini-Tn*5*-Km sequence in pAKC973 (Table 1) were cloned into the *Cla*I-*Hin*dIII sites of pBluescript SK(+). The resulting plasmids, pAKC976 and pAKC977, were used for producing unidirectional deletions into the *rsmC* sequence with the Erase-a-Base system (Promega Biotec). Plasmids carrying deletions were used for sequence analysis using Sequenase version 2.0 (U.S. Biochemical, Cleveland, Ohio). Oligonucleotide primers were also used in nucleotide sequence determinations. DNA and protein sequence analyses were performed with the PC gene software (IntelliGenetics, Inc., Mountain View, Calif.).

RNA assays. Total RNA was extracted by the method of Aiba et al. (1) from *E. carotovora* subsp. *carotovora* strains grown at 28°C in minimal salts medium plus sucrose (0.5% wt/vol) or in this medium supplemented with appropriate drugs.

Northern blot analyses were performed as described by Liu et al. (19). The probes used in this study were the 304-bp *Eco*RV-*Hin*dIII fragment of *rsmC* from pAKC975 (see Fig. 3A), the 314-bp *Eco*RV-*Kpn*I fragment of *pel-1* from pAKC783 (18), the 743-bp *Hin*dIII fragment of *peh-1* from pAKC781 (18), the 200-bp *Eco*RI fragment of *celV* from pACK1034 (19), the 779-bp *Eco*RV-*Sma*I fragment of $hrpN_{Ecc}$ from pAKC924 (7), the 183-bp *Nde*I-*Sal*I fragment of *rsmA* from pAKC882, and the 321-bp *Bam*HI-*Hin*dIII fragment of *rsmB* from pAKC1004. DNA probes were labeled with [α-^{32}P]dATP by using the Prime-a-Gene labeling system (Promega Biotec) according to the manufacturer's instructions.

Primer extension assay was performed as instructed by the manufacturer (Promega Biotec) with primer rsmC1 (see Fig. 3A) and 20 μg of RNA.

Identification of the *rsmC* product. *E. coli* JM109(DE3) carrying the cloning vector, pET28a(+), or pAKC978 (Table 1), which contains the coding region of *rsmC* in the expression vector pET28a(+), were grown at 37°C in LB medium containing kanamycin. When the cultures reached an A_{600} of 0.7, each culture was divided into two parts; isopropyl-β-D-thiogalactopyranoside (IPTG) was added to one part to yield a final concentration of 1.0 mM, and the other part served as the control. Following an additional 3 h of incubation, cells were collected by centrifugation. Double-strength sodium dodecyl sulfate (SDS) loading buffer (100 mM Tris-HCl [pH 6.8], 200 mM dithiothreitol, 4% [wt/vol] SDS, 0.2% [wt/vol] bromophenol blue, 20% [vol/vol] glycerol) was added, and the samples were boiled for 5 min. Proteins were fractionated by 0.1% (wt/vol) SDS–15% (wt/vol) polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie brilliant blue.

Western blot analysis. *E. carotovora* subsp. *carotovora* Ecc71, AC5053, AC5054, and AC5071 were grown at 28°C in minimal salts medium plus sucrose to an A_{600} value of 2.3. Total bacterial protein was precipitated with trichloroacetic acid at a final concentration of 10% (vol/vol) and resuspended in 1× SDS loading buffer. The protein concentrations were determined with a bicinchoninic acid protein assay kit (Pierce Corp., Rockford, Ill.) according to the manufacturer's specifications. Western blot analysis of the total bacterial protein was carried out as described by Mukherjee et al. (24). The antibody raised against the harpin from strain *E. chrysanthemi* (3) was used as the probe for $harpin_{Ecc}$. The anti-RsmA antiserum produced against a synthesized peptide from amino acids 48 to 61 of RsmA (6) in rabbit by Genemed Biotechnologies Inc. (San Francisco, Calif.) was used as the probe for RsmA.

Construction of *rsmA-lacZ* and *csrA-lacZ* fusions and β-galactosidase assay. To construct *lacZ* fusions of the *rsmA* genes of *E. amylovora*, *E. carotovora* subsp. *carotovora*, and *E. herbicola* pv. gypsophilae and the *csrA* gene of *E. coli*, the promoter regions of these genes were amplified by PCR from the chromosomal DNAs of *E. amylovora* E9, *E. carotovora* subsp. *carotovora* Ecc71, *E. herbicola* pv. gypsophilae PD713, and *E. coli* MC4100 with primers designed from the nucleotide sequences of these genes (6, 25, 35). PCR products were digested with *Eco*RI and *Bam*HI and cloned into the promoter-probe vector pNM481Sp^r to yield pAKC887, pAKC888, pAKC889, and pAKC890 (Table 1). *E. carotovora* subsp. *carotovora* AC5047 and AC5050 carrying these constructs were grown at 28°C in minimal salts medium plus sucrose and spectinomycin to an A_{600} of 2.0, and culture samples were assayed for β-galactosidase activity as described by Miller (22).

Construction of RsmA$^-$ RsmC$^-$ and RsmC$^-$ Ohl$^-$ double mutants. To inactivate *rsmC* in the RsmC$^+$ plasmid pAKC979, the omega Sp^r DNA fragment was inserted at the *Eco*RV site (see Fig. 3A) to yield pAKC980. To confirm that *rsmC* was inactivated in pAKC980, this plasmid was transformed into Ecc71 and AC5053, and the exoenzyme levels were checked. The assay data showed that pAKC980 did not suppress levels of Pel, Peh, Cel, and Prt, indicating that *rsmC* was inactivated. To construct an RsmA$^-$ RsmC$^-$ double mutant, pAKC980 was transferred into the RsmA$^-$ strain AC5071 by using the helper plasmid pRK2013. To construct an RsmC$^-$ Ohl$^-$ double mutant, pAKC855 (Table 1) was transferred into the RsmC$^-$ strain AC5050 by using the helper plasmid pRK2013. Transconjugants were selected on minimal salts agar plus sucrose (0.2%, wt/vol) and spectinomycin, and Sp^r Tc^s isolates were obtained. The inactivation of *rsmC* in AC5054 was confirmed by Northern analysis. The inactivation of *ohl* in AC5051 was determined by assaying for OHL production (4).

Plant tissue maceration. The celery petiole assays were previously described (29). The extent of tissue maceration was estimated visually.

Nucleotide sequence accession number. The GenBank accession number for *rsmC* is AF178852.

RESULTS

Isolation of mini-Tn5-Km insertion RsmC$^-$ mutants. *E. carotovora* subsp. *carotovora* AC5047 was mutagenized with mini-Tn*5*-Km, and the Km^r transconjugants were screened for increased protease activity on nutrient gelatin agar medium. Colonies with high protease activity were subsequently tested for the levels of pectinases and cellulase. The characteristics of one class of mutants represented by AC5070 and AC5071 and designated as RsmA$^-$ have been described elsewhere (4, 6, 24). Here we report the characteristics of another class of derepressed mutant and the corresponding gene, *rsmC*. The mutant strain, AC5050, and its parent strain, AC5047, were grown in minimal salts medium supplemented with sucrose, and culture samples were assayed for the levels of extracellular enzymes. The data in Fig. 1A show that the levels of Pel, Peh, Cel, and Prt activities in AC5050 are markedly higher than

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
E. carotovora subsp. *carotovora*		
Ecc71	Wild type	45
AC5047	Nal^r derivative of AC5006	4
AC5050	RsmC$^-$ derivative of AC5047 by mini-Tn*5*-Km mutagenesis	This work
AC5051	Ohl$^-$ derivative of AC5050 by marker exchange with pAKC855	This work
AC5053	RsmC$^-$ derivative of Ecc71 by marker exchange with pAKC970	This work
AC5054	RsmC$^-$ derivative of AC5071 by marker exchange with pAKC980	This work
AC5070	RsmA$^-$ derivative of AC5047	4
AC5071	RsmA$^-$ derivative of Ecc71	24
AC5091	Ohl$^-$ derivative of AC5047	4
SCRI193	Wild type	37
E. carotovora subsp. *atroseptica* Eca12	Wild type	45
E. carotovora subsp. *betavasculorum* Ecb11129	Wild type	J. E. Loper
E. herbicola pv. gypsophilae PD713	Wild type	17
Escherichia coli		
DH5α	ϕ80*lacZ*Δ*M15*Δ(*lacZYA-argF*) *U169 hsdR17 recA1 endA1 thi-1*	Gibco BRL
JM109(DE3)	*endA1 recA1 gyrA96 hsdR17 supE44 relA1 thi* Δ(*lac-pro*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*) λ*c*I857 *ind1 Sam7 lacU5*-T7 gene 1	Promega Biotec
MC4100	*araD139* Δ(*lacIPOZYA*)*U169 recA1 thi-1* Str^r	18
Plasmids		
pBluescript SK(+)	Ap^r	Stratagene
pCL1920	Sp^r, Sm^r	16
p34S-Gm	Gm^r; source of Gm^r cassette	8
pCL1920Gm^r	Gm^r, Sp^r, Sm^r, Gm^r cassette inserted at the *Bam*HI site of pCL1920	This work
pET-28a(+)	Km^r	Novagen
pHP45Ω	Source of Sp^r omega fragment	33
pLARF5	Tc^r	15
pMP220	Tc^r; promoter-probe vector	40
pNM481	Ap^r, promoter-probe vector	23
pNM481Sp^r	Sp^r, Sp^r omega fragment inserted at the *Sca*I site of pNM481	This work
pRK415	Tc^r	15
pRK2013	Mob$^+$, Tra$^+$, Km^r	10
pAKC781	Ap^r, *peh-1*$^+$	18
pAKC783	Ap^r, *pel-1*$^+$	18
pAKC882	Ap^r, pT7-*rsmA*	26
pAKC855	Sp^r, Tc^r, Ohl$^-$	4
pAKC887	Sp^r, *rsmA*$_{Ecc}$-*lacZ*, 279-bp (245 bases upstream of translational start site to 34 bases downstream of translational start site) PCR product from Ecc71 in pNM481Sp^r	This work
pAKC888	Sp^r, *rsmA*$_{Ea}$-*lacZ*, 317-bp (283 bases upstream of translational start site to 34 bases downstream of translational start site) PCR product from E9 in pNM481Sp^r	This work
pAKC889	Sp^r, *rsmA*$_{Ehg}$-*lacZ*, 300-bp (266 bases upstream of translational start site to 34 bases downstream of translational start site) PCR product from PD713 in pNM481Sp^r	This work
pAKC890	Sp^r, *csrA-lacZ*, 287 bp (252 bases upstream of translational start site to 34 bases downstream of translational start site) PCR product from MC4100 in pNM481Sp^r	This work
pAKC924	Ap^r, *hrpN*$_{Ecc}$ in pBluescript SK(+)	7
pAKC970	Km^r, Tc^r; pLARF5 containing mini-Tn*5*-Km and flanking chromosomal DNA from AC5050	This work
pAKC971	Tc^r; pLARF5 containing *rsmC* from genomic library of Ecc71	This work
pAKC972	Tc^r; pLARF5 containing *rsmC* from genomic library of Ecc71	This work
pAKC973	Ap^r, 4.0-kb *Cla*I fragment of pAKC970 containing mini-Tn*5*-Km and flanking DNA in pBluescript SK (+)	This work
pAKC974	Ap^r; RsmC$^+$, 2.0-kb *Cla*I fragment of pAKC971 in pBluescript SK (+)	This work
pAKC975	Sp^r, RsmC$^+$, 2.0-kb *Cla*I fragment of pAKC971 in pCL1920	This work
pAKC976	Ap^r, 1.5-kb *Cla*I-*Hin*dIII fragment of pAKC973 (flanking DNA of mini-Tn*5*-Km) in pBluescript SK (+)	This work
pAKC977	Ap^r, 0.5-kb *Cla*I-*Hin*dIII fragment of pAKC973 (flanking DNA of mini-Tn*5*-Km) in pBluescript SK (+)	This work
pAKC978	Km^r, 371-bp *Eco*RV-*Hin*dIII fragment of pAKC974 in pET28a(+)	This work
pAKC979	Tc^r, RsmC$^+$, 2.0-kb *Cla*I fragment of pAKC971 in pRK415	This work
pAKC980	Tc^r, Sp^r; RsmC was inactivated in pAKC979 by inserting an omega fragment at the *Eco*RV site	This work
pAKC1002	Tc^r, *rsmB-lacZ* in pMP220	19
pAKC1004	Sp^r, *plac-rsmB′* in pCL1920	19
pAKC1004Gm^r	Gm^r, Sp^r, Gm^r cassette inserted at the *Bam*HI site of pAKC1004	This work
pAKC1034	Ap^r, 200-bp DNA fragment of *celV* from Ecc71 in pGEM-T Easy	19

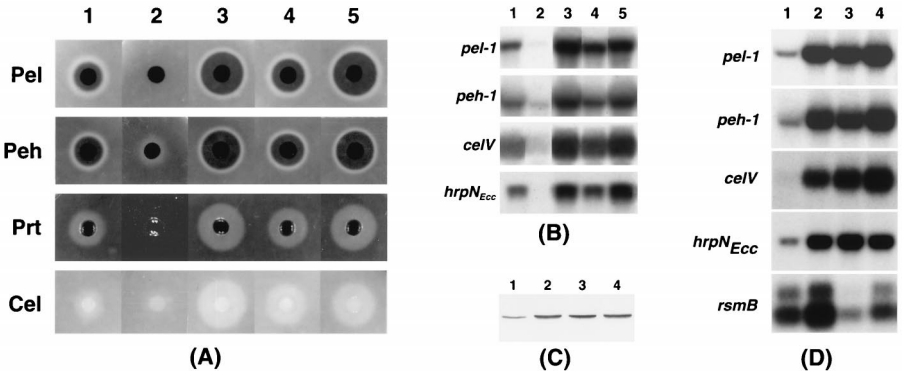

FIG. 1. (A) Agarose plate assays for Pel, Peh, Prt, and Cel activities of *E. carotovora* subsp. *carotovora* AC5047 (column 1), the Ohl$^-$ derivative of AC5047 (AC5091; column 2), the RsmC$^-$ mutant (AC5050; column 3), the Ohl$^-$ derivative of AC5050 (AC5051; column 4), and the RsmA$^-$ mutant of AC5047 (AC5070; column 5). Bacteria were grown at 28°C in minimal salts medium plus sucrose and harvested at an A_{600} value of 2.5. Culture supernatants (10 μl) were added to each well. After 18 h of incubation at 28°C, the Pel and Peh assay plates were developed with 4 N HCl, and the Cel assay plate was developed with Congo red and NaCl solutions. Halos around the wells in the Prt assay plate became visible within 24 h without any further treatment. (B) Northern blot analysis of *pel-1*, *peh-1*, *celV*, and *hrpN*$_{ECC}$ mRNA in *E. carotovora* subsp. *carotovora* AC5047 (RsmA$^+$ RsmC$^+$ Ohl$^+$; lane 1), AC5091 (RsmA$^+$ RsmC$^+$ Ohl$^-$; lane 2), AC5050 (RsmA$^+$ RsmC$^-$ Ohl$^+$; lane 3), AC5051 (RsmA$^+$ RsmC$^-$ Ohl$^-$; lane 4), and AC5070 (RsmA$^-$ RsmC$^+$ Ohl$^+$; lane 5). Bacteria were grown at 28°C in minimal salts medium plus sucrose to an A_{600} of 2.0 for total RNA extraction. Each lane contained 10 μg of total RNA. (C) Western blot analysis of harpin$_{Ecc}$ produced by *E. carotovora* subsp. *carotovora* Ecc71 (RsmA$^+$ RsmC$^+$; lane 1), AC5053 (RsmA$^+$ RsmC$^-$; lane 2), AC5071 (RsmA$^-$ RsmC$^+$; lane 3), and AC5054 (RsmA$^-$ RsmC$^-$; lane 4). Each lane contained 20 μg of total bacterial protein. (D) Northern blot analysis of *pel-1*, *peh-1*, *celV*, *hrpN*$_{ECC}$, and *rsmB* transcripts in *E. carotovora* subsp. *carotovora* Ecc71 (RsmA$^+$ RsmC$^+$; lane 1), AC5053 (RsmA$^+$ RsmC$^-$; lane 2), AC5071 (RsmA$^-$ RsmC$^+$; lane 3), and AC5054 (RsmA$^-$ RsmC$^-$; lane 4). Bacteria were grown at 28°C in minimal salts medium plus sucrose to an A_{600} of 2.0 for total RNA extraction. Each lane contained 10 μg of total RNA.

those in the parent strain, AC5047. Moreover, the results of Northern hybridization analysis using *pel-1*, *peh-1*, *celV*, and *hrpN*$_{Ecc}$ as probes revealed that the levels of transcripts of these genes are considerably higher in the RsmC$^-$ mutant than in the parent strain (Fig. 1B). These findings demonstrate that the negative effect of *rsmC* on Pel, Peh, and Cel production is due to the modulation of transcript levels. Further support for this conclusion comes from the findings with an RsmC$^-$ mutant of *E. carotovora* subsp. *carotovora* Ecc71 constructed by marker exchange and the transdominant effects of the cloned *rsmC*$^+$ DNA (see below).

Since the phenotypes of AC5050 and AC5070 are very similar (Fig. 1A, columns 3 and 5; also see references 4, 6, and 7), it was initially deemed important to determine if the mutants were genetically different. For this, we first localized the sites of mini-Tn*5*-Km insertion in these mutants. Chromosomal DNAs of the mutants and the parent strain, AC5047, were digested with the restriction enzyme *Cla*I, which does not have a recognition site in the mini-Tn*5*-Km DNA. The fragments were resolved in an agarose gel, transferred to a nylon membrane, and hybridized with the labeled transposon DNA under stringent conditions. Results (data not shown) indicated that while there were no hybridizing signals with AC5047, the probe hybridized to DNA fragments of about 11 kb of AC5070 and 4 kb of AC5050. Since *rsmA* does not possess a *Cla*I site (4), these data indicated that the mutants have insertions in different genes. To determine if the disruption of a functional gene due to the insertion of the transposon in the chromosome of AC5050 was in fact responsible for the higher levels of enzymatic activities, we cloned the mini-Tn*5*-Km DNA along with flanking AC5050 DNA into the cosmid vector pLAFR5, yielding pAKC970 (Table 1). This plasmid was transferred to Ecc71, and a spontaneous Km^r Tc^s derivative, AC5053, was obtained according to the procedures described by Chatterjee et al. (4). The absence of a functional *rsmC*$^+$ allele in AC5053 due to its replacement by *rsmC*::mini-Tn*5* DNA was confirmed by Northern analysis (data not shown). The parent strain, Ecc71, and its RsmC$^-$ derivative, AC5053, were grown in minimal salts medium supplemented with sucrose, and culture samples were assayed for Pel, Peh, Cel, and Prt activities as well as harpin$_{Ecc}$. The data (Table 2; Fig. 1C, lanes 1 and 2) show that the levels of Pel, Peh, Cel, Prt, and harpin$_{Ecc}$ in

TABLE 2. Levels of Pel, Peh, Prt, and Cel produced by *E. carotovora* subsp. *carotovora* strains

Strain[a]	Relevant phenotype[b]	Sp act (U/ml/A_{600} unit; mean ± SD)[c]			
		Pel	Peh	Prt	Cel
Ecc71	RsmA$^+$ RsmC$^+$	0.19 ± 0.03	66.2 ± 3.21	1.4 ± 0.28	ND
AC5053	RsmA$^+$ RsmC$^-$	5.00 ± 0.19	441.8 ± 8.64	57.9 ± 2.32	1.32 ± 0.26
AC5071	RsmA$^-$ RsmC$^+$	6.97 ± 0.23	391.8 ± 2.31	23.8 ± 2.74	0.90 ± 0.03
AC5054	RsmA$^-$ RsmC$^-$	15.77 ± 0.43	744.5 ± 18.69	77.8 ± 4.15	3.32 ± 0.09
AC5047(pCL1920)	RsmA$^+$ RsmC$^+$ (none [vector])	0.17 ± 0.02	10.3 ± 0.26	—	—
AC5047(pAKC975)	RsmA$^+$ RsmC$^+$ (RsmC$^+$)	0.06 ± 0.01	ND	—	—
AC5050(pCL1920)	RsmA$^+$ RsmC$^-$ (none [vector])	2.10 ± 0.11	341.0 ± 9.70	—	—
AC5050(pAKC975)	RsmA$^+$ RsmC$^-$ (RsmC$^+$)	0.11 ± 0.01	36.7 ± 1.70	—	—

[a] Bacteria were grown at 28°C in minimal salts medium plus sucrose (0.5% [wt/vol]) for Pel, Peh, and Cel assays or in this medium plus celery extract for Prt assay. Strains carrying plasmids were grown in minimal salts medium plus sucrose and spectinomycin. Cultures were harvested at an A_{600} value of 2.5, and the culture supernatants were used for enzyme assays.

[b] Relevant phenotypes conferred by the plasmids are indicated in parentheses.

[c] ND, not detectable; —, not done.

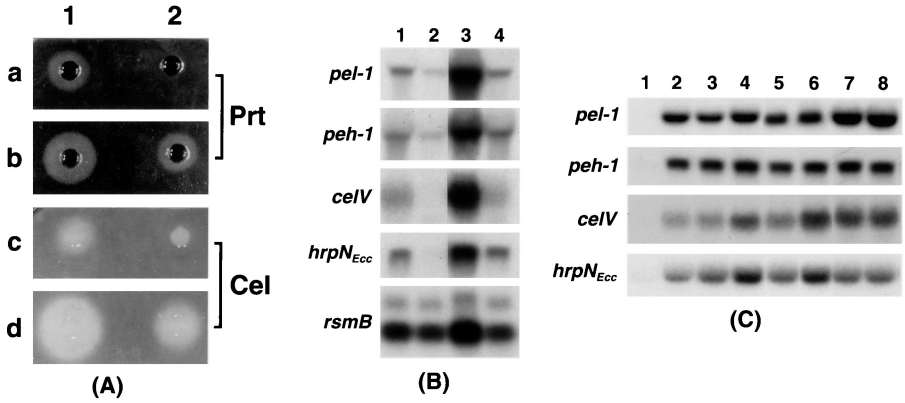

FIG. 2. (A) Agarose plate assays for Prt and Cel activities of *E. carotovora* subsp. *carotovora* AC5047 (a and c) and its RsmC$^-$ mutant (AC5050; b and d) carrying the cloning vector, pCL1920 (column 1) or the RsmC$^+$ plasmid, pAKC975 (column 2). Bacteria were grown at 28°C in minimal salts medium plus sucrose and spectinomycin and harvested at an A_{600} of 2.5. Culture supernatants (10 μl) were added to each well. (B) Northern blot analysis of *pel-1*, *peh-1*, *celV*, *hrpN$_{Ecc}$*, and *rsmB* transcripts produced by *E. carotovora* subsp. *carotovora* AC5047 (RsmC$^+$) and AC5050 (RsmC$^-$) carrying the cloning vector pCL1920 or the RsmC$^+$ plasmid pAKC975. Lane 1, AC5047 carrying pCL1920; lane 2, AC5047 carrying pAKC975; lane 3, AC5050 carrying pCL1920; lane 4, AC5050 carrying pAKC975. Total RNAs were isolated from bacteria grown at 28°C in minimal salts medium plus sucrose and spectinomycin to an A_{600} of 2.0. Each lane contained 10 μg of total RNA. (C) Northern blot analysis of *pel-1*, *peh-1*, *celV*, and *hrpN$_{Ecc}$* mRNA produced by *E. carotovora* subsp. *carotovora* Ecc71 (RsmA$^+$ RsmC$^+$), AC5053 (RsmA$^+$ RsmC$^-$), AC5071 (RsmA$^-$ RsmC$^+$), and AC5054 (RsmA$^-$ RsmC$^-$) carrying the cloning vector pCL1920Gm^r or the *rsmB*$^+$ plasmid pAKC1004Gm^r. Lane 1, Ecc71/pCL1920Gm^r; lane 2, Ecc71/pAKC1004Gm^r; lane 3, AC5053/pCL1920Gm^r; lane 4, AC5053/pAKC1004Gm^r; lane 5, AC5071/pCL1920Gm^r; lane 6, AC5071/pAKC1004Gm^r; lane 7, AC5054/pCL1920Gm^r; lane 8, AC5054/pAKC1004Gm^r. Total RNAs were isolated from bacteria grown at 28°C in minimal salts medium plus sucrose and gentamicin to an A_{600} of 2.0. Each lane contained 10 μg of total RNA.

AC5053 are markedly higher than those in the parent strain, Ecc71. Moreover, the results of Northern hybridization analysis (Fig. 1D, lanes 1 and 2) reveal that the levels of transcripts of *pel-1*, *peh-1*, *celV* and *hrpN$_{Ecc}$* are considerably higher in the RsmC$^-$ mutant than in the parent strain.

Cloning of the wild-type *rsmC*$^+$ allele. The 4-kb *Cla*I fragment from pAKC970 encompassing the transposon sequence and the flanking DNA (Table 1) was used as the probe in colony hybridization of Ecc71 genomic library, yielding two hybridizing plasmids, pAKC971 and pAKC972. The parent strain (AC5047) and its RsmC$^-$ mutant (AC5050) carrying these plasmids produced levels of extracellular enzymes much lower than the levels produced by these strains carrying the cloning vector pLAFR5 (data not shown). The 2-kb wild-type *Cla*I fragment of pAKC971 containing the *rsmC*$^+$ DNA was subcloned into plasmid pCL1920, yielding pAKC975. *E. carotovora* subsp. *carotovora* AC5047 and AC5050 were transformed with pAKC975 or the cloning vector pCL1920, and the bacterial constructs were grown in minimal salts medium supplemented with sucrose and spectinomycin. The culture supernatants were assayed for Pel, Peh, Cel, and Prt activities. It is apparent that multiple copies of *rsmC*$^+$ DNA caused the repression of Pel and Peh production (Table 2) as well as Prt and Cel levels (Fig. 2A) in both the parent strain (AC5047) and the RsmC$^-$ (AC5050). Moreover, these strains carrying multiple copies of the *rsmC*$^+$ DNA produced significantly lower levels of transcripts of *pel-1*, *peh-1*, *celV*, and *hrpN$_{Ecc}$* than the strains carrying the vector (Fig. 2B). A very similar effect of multiple copies of the *rsmC*$^+$ DNA was noted with Ecc71 and its RsmC$^-$ mutant, AC5053 (data not shown). This transdominant effect of the cloned DNA taken along with other findings described above indicated that the phenotypes of AC5050 and AC5053 resulted from the inactivation of a regulatory gene which negatively controls harpin and extracellular enzyme production.

Analysis of the nucleotide sequence of *rsmC* and identification of the gene product. Sequence analysis of the DNA regions flanking mini-Tn*5*-Km in pAKC973 revealed that the minitransposon had inserted into an open reading frame (ORF) (Fig. 3A) which could encode a protein of 130 amino acid residues. Ten bases upstream of the transcriptional start site (Fig. 3B), there is a typical −10 consensus sequence of a sigma-70-type promoter (Fig. 3A). Database (BLAST 2.0) search for sequences homologous to RsmC revealed no protein with significant homology. However, a segment of RsmC (amino acid residues 33 to 99) has 43% identity with a segment (amino acid residues 734 to 800) of a putative transcriptional adaptor of *Caenorhabditis elegans* (GenBank accession no. U20864). To establish that the ORF deduced from the sequence data is actually functional, we identified the transcripts and the corresponding protein product. Total RNA samples from the parent strain (Ecc71) and the RsmC$^-$ mutant (AC5053) grown in minimal medium were hybridized with *rsmC*. The results (Fig. 3C) show that Ecc71 produced an *rsmC* transcript of about 600 bases, while AC5053 produced a diffuse weak signal of an apparently truncated transcript. The size of the transcript produced by Ecc71 is consistent with the size of the *rsmC* ORF. To identify the product of *rsmC*, the 371-bp *Eco*RV-*Hin*dIII DNA fragment containing the coding region of *rsmC* (Fig. 3A) was cloned into the expression vector pET28a(+), making pAKC978, where *rsmC* is under the transcriptional control of T7 promoter. *E. coli* JM109(DE3) carrying pAKC978 and the cloning vector were grown at 37°C in LB medium containing kanamycin and induced by IPTG. Bacterial cells were collected and the total bacterial protein samples were assayed by SDS-PAGE in a 15% (wt/vol) polyacrylamide gel. After IPTG induction, a protein of ca. 15 kDa was produced by JM109(DE3) carrying pAKC978 (Fig. 3D, lane 4) but not by JM109(DE3) carrying the cloning vector pET28a(+) (Fig. 3D, lane 2). The apparent molecular mass of 15 kDa of the overproduced protein matches the mass of 14.5 kDa of the polypeptide deduced from the *rsmC* sequence, further indicating that this protein band is the product of *rsmC*. Without IPTG induction, this protein band was not detected with JM109(DE3) carrying pET28a(+) (Fig. 3D, lane 1), although a faint band was visible at the same position with JM109(DE3) carrying pAKC978 (Fig. 3D, lane 3). This may have resulted

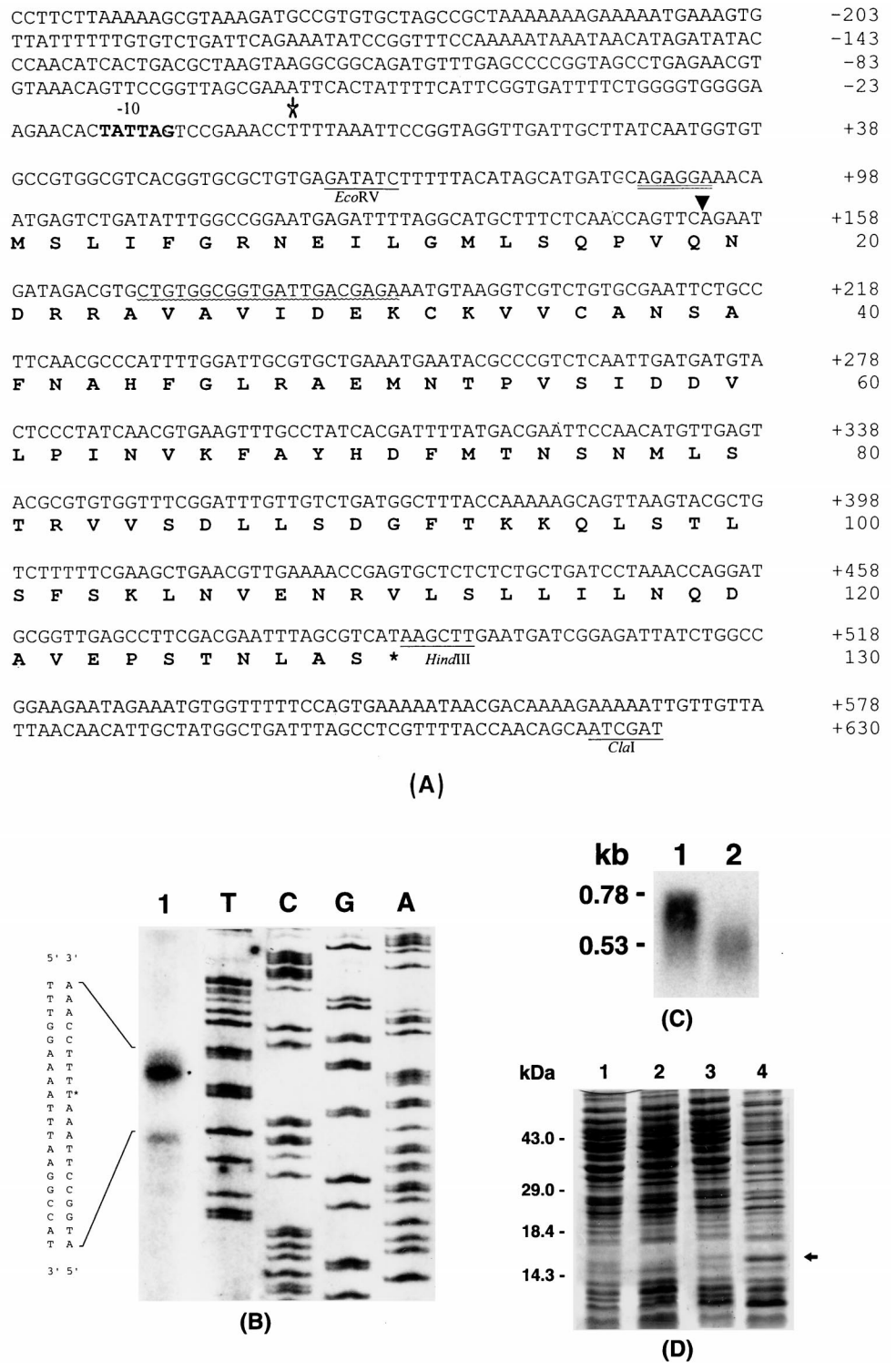

FIG. 3. (A) Nucleotide and deduced amino acid sequences of *E. carotovora* subsp. *carotovora rsmC*. The putative ribosome binding site is double underlined. The transcriptional start site at the thymine residue is indicated with an asterisk. The −10 consensus sequences are shown in boldface. The nucleotide sequence used for the synthesis of a complementary oligonucleotide for the primer extension assay is underlined with a wavy line. The position of mini-Tn*5*-Km insertion in the RsmC$^-$ mutants AC5050 and AC5053 is indicated with an arrowhead. Some restriction endonuclease sites are also shown. Numbers on the right refer to positions of the nucleotides and the amino acid residues for each line. (B) Primer extension analysis of *rsmC* mRNA. Lane 1, 20 μg of total RNA sample from *E. carotovora* subsp. *carotovora* Ecc71 grown at 28°C in minimal salts medium plus sucrose to an A_{600} of 2.0. The products of the primer extension reaction were run alongside a regular sequencing gel. Nucleotides on the left refer to the nucleotide sequence beyond the transcriptional start site; the asterisk denotes the thymine residue at which transcription was initiated. (C) Northern blot analysis of *rsmC* mRNA in *E. carotovora* subsp. *carotovora* Ecc71 (RmsC$^+$; lane 1) and its RsmC$^-$ derivative, AC5053 (lane 2). Total RNAs were isolated from bacteria grown at 28°C in minimal salts medium plus sucrose to an A_{600} of 2.0. Each lane contained 20 μg of total RNA. (D) Overproduction of RsmC in *E. coli* strain JM109(DE3). Bacteria carrying the cloning vector pET28a(+) or the *rsmC*$^+$ plasmid pAKC978 were grown in LB medium plus kanamycin with or without IPTG as described in Materials and Methods. Each lane contained 10 μg of total bacterial protein. The arrow indicates the 15-kDa overproduced RsmC protein. Lane 1, pET28(+), no IPTG; lane 2, pET28(+), with IPTG (final concentration, 1 mM); lane 3, pAKC978, no IPTG; lane 4, pAKC978 with IPTG (final concentration, 1 mM).

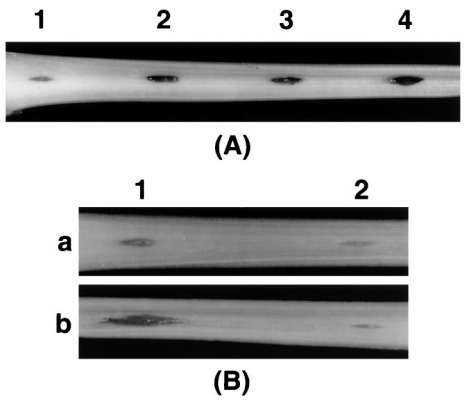

FIG. 4. (A) Maceration of a celery petiole by *E. carotovora* subsp. *carotovora* Ecc71 (RsmA$^+$ RsmC$^+$; site 1), AC5053 (RsmA$^+$ RsmC$^-$; site 2), AC5071 (RsmA$^-$ RsmC$^+$; site 3), and AC5054 (RsmA$^-$ RsmC$^-$; site 4). (B) Maceration of celery petioles by *E. carotovora* subsp. *carotovora* AC5047 (RsmA$^+$ RsmC$^+$; a) and AC5050 (RsmA$^+$ RsmC$^-$; b) carrying the cloning vector pCL1920 (site 1) or the RsmC$^+$ plasmid pAKC975 (site 2). About 2×10^8 cells were injected into the celery petiole at each inoculation site and covered with petroleum jelly. The inoculated petioles were incubated in a moist chamber at 25°C for 24 h.

from a leaky RsmC production due to the activity of T7 promoter.

Exoenzyme production by the RsmC$^-$ mutant in the absence of a quorum sensing signal. Our studies had shown that RsmA$^-$ mutants of *E. carotovora* subsp. *carotovora* do not require the cell density/quorum sensing signal OHL for enzyme and harpin$_{Ecc}$ overproduction, pathogenicity, and elicitation of a hypersensitive reaction (4, 6). Since AC5050, like AC5070, overproduces enzymes and harpin$_{Ecc}$, we wanted to determine if this mutant also is OHL independent. For this, we made an Ohl$^-$ and RsmC$^-$ double mutant, AC5051, by replacing *ohlI*$^+$ of AC5050 with *ohlI*::omega (Sp^r) of pAKC855 (Table 1). The results (Fig. 1A) show that AC5051 produced less Pel, Peh, Cel, and Prt than AC5050, although these levels were somewhat higher than the levels in the RsmC$^+$ Ohl$^+$ strain (Fig. 1A, column 1) and considerably higher than the levels in its RsmC$^+$ Ohl$^-$ derivative (Fig. 1A, column 2; also see reference 4). We noted a similar effect of OHL deficiency on the levels of *pel-1*, *peh-1*, *celV*, and *hrpN*$_{Ecc}$ transcripts in RsmC$^+$ and RsmC$^-$ bacteria (Fig. 1B). These observations demonstrate that in the RsmC$^-$ mutant, the requirement for OHL is partially relieved.

Pathogenicity assays. Figure 4A shows that AC5053 caused more extensive maceration of celery petioles than Ecc71. This observation was expected since the mutant produced higher levels of extracellular enzymes and harpin$_{Ecc}$ than the RsmA$^+$ RsmC$^+$ parent. Also, AC5053 carrying multiple copies of *rsmC* caused significantly less maceration than the same strains carrying the vector (Fig. 4B), most likely due to the inhibition of extracellular protein production (see above).

***rsmC* positively controls RsmA production.** The similarities in phenotypes of RsmA$^-$ and RsmC$^-$ mutants strongly suggested that the RsmC effect could partly manifest itself by modulating RsmA levels. To confirm this, we compared the levels of *rsmA* transcripts, the expression of a *rsmA-lacZ* gene fusion, and the levels of RsmA protein in RsmC$^+$ and RsmC$^-$ *E. carotovora* subsp. *carotovora* strains. Unlike *pel-1*, *peh-1*, *celV*, and *hrpN*$_{Ecc}$ transcripts (see above), the levels of *rsmA* transcript produced by AC5053 were lower than the levels produced by the parent strain Ecc71 (Fig. 5A). The data in Fig. 6A show that the level of β-galactosidase produced by the RsmC$^+$ strain AC5047 carrying the fusion plasmid pAKC887 was 10- to 12-fold higher than the level produced by the RsmC$^-$ strain AC5050 carrying the fusion plasmid. Similarly, the results of Western blot analysis (Fig. 5B) also show that the level of RsmA polypeptide is higher in the RsmC$^+$ strain than in the RsmC$^-$ mutant. Moreover, the data shown in Fig. 5C revealed that Ecc71 and AC5053 carrying multiple copies of *rsmC* produced higher levels of *rsmA* transcript than these bacteria carrying the cloning vector, pCL1920. These observations establish that RsmC has a positive effect on RsmA production and that the RsmC effect on exoprotein production may be attributed, at least in part, to this regulatory effect. To strengthen the latter conclusion, we constructed an RsmA$^-$ RsmC$^-$ double mutant and assayed for the levels of exoproteins and transcripts (Table 2; Fig. 1D). The levels of Pel, Peh, Cel, and Prt were even higher in the double mutant than in the RsmA$^-$ RsmC$^+$ parent. Similarly, the levels of *pel-1*, *peh-1*, and *celV* transcripts were higher in the double mutant (Fig. 1D, column 4) than in the RsmA$^-$ RsmC$^+$ parent (Fig. 1D, column 3). These findings raised the possibility that RsmC acted on a regulatory component in addition to RsmA. We should note that the levels of *hrpN*$_{Ecc}$ transcripts (Fig. 1D) and harpin$_{Ecc}$ protein (Fig. 1C) were comparable in RsmA$^-$ RsmC$^+$ and RsmA$^-$ RsmC$^-$ mutants. These results suggested that the RsmC effect on *hrpN*$_{Ecc}$ expression occurs primarily by its effect on RsmA, whereas the effect of RsmC on exoenzyme production is mediated via multiple regulators.

rsmA or *rsmA*-like genes have been cloned from several enterobacterial species, including *E. amylovora* (designated *rsmA*$_{Ea}$ [25]), *E. herbicola* pv. gypsophilae (designated *rsmA*$_{Ehg}$ [25]), and *E. coli* (designated *csrA* [35]). To test the effects of RsmC of *E. carotovora* subsp. *carotovora* Ecc71 on the expression of these heterologous *rsmA* (*csrA*) species, we made *lacZ* gene fusions of *rsmA*$_{Ea}$, *rsmA*$_{Ehg}$, and *csrA*. Plasmids carrying these fusions were transferred to RsmC$^+$ strain AC5047 and RsmC$^-$ strain AC5050. These strains carrying the *rsmA*$_{Ecc}$-*lacZ* gene fusion served as the controls. The data shown in Fig. 6A clearly indicate that with each fusion, higher levels of β-galactosidase were produced in the RsmC$^+$ strain than in the RsmC$^-$ strain. Although the degree of stimulation was variable depending on the source of the *rsmA* gene, these results demonstrate that RsmC stimulates the expression of *rsmA* homologs in *E. carotovora* subsp. *carotovora*.

Effect of RsmC on *rsmB* expression. We have shown that *rsmB* specifies a regulatory RNA which positively controls exoprotein production and various secondary metabolites (19). The similarities in phenotypes due to RsmC deficiency (see above) and the dosage of *rsmB* RNA (19) suggested that the

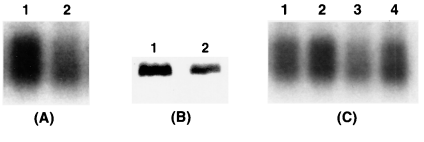

FIG. 5. (A) Northern analysis of *rsmA* transcripts produced by *E. carotovora* subsp. *carotovora* Ecc71 (lane 1) and its RsmC$^-$ derivative, AC5053 (lane 2). Total RNAs were extracted from bacteria grown in minimal salts medium plus sucrose at 28°C to an A_{600} of 2.0. Each lane contained 10 μg of total RNA. (B) Western blot analysis of RsmA produced by *E. carotovora* subsp. *carotovora* Ecc71 (RsmC$^+$; lane 1) and AC5053 (RsmC$^-$; lane 2). Each lane contained 20 μg of total bacterial protein. (C) Levels of *rsmA* transcripts in *E. carotovora* subsp. *carotovora* Ecc71 (RsmC$^+$) carrying the cloning vector pCL1920 (lane 1) or the *rsmC*$^+$ plasmid pAKC975 (lane 2) and AC5053 (RsmC$^-$) carrying the cloning vector pCL1920 (lane 3) or the *rsmC*$^+$ plasmid pAKC975 (lane 4). Total RNAs were extracted from bacteria grown in minimal salts medium plus sucrose and spectinomycin at 28°C to an A_{600} of 2.0 and subjected to Northern analysis. Each lane contained 10 μg of total RNA.

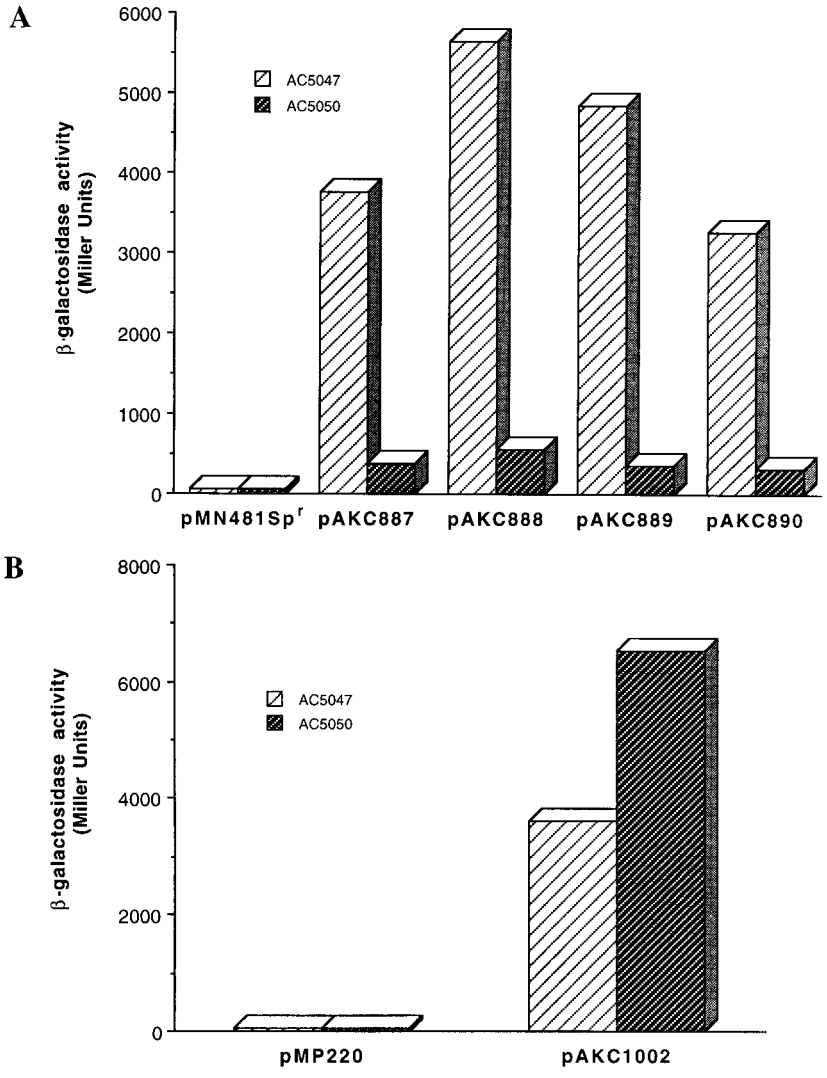

FIG. 6. (A) β-Galactosidase assays of *E. carotovora* subsp. *carotovora* AC5047 or its RsmC$^-$ mutant AC5050 carrying the cloning vector pNM481Sp^r, pAKC887 (*rsmA*$_{Ecc}$-*lacZ* fusion), pAKC888 (*rsmA*$_{Ea}$-*lacZ* fusion), pAKC889 (*rsmA*$_{Ehg}$-*lacZ* fusion), and pAKC890 (*csrA-lacZ* fusion). Bacteria were grown at 28°C in minimal salts medium plus sucrose and spectinomycin to an A_{600} of 2.0 and assayed for β-galactosidase activity. (B) β-Galactosidase assays of *E. carotovora* subsp. *carotovora* AC5047 or its RsmC$^-$ mutant AC5050 carrying the cloning vector pMP220 or the *rsmB-lacZ* plasmid pAKC1002. Bacteria were grown at 28°C in minimal salts medium plus sucrose and tetracycline to an A_{600} of 2.0 and assayed for β-galactosidase activity.

RsmC effect could be modulated via *rsmB* RNA. To test this idea, we examined the levels of *rsmB* transcripts in RsmC$^-$ and RsmC$^+$ strains as well as in the RsmC$^-$ strain carrying multiple copies of *rsmC*$^+$ DNA. The data in Fig. 1D and 2B show that (i) *rsmB* transcript levels are lower in the RsmC$^+$ parent than in the RsmC$^-$ mutant and (ii) multiple copies of *rsmC*$^+$ DNA inhibit the production of *rsmB* transcripts. The expression of a *rsmB-lacZ* transcriptional fusion in RsmC$^+$ and RsmC$^-$ bacteria (Fig. 6B) also demonstrates a negative effect of RsmC on *rsmB* transcription.

We should note that the level of *rsmB* RNA in the RsmA$^-$ RsmC$^+$ strain was about 10% of the level found in its RsmA$^+$ RsmC$^+$ parent strain (Fig. 1D). We have determined that this reduced level of *rsmB* RNA is primarily due to shorter half-life of the transcripts in RsmA$^-$ mutants than in the RsmA$^+$ strains (25). Thus, it appears that RsmA contributes to the stability of *rsmB* RNA, perhaps by forming a ribonucleoprotein complex. This effect notwithstanding, it is evident that as in RsmA$^+$ bacteria, the deficiency of RsmC in the RsmA$^-$ mutant stimulated the production of *rsmB* RNA.

We next examined the effects of the dosage of *rsmB*$^+$ DNA in *E. carotovora* subsp. *carotovora* Ecc71 (RsmA$^+$ RsmC$^+$ RsmB$^+$), AC5053 (RsmA$^+$ RsmC$^-$ RsmB$^+$), AC5071 (RsmA$^-$ RsmC$^+$ RsmB$^+$), and AC5054 (RsmA$^-$ RsmC$^-$ RsmB$^+$). The data in Table 3 show that Pel levels were 20-fold higher in Ecc71 and two- to threefold higher in AC5053 and AC5054 carrying the *rsmB*$^+$ plasmid than in bacteria carrying the cloning vector. Similar effects were noted with Peh, Cel, and Prt activities (data not shown) and the transcripts of *pel-1*, *peh-1*, and *celV* genes (Fig. 2C). A notable exception was the strain AC5054, which is deficient in both RsmA and RsmC, in that the enzyme and transcript levels were comparable in bacteria carrying a single copy of *rsmB* or multiple copies of *rsmB* (Table 3; Fig. 2C, columns 7 and 8).

***rsmC* homologs occur in *E. carotovora* subspecies.** Southern hybridization analysis using the *rsmC* DNA as the probe re-

TABLE 3. Levels of Pel produced by *E. carotovora* subsp. *carotovora* strains carrying multiple copies of *rsmB*$^+$ DNA

Bacterial construct[a]	Relevant phenotype[b]	Pel sp act (U/ml/A_{600} unit; mean ± SD)
Ecc71(pCL1920Gm^r)	RsmA$^+$ RsmB$^+$ RsmC$^+$ (none [vector])	0.2 ± 0.00
Ecc71(pAKC1004Gm^r)	RsmA$^+$ RsmB$^+$ RsmC$^+$ (RsmB$^+$)	4.2 ± 0.08
AC5053(pCL1920Gm^r)	RsmA$^+$ RsmB$^+$ RsmC$^-$ (none [vector])	4.6 ± 0.04
AC5053(pAKC1004Gm^r)	RsmA$^+$ RsmB$^+$ RsmC$^-$ (RsmB$^+$)	10.6 ± 0.58
AC5071(pCL1920Gm^r)	RsmA$^-$ RsmB$^+$ RsmC$^+$ (none [vector])	7.8 ± 0.14
AC5071(pAKC1004Gm^r)	RsmA$^-$ RsmB$^+$ RsmC$^+$ (RsmB$^+$)	19.1 ± 0.08
AC5054(pCL1920Gm^r)	RsmA$^-$ RsmB$^+$ RsmC$^-$ (none [vector])	22.9 ± 0.36
AC5054(pAKC1004Gm^r)	RsmA$^-$ RsmB$^+$ RsmC$^-$ (RsmB$^+$)	21.3 ± 0.03

[a] Bacteria were grown at 28°C in minimal salts medium plus sucrose (0.5% [wt/vol]) and gentamicin to an A_{600} value of 2.5, and culture supernatants were used for enzyme assays.

[b] Relevant phenotypes conferred by the plasmids are given in parentheses.

vealed that *rsmC* homologs exist in strains of *E. carotovora* subspecies *carotovora*, *atroseptica*, and *betavasculorum* (Fig. 7) but not in the other *Erwinia* or enterobacterial species (data not shown). In addition, using synthetic oligonucleotide primers specific to the internal sequences of *rsmC*, we conducted PCR analysis of DNA preparations of strains of *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *betavasculorum*, *E. amylovora*, *E. rhapontici*, *E. stewartii*, *E. chrysanthemi*, and *E. herbicola*, as well as *E. coli*, *Salmonella*, *Serratia*, *Yersinia*, and *Shigella* strains. The PCR-amplified DNA segments similar in size to the expected product from Ecc71 were detected only with *E. carotovora* subspecies and not with the other bacteria. These data suggest that *rsmC* sequences have been conserved in the *E. carotovora* group of the soft-rotting *Erwinia* species.

DISCUSSION

The data presented here show that RsmC positively regulates the expression of *rsmA*, which encodes an RNA-binding protein (6) and negatively regulates *rsmB*, which specifies a regulatory RNA (19). For example, the RsmC$^-$ mutant produces low levels of *rsmA* transcripts and RsmA protein, but when provided with the *rsmC*$^+$ DNA in *trans* it produces high levels of these transcripts and RsmA. The levels of *rsmB* transcripts, on the other hand, are higher in RsmC$^-$ bacteria than in RsmC$^+$ strains. These observations were also confirmed by following the expression of *rsmA-lacZ* and *rsmB-lacZ* fusions. We do not yet know how RsmC interacts with the RNA polymerase holoenzyme to regulate gene expression. The absence of a DNA-binding motif in RsmC suggests that it probably does not function as a classical transcriptional factor by directly interacting with *rsmA* and *rsmB* sequences. It is perhaps significant that a stretch of RsmC has sequence homology with eukaryotic transcriptional adapters (9). Such adapters have been found to activate transcription by interacting with components of transcriptional machinery, i.e., via protein-protein interactions. By extrapolating from those observations, we postulate that RsmC interacts with RNA polymerase holoenzyme and this ternary complex modulates transcription. At this juncture we have to entertain the possibility that RsmC acts directly or indirectly both as a positive regulator of *rsmA* and a negative regulator of *rsmB* transcription. We have initiated in vitro transcription studies and mutational analysis of *E. carotovora* subsp. *carotovora* to identify the targets of RsmC and to elucidate its mode of action.

In a series of studies (4, 6, 19, 26), we have documented that RsmA and *rsmB* RNA control the production of exoproteins, polysaccharides, and an assortment of secondary metabolites as well as various virulence factors in *Erwinia* species. Those observations and the data presented here demonstrate that there is a correlation between expression of *rsmA* and *rsmB* and levels of exoenzymes, harpin$_{Ecc}$, and plant virulence. Indeed, the following findings establish that the pleiotropic effect of RsmC deficiency is mainly if not solely directed via the RsmA-*rsmB* regulatory pathway, i.e., due to the reduced levels of RsmA and high levels of *rsmB* RNA. (i) *rsmC*$^+$ DNA stimulates RsmA production and concomitantly causes a severe repression of exoprotein production. (ii) The levels of exoenzymes are higher in the RsmA$^-$ RsmC$^-$ double mutant than in the RsmA$^-$ RsmC$^+$ strain, indicating that a regulatory factor in addition to RsmA is also affected in the RsmC$^-$ mutant. (iii) The pleiotropic phenotypes of the RsmC$^-$ mutant and the RsmA$^-$ RsmC$^-$ double mutant are reminiscent of the effects of multiple copies of *rsmB* DNA (19). (iv) *rsmB* expression is much higher in the RsmC$^-$ mutant than in the RsmC$^+$ parent. Furthermore, multiple copies of *rsmC*$^+$ DNA lower the levels of *rsmB* RNA. (v) Multiple copies of *rsmB*$^+$ DNA further stimulate the levels of exoproteins in RsmA$^-$ bacteria but not in the RsmA$^-$ RsmC$^-$ double mutant. (vi) The levels of *rsmA* and *rsmB* RNA, but not the transcripts of other known global regulator genes, such as *kdgR* (20), *hexA* (13), *rpoS* (27), *hor* (42), and *ohlI* (4), are affected in the RsmC$^-$ mutant (25).

Since RsmC positively regulates *rsmA* expression, it was expected that the phenotypes of RsmC$^-$ mutants would closely resemble those of the RsmA$^-$ strains. That this indeed is the case is evident from exoenzyme overproduction, hypervirulence, and overexpression of *hrpN*$_{Ecc}$. It was, therefore, surprising that the RsmC$^-$ mutant at least partially requires the quorum sensing signal, OHL, for exoenzyme overproduction (Fig. 1). This contrasts with the finding (4) that RsmA$^-$ mutants can overproduce exoenzymes in the absence of OHL. Since we do not yet know how OHL activates the expression of exoenzyme genes in *E. carotovora* subsp. *carotovora*, these findings with the RsmC$^-$ and RsmA$^-$ mutants are difficult to explain. We should, however, note that there are reports describing LuxR homologs that in conjunction with OHL could

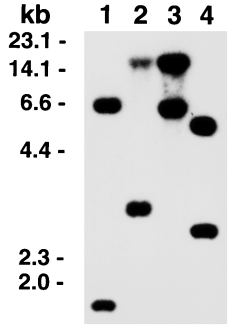

FIG. 7. Southern blot analysis of *Eco*RI-digested chromosomal DNAs of *E. carotovora* subspecies with *rsmC* of *E. carotovora* subsp. *carotovora* Ecc71. Lane 1, *E. carotovora* subsp. *atroseptica* Eca12; lane 2, *E. carotovora* subsp. *betavasculorum* Ecb11129; lanes 3 and 4, *E. carotovora* subsp. *carotovora* strains Ecc71 and SCRI193.

affect gene expression in soft-rotting *Erwinia*. For example, CarR (a regulator of carbapenem antibiotic production) together with OHL activates carbapenem biosynthetic genes, *carA* to *carH*, in *E. carotovora* subsp. *carotovora* GS101, but CarR does not affect exoenzyme production (21). Another *luxR* homolog, *expR*, has been detected in *E. carotovora* subsp. *carotovora* SCC3193 (32). However, ExpR$^-$ mutant does not have any effect on OHL or exoenzyme production. Moreover, a genetic homolog of *expR* has not been found in *E. carotovora* subsp. *carotovora* Ecc71, although OHL plays a key role in regulating exoenzymes and harpin$_{Ecc}$ in this bacterium (4, 6, 25). Nasser et al. (30) reported that ExpR of *E. chrysanthemi* did bind sequences of a *pel* gene of *E. carotovora* subsp. *carotovora* SCRI193 and several *pel* genes of *E. chrysanthemi* 3193. The physiological significance of this physical interaction is not apparent since the ExpR$^-$ knockout mutants of *E. chrysanthemi*, like the ExpR$^-$ mutants of *E. carotovora* subsp. *carotovora*, were not affected in OHL and exoenzyme production. These uncertainties notwithstanding, the data presented here and in our previous publications (4, 19, 24) support the idea that the regulatory effects of RsmC and OHL are directed via common as well as nonoverlapping steps. As a prelude to understanding the basis for OHL independence in *E. carotovora* subsp. *carotovora* mutants, we have begun testing various strategies that would allow the identification of a regulator(s) which is responsible for the OHL effect and is affected by RsmA and RsmC.

Several lines of evidence have established that *rsmA*-like genes occur and are expressed in many enterobacteria (6, 26). In this report we have documented that RsmC is a positive regulator not only of Ecc71 *rsmA* but also of *rsmA* genes non-soft-rotting bacteria such as *E. amylovora*, *E. herbicola* pv. gypsophilae, and *E. coli*. For example, (i) the levels of Ecc71 *rsmA* transcript are higher in RsmC$^+$ than in RsmC$^-$ bacteria and (ii) the expression of *lacZ* driven by promoters of *rsmA* genes of *E. amylovora*, *E. herbicola* pv. gypsophilae, *E. coli*, and *E. carotovora* subsp. *carotovora* is consistently higher in RsmC$^+$ than in RsmC$^-$ *E. carotovora* subsp. *carotovora* strains. However, we were surprised to find that genetic homologs of *rsmC* are present in *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *betavasculorum*, and *E. carotovora* subsp. *carotovora* but not in other bacteria, including non-soft-rotting *Erwinia* species and *E. coli*. We have initiated a search for functional homologs of RsmC of *E. coli* and non-soft-rotting *Erwinia* species by testing for heterologous complementation.

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