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RsmA and the Quorum-Sensing Signal, *N*-[3-Oxohexanoyl]-L-Homoserine Lactone, Control the Levels of *rsmB* RNA in *Erwinia carotovora* subsp. *carotovora* by Affecting Its Stability

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RsmA (for regulator of secondary metabolism), RsmC, and *rsmB* RNA, the components of a posttranscriptional regulatory system, control extracellular protein production and pathogenicity in *Erwinia carotovora* subsp. *carotovora*. RsmA, an RNA binding protein, acts as a negative regulator by promoting message decay. *rsmB* RNA, on the other hand, acts as a positive regulator by neutralizing the effect of RsmA. RsmC modulates the levels of RsmA and *rsmB* RNA by positively regulating *rsmA* and negatively controlling *rsmB*. The level of *rsmB* RNA is substantially higher in RsmA$^+$ bacteria than in RsmA$^-$ mutants. We show that *rsmB* RNA is more stable in the presence of RsmA than in its absence. RsmA does not stimulate the expression of an *rsmB-lacZ* transcriptional fusion; in fact, the β-galactosidase level is somewhat higher in RsmA$^-$ bacteria than in RsmA$^+$ bacteria. We also investigated the basis for increased levels of *rsmA* and *rsmB* RNAs in the absence of the quorum-sensing signal, *N*-[3-oxohexanoyl]-L-homoserine lactone (OHL). The absence of OHL activates transcription of *rsmA* but not of *rsmB*. Instead, increased stability of *rsmB* RNA in the presence of RsmA accounts for the elevated levels of the *rsmB* RNA in OHL$^-$ bacteria. Mutant studies disclosed that while RsmA, OHL, and RsmC control the levels of *rsmB* RNA, high levels of *rsmB* RNA occur in the absence of RsmC or OHL only in RsmA$^+$ bacteria, indicating a critical role for RsmA in modulating the levels of *rsmB* RNA. The findings reported here firmly establish that the quorum-sensing signal is channeled in *E. carotovora* subsp. *carotovora* via the *rsmA-rsmB* posttranscriptional regulatory system.

Rsm (for regulator of secondary metabolism) is a novel type of posttranscriptional regulatory system which has a profound effect on bacterial metabolism and behavior. Rsm of *Erwinia carotovora* subsp. *carotovora* consists of three major components: an RNA binding protein, RsmA, which promotes RNA decay (8, 11); an untranslated RNA molecule, *rsmB*, which neutralizes RsmA action, apparently by sequestering RsmA (29); and RsmC, which positively regulates *rsmA* and negatively controls *rsmB* RNA levels (13). It is now apparent that Rsm and Rsm-like systems control diverse phenotypes in many prokaryotic species. In *Erwinia*, this regulatory system plays a critical role by affecting plant interaction, extracellular enzyme production, extracellular polysaccharide synthesis, swarming motility, secondary metabolite production, and the quorum-sensing system (8, 11, 12, 31, 34, 35). In *Escherichia coli*, a homologous system comprising CsrA (equivalent to RsmA) and *csrB* (equivalent to *rsmB*) RNA, controls glycogen synthesis, metabolism of acetate, motility and flagellum biosynthesis, and biofilm formation (21, 27, 40). There are substantial data indicating the existence of the regulatory system in various enterobacterial species, including human pathogens (2, 3, 5, 9, 14, 40). Moreover, recent studies of several *Pseudomonas* species have demonstrated that RsmA-*rsmB* regulatory systems control secondary metabolite production and plant interaction. For example, in *Pseudomonas aeruginosa*, overexpression of *rsmA* reduces the levels of protease (Prt), elastase, and staphylolytic (LasA Prt) activity, as well as those of the PA-IL lectin, hydrogen cyanide, and pyocyanin. In *Pseudomonas fluorescens*, overexpressed *prrB*, which is structurally similar to *rsmB*, increases the production of Phl (2,4-diacetylphloroglucinol) and hydrogen cyanide (1, 6, 7, 38). In *Pseudomonas syringae* pv. tomato strain DC3000, RsmA reduces the production of extracellular proteins, causes attenuation of pathogenicity in *Arabidopsis thaliana*, and lowers the efficiency of the induction of the hypersensitive reaction in tobacco (A. Chatterjee, Y. Cui, H. Yang, J. Alfano, A. Collmer, and A. K. Chatterjee, *Pseudomonas* 2001, abstr. PS 8, 2001). Thus, the RsmA-*rsmB* regulatory system appears to have been conserved in many prokaryotes.

As this system is so critical, it was predicted that *rsmA* and *rsmB* expression would be rigorously controlled. Indeed, studies of *E. carotovora* subsp. *carotovora* have disclosed that several transcriptional factors control the expression of the *rsmA* and *rsmB* genes. Examples include HexA, a LysR-type regulator (17, 36); RsmC, a putative transcriptional adapter (13); and KdgR, an IcIR-type repressor (20, 30). A remarkable feature of these regulators is their dual action: they positively control RsmA production and negatively control the levels of *rsmB* RNA (13, 20, 30, 36). GacS and GacA, members of a two-component system (19), control *rsmB* transcription in *E. carotovora* subsp. *carotovora* (10) and *rsmZ* transcription in *P. aeruginosa* (18).

We have consistently noted that RsmA$^+$ bacteria contain

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. carotovora subsp. *carotovora*		
Ecc71	Wild type	44
AC5047	Nal^r derivative of AC5006	8
AC5050	RsmC$^-$ derivative of AC5047; Km^r	13
AC5070	RsmA$^-$ derivative of AC5047; Km^r	8
AC5071	RsmA$^-$ derivative of Ecc71; Km^r	35
AC5053	RsmC$^-$ derivative of Ecc71; Km^r	13
AC5054	RsmC$^-$ derivative of AC5071; Km^r Sp^r	13
AC5088	RsmB$^-$ derivative of Ecc71; Cm^r	Laboratory collection
AC5090	Ohl$^-$ derivative of AC5070; Km^r Sp^r	8
AC5091	Ohl$^-$ derivative of AC5047; Km^r	8
AC5094	Ohl$^-$ derivative of Ecc71; Km^r	8
E. coli DH5α	ϕ80*lacZ*Δ*M15* Δ(*lacZYA-argF*) *U169 hsdR17 recA1 endA1 thi-1*	Gibco BRL
Plasmids		
pBluescript SK(+)	Ap^r	Stratagene
pDK7	Cm^r	24
pMP220	Tc^r; promoter-probe vector	42
pRK415	Tc^r	23
pCL1920	Sp^r Sm^r	26
pAKC783	Ap^r *pel-1*$^+$ DNA in pBluescript SK(+)	28
pAKC856	Ap^r *ohlI* DNA in pBluescript SK(+)	8
pAKC861	Sp^r *lacp-ohlI* in pCL1920	Laboratory collection
pAKC878	Tc^r *rsmA*$^+$ DNA in pRK415	29
pAKC975	Sp^r *rsmC*$^+$ in pCL1920	13
pAKC979	Tc^r *rsmC*$^+$ in pRK415	13
pAKC882	Ap^r *rsmA* in pT7-7	35
pAKC1002	Tc^r $rsmB_{Ecc}$-*lacZ* in pMP220	29
pAKC1004	Sp^r *rsmB* DNA in pCL1920	29
pAKC1047	Tc^r $rsmB_{Ea}$-*lacZ* in pMP220	10
pAKC1048	Tc^r $rsmB_{Ehg}$-*lacZ* in pMP220	10
pAKC1049	Sp^r *lacp-rsmB* in pCL1920	31
pAKC1100	Tc^r $rsmA_{Ecc}$-*lacZ* in pMP220	This work

significantly higher levels of *rsmB* RNA than RsmA$^-$ bacteria. Since RsmA acts as a negative regulator by promoting message decay, the occurrence of higher levels of *rsmB* RNA was deemed unusual, prompting further analysis. While this work was in progress, Gudapaty et al. (16) attributed a similar effect of CsrA on *csrB* RNA in *E. coli* to a positive regulation of *csrB* by CsrA. The CsrA effect is not due to differences in stability of *csrB* RNA in CsrA$^+$ and CsrA$^-$ bacteria. In fact, studies with a *csrB-lacZ* fusion demonstrated that CsrA, by a yet-undetermined mechanism, controls *csrB* transcription. Since we have never noted a positive regulatory effect of RsmA in *Erwinia*, the results with *E. coli* were quite intriguing and warranted further analysis of the Rsm system.

The quorum-sensing signal, *N*-[3-oxohexanoyl]-L-homoserine lactone (OHL), is required for extracellular enzyme production, expression of the Hrp regulon, and the pathogenicity of *E. carotovora* subsp. *carotovora* (8, 12, 15, 22, 39). The findings (8, 12) that RsmA$^-$ mutants of *E. carotovora* subsp. *carotovora* in the absence of OHL produce high levels of extracellular enzymes, overexpress the Hrp regulon, and exhibit enhanced virulence have raised the possibility that the OHL response is channeled via the RsmA-*rsmB* regulatory system. Indeed, a recent report documents the fact that OHL deficiency led to increased production of both *rsmA* and *rsmB* RNAs (25). While this finding supports the idea that OHL acts via the RsmA-*rsmB* pathway, it was not clear (i) how OHL affected *rsmB* RNA levels and (ii) why OHL deficiency inhibited extracellular enzyme production and negatively affected other traits. Here, we present data that provide answers to those questions.

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 (LB) agar containing appropriate antibiotics. The wild-type strains were maintained on LB agar. The recipes for LB medium and minimal-salts medium have been described previously (8, 37). When required, antibiotics were added as follows (in micrograms per milliliter): ampicillin (Ap), 100; chloramphenicol (Cm), 20; kanamycin (Km), 50; spectinomycin (Sp), 50; and tetracycline (Tc), 10. The media were solidified by the addition of 1.5% agar.

DNA techniques. Standard procedures were used in the isolation of plasmid and chromosomal DNAs, electroporation, restriction endonuclease digestion, gel electrophoresis, and DNA ligation (41). PCR was performed as described by Liu et al. (29). The Primer-a-Gene DNA labeling system and restriction and modifying enzymes were obtained from Promega (Madison, Wis.).

RNA preparation and Northern hybridization experiments. Total RNA was isolated from *E. carotovora* subsp. *carotovora* constructs grown in minimal-salts medium plus sucrose (0.5% [wt/vol]) with or without drugs at 28°C. To determine the stability of *rsmB* mRNA in RsmA$^+$ Ohl$^+$ (Ecc71), RsmA$^-$ Ohl$^+$ (AC5070 and AC5071), RsmA$^+$ Ohl$^-$ (AC5094), and RsmA$^-$ Ohl$^-$ (AC5090) strains, the bacteria were grown to a Klett value of ca. 150, at which point rifampin (0.2 mg/ml) was added to block further initiation. Culture samples (8 ml each) were then withdrawn at various time points into tubes containing 5 ml of ice-cold water, and total RNA was extracted.

The probes used in this work were the 314-bp *Eco*RV-*Kpn*I fragment of *pel-1* from pAKC783 (28), the 183-bp *Nde*I-*Sal*I fragment of *rsmA* from pAKC882

(35), the 304-bp *Eco*RV-*Hin*dIII fragment of *rsmC* from pAKC975 (13), the 321-bp *Bam*HI-*Hin*dIII fragment of *rsmB* from pAKC1004 (29), and the 386-bp *Dra*I-*Eco*RI fragment of *ohlI* from pAKC856 (8). DNA probes (Promega) were labeled with [α-^{32}P]dATP by random priming according to the manufacturer's instructions. Prehybridization (6 h at 65°C) and hybridization (18 h at 65°C) were performed in prehybridization buffer (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 2× Denhardt's solution, 0.1% sodium dodecyl sulfate [SDS], 100 μg of denatured salmon sperm DNA/ml). After hybridization, the membranes were washed twice for 20 min at 65°C in 2× SSC–0.5% SDS and then for 30 min at 65°C in 0.5× SSC–0.5% SDS and examined by autoradiography with X-ray film (Kodak, Rochester, N.Y.). The densitometric analysis of autoradiography was performed by using the MetaMorph imaging system (version 4.6r3; Universal Imaging Corp.).

Western blot analysis of RsmA. *E. carotovora* subsp. *carotovora* strains Ecc71 and AC5094 were grown in minimal-salts medium plus sucrose (0.5% [wt/vol]) at 28°C to a Klett value of ca. 150. Total bacterial proteins were precipitated with trichloroacetic acid at a final concentration of 10% (vol/vol), centrifuged, and resuspended in 1× SDS loading buffer (41). Western blot analysis of RsmA was performed according to the method of Mukherjee et al. (34). The anti-RsmA antiserum raised against the synthesized peptide from amino acids 48 to 61 of RsmA in rabbit by Genemed Biotechnologies Inc. (San Francisco, Calif.) was used as the probe.

β-Galactosidase assays. A DNA segment containing nucleotides −117 to +145 from the transcriptional start site of *E. carotovora* subsp. *carotovora rsmA* ($rsmA_{Ecc}$) was amplified by PCR and cloned into the promoter-probe vector pMP220 to produce the $rsmA_{Ecc}$-*lacZ* fusion, pAKC1100. The $rsmB_{Ecc}$-*lacZ* (pAKC1002), *rsmB* gene of *Erwinia amylovora* ($rsmB_{Ea}$)-*lacZ* (pAKC1047), and *rsmB* gene of *Erwinia herbicola* pv. *gypsophilae* ($rsmB_{Ehg}$)-*lacZ* (pAKC1048) fusions used in this experiment have been described previously (10, 29). *E. carotovora* subsp. *carotovora* strains carrying these constructs were grown in minimal-salts medium plus sucrose (0.5% [wt/vol]) and tetracycline at 28°C to a Klett value of ca. 200 and harvested for β-galactosidase assays. The β-galactosidase assays were carried out according to the method of Miller (33).

Extracellular-enzyme assays. The quantitative assay conditions of pectate lyase (Pel) are described in our previous publication (37). For estimation of the levels of extracellular polygalacturonase (Peh), cellulase (Cel), and Prt activities in culture supernatants, we used agarose semiquantitative plate assay procedures (8).

Bioluminescence assay for OHL production. Ecc71 was grown in minimal-salts medium plus sucrose (0.5% [wt/vol]) at 28°C to Klett values of 150 and 200. Culture supernatants were used for assays by using an *E. coli*-based bioassay system as described previously (8). There is a linear relationship between the quantity of OHL and the emission of bioluminescence expressed as relative light units (RLU) per minute per milliliter.

The experiments were performed at least two to three times, and the results were reproducible.

RESULTS AND DISCUSSION

RsmA affects the stability of *rsmB* RNA. The data in Fig. 1A show that *rsmB* RNA levels are at least four times higher in the RsmA$^+$ strain (lane 1) than in the RsmA$^-$ strain (lane 3). In fact, *rsmB* transcript levels are low in the absence of RsmA. This contrasts with about three- to fourfold-higher levels of *rsmC* transcripts in RsmA$^-$ bacteria than in RsmA$^+$ bacteria (Fig. 1B).

To understand the basis for higher levels of *rsmB* RNA in RsmA$^+$ bacteria, we compared the stabilities of *rsmB* RNA in RsmA$^+$ and RsmA$^-$ bacteria. The data shown in Fig. 2A clearly establish that in the absence of RsmA, *rsmB* RNA is quite unstable. The positive effect of RsmA on *rsmB* RNA stability in *E. carotovora* subsp. *carotovora* is very different from the findings in *E. coli*, as the presence or absence of CsrA had no apparent effect on the stability of *csrB* RNA (16). The observations with *E. coli* and *E. carotovora* subsp. *carotovora* are hard to reconcile in the present state of our understanding of the Rsm and Csr systems. However, the findings clearly indicate a fundamental difference in the regulation of *rsmB* and *csrB* in these bacteria that inhabit different ecosystems.

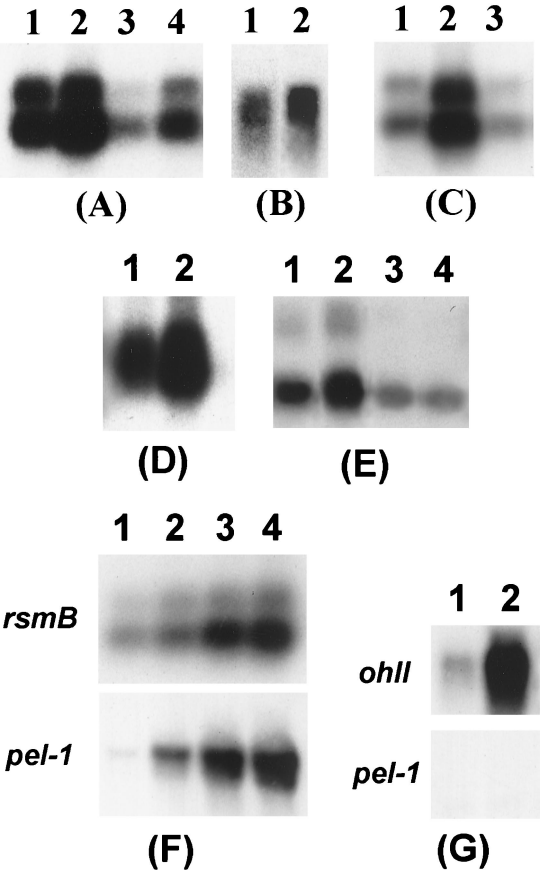
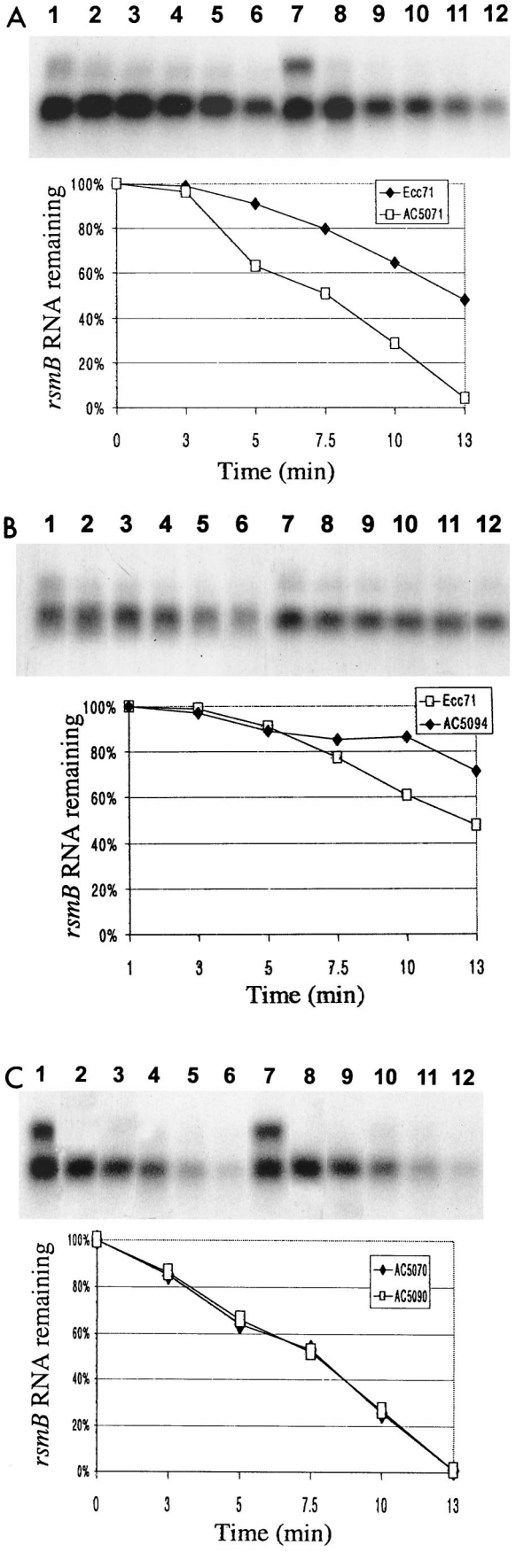


FIG. 1. Northern blot analysis of *rsmA*, *rsmB*, *rsmC*, *pel-1*, and *ohlI* transcripts in *E. carotovora* subsp. *carotovora* strains. (A) *rsmB* RNA in Ecc71 (wild-type parent; lane 1), AC5053 (RsmA$^+$ RsmC$^-$; lane 2), AC5071 (RsmA$^-$ RsmC$^+$; lane 3), and AC5054 (RsmA$^-$ RsmC$^-$; lane 4). (B) *rsmC* RNA in Ecc71 (lane 1) and AC5071 (lane 2). (C) *rsmB* RNA in AC5054 carrying pRK415 (vector; lane 1), AC5054 carrying pAKC878 (RsmA$^+$; lane 2), and AC5054 carrying pAKC979 (RsmC$^+$; lane 3). (D) *rsmA* RNA in Ecc71 (lane 1) and AC5094 (Ohl$^-$; lane 2). (E) *rsmB* RNA in Ecc71 (lane 1), AC5094 (RsmA$^+$ Ohl$^-$; lane 2), AC5071 (RsmA$^-$ Ohl$^+$; lane 3), and AC5090 (RsmA$^-$ Ohl$^-$; lane 4). (F) *rsmB* and *pel-1* RNAs in AC5094 (Ohl$^-$) carrying pDK7 and pCL1920 (vectors; lanes 1), AC5094 carrying pDK7 and pAKC1049 (*plac-rsmB*; lanes 2), AC5094 carrying pDK7 and pAKC1049 with 0.1 mM IPTG treatment (lanes 3), and AC5094 carrying pDK7 and pAKC1049 with 1.0 mM IPTG treatment (lanes 4). (G) *ohlI* and *pel-1* RNAs in AC5088 (RsmB$^-$) carrying pCL1920 (vector; lanes 1) or pAKC861 (*plac-ohlI*; lanes 2). For *rsmB*, each lane contained 5 μg of total RNA, and for *rsmA*, *rsmC*, *pel-1*, and *ohlI*, each lane contained 15 μg of total RNA.

Effect of RsmA on *rsmB* transcription. As stated above, the data for *E. coli* strongly suggest a positive effect of CsrA on *csrB* transcription. To ascertain if transcription could account at least in part for the differences in RsmA$^+$ and RsmA$^-$ bacteria, we examined the expression of an $rsmB_{Ecc}$-*lacZ* transcriptional fusion (29). In this fusion, 221 bp of $rsmB_{Ecc}$ DNA has been cloned in front of *lacZ* in a low-copy-number promoter-probe vector, pMP220 (42). The *lacZ* construct or the vector was transferred into RsmA$^+$ strain AC5047, its RsmA$^-$ derivative, AC5070, and its RsmC$^-$ derivative, AC5050. The



data (Table 2) reveal that the expression of $rsmB_{Ecc}$-*lacZ* is slightly (ca. 1.3 times) higher in RsmA$^-$ bacteria than RsmA$^+$ bacteria. These observations are very different from those reported for *E. coli*, where substantial (ca. 20-fold) stimulation was noted in CsrA$^+$ bacteria compared to CsrA$^-$ bacteria. More importantly, our data do not indicate a positive effect of RsmA on *rsmB* transcription.

We extended these findings by examining the expression of *lacZ* transcriptional fusions of $rsmB_{Ea}$ and $rsmB_{Ehg}$ (see reference 31 for the characteristics of these genes). The fusions were transferred to RsmA$^+$ and RsmA$^-$ *E. carotovora* subsp. *carotovora* strains and assayed for β-galactosidase activity. The data (Table 2) show that RsmA did not stimulate *rsmB* transcription. Although somewhat limited in scope, our findings with three different plant-pathogenic *Erwinia* species and those of Gudapaty et al. (16) with *E. coli* raise the possibility that *rsmB* RNA production is differently regulated in plant-pathogenic and non-plant-pathogenic bacteria.

RsmC deficiency in RsmA$^+$ bacteria leads to *rsmB* RNA overproduction. We considered the possibility that the RsmA effect on *rsmB* RNA was exerted via another regulator controlled by RsmA. We considered RsmC as the likely candidate for the following reasons: (i) *rsmC* RNA levels are higher in RsmA$^-$ bacteria than in RsmA$^+$ bacteria (Fig. 1B); (ii) RsmC is known to negatively regulate *rsmB* RNA production (13; also, Table 2 shows the effect of RsmC deficiency on the expression of an $rsmB_{Ecc}$-*lacZ* transcriptional fusion); and (iii) RsmA does not affect the levels of GacA, HexA, and KdgR (A. Chatterjee, unpublished data), known to affect RsmA and *rsmB* RNA production (10, 20, 30, 36). The results (Fig. 1A) with RsmA$^+$ RsmC$^+$ (lane 1), RsmA$^+$ RsmC$^-$ (lane 2), RsmA$^-$ RsmC$^+$ (lane 3), and RsmA$^-$ RsmC$^-$ (lane 4) strains reveal that the level of *rsmB* RNA is highest in RsmA$^+$ RsmC$^-$ bacteria, followed by the levels in RsmA$^+$ RsmC$^+$, RsmA$^-$ RsmC$^-$, and RsmA$^-$ RsmC$^+$ strains. We attribute the presence of the highest level of *rsmB* RNA in the RsmA$^+$ RsmC$^-$ strain to the cumulative effects of the absence of the negative effect of RsmC and the stability of *rsmB* RNA in the presence of RsmA. By contrast, the marked reduction in the level of *rsmB* RNA in RsmA$^-$ RsmC$^+$ bacteria is due the presence of RsmC and the instability of the RNA in the absence of RsmA.

To assess the relative importance of RsmA and RsmC in *rsmB* RNA production, we transferred the low-copy-number vector pRK415; the RsmA$^+$ plasmid, pAKC878; or the RsmC$^+$ plasmid, pAKC979, into the RsmA$^-$ RsmC$^-$ strain

FIG. 2. Stabilities of *rsmB* transcript in *E. carotovora* subsp. *carotovora* strains. Samples were collected 0, 3, 5, 7.5, 10, and 13 min after the addition of rifampin. The densitometeric scanning results (percentage of remaining mRNA) were plotted against time after rifampin treatment. (A) Ecc71 (lanes 1 to 6; each lane contained 5 μg of total RNA) and AC5071 (RsmA$^-$) (lanes 7 to 12; each lane contained 15 μg of total RNA). The X-ray film was exposed for 5 h at −80°C. (B) Ecc71 (lanes 1 to 6; each lane contained 15 μg of total RNA) and AC5094 (Ohl$^-$) (lanes 7 to 12; each lane contained 7 μg of total RNA). The X-ray film was exposed for 1 h at −80°C. (C) AC5070 (RsmA$^-$ Ohl$^+$) (lanes 1 to 6) and AC5090 (RsmA$^-$Ohl$^-$) (lanes 7 to 12). Each lane contained 15 μg of total RNA. The X-ray film was exposed for 5 h at −80°C.

TABLE 2. β-Galactosidase activities of RsmA$^-$, RsmC$^-$, and Ohl$^-$ *E. carotovora* subsp. *carotovora* strains carrying *rsmA-lacZ* and *rsmB-lacZ* fusions[a]

Strain	Relevant phenotype	β-Galactosidase activity (Miller units)
AC5047/pMP220	RsmA$^+$ RsmC$^+$ Ohl$^+$/(vector)	61
AC5070/pMP220	RsmA$^-$ RsmC$^+$ Ohl$^+$/(vector)	71
AC5047/pAKC1002	RsmA$^+$ RsmC$^+$ Ohl$^+$/(*rsmB*$_{Ecc}$-*lacZ*)	2,150
AC5070/pAKC1002	RsmA$^-$ RsmC$^+$ Ohl$^+$/(*rsmB*$_{Ecc}$-*lacZ*)	2,900
AC5050/pMP220	RsmA$^+$ RsmC$^-$ Ohl$^+$/(vector)	72
AC5050/pAKC1002	RsmA$^+$ RsmC$^-$ Ohl$^+$/(*rsmB*$_{Ecc}$-*lacZ*)	3,817
AC5047/pMP220	RsmA$^+$ RsmC$^+$ Ohl$^+$/(vector)	88
AC5070/pMP220	RsmA$^-$ RsmC$^+$ Ohl$^+$/(vector)	96
AC5047/pAKC1047	RsmA$^+$ RsmC$^+$ Ohl$^+$/(*rsmB*$_{Ea}$-*lacZ*)	1,402
AC5070/pAKC1047	RsmA$^-$ RsmC$^+$ Ohl$^+$/(*rsmB*$_{Ea}$-*lacZ*)	2,712
AC5047/pAKC1048	RsmA$^+$ RsmC$^+$ Ohl$^+$/(*rsmB*$_{Ehg}$-*lacZ*)	1,530
AC5070/pAKC1048	RsmA$^-$ RsmC$^+$ Ohl$^+$/(*rsmB*$_{Ehg}$-*lacZ*)	2,097
AC5047/pMP220	RsmA$^+$ Ohl$^+$/(vector)	49
AC5091/pMP220	RsmA$^+$ Ohl$^-$/(vector)	69
AC5047/pAKC1100	RsmA$^+$ Ohl$^+$/(*rsmA*$_{Ecc}$-*lacZ*)	810
AC5091/pAKC1100	RsmA$^+$ Ohl$^-$/(*rsmA*$_{Ecc}$-*lacZ*)	1,868
AC5047/pMP220	RsmA$^+$ Ohl$^+$/(vector)	57
AC5091/pMP220	RsmA$^+$ Ohl$^-$/(vector)	63
AC5047/pAKC1002	RsmA$^+$ Ohl$^+$/(*rsmB*$_{Ecc}$-*lacZ*)	2,901
AC5091/pAKC1002	RsmA$^+$ Ohl$^-$/(*rsmB*$_{Ecc}$-*lacZ*)	2,662

[a] Bacteria were grown in minimal-salts medium plus sucrose and tetracycline at 28°C to a Klett value of ca. 200 for the assays.

and compared the levels of *rsmB* RNA. The data in Fig. 1C show that while RsmA had a marked effect on the levels of *rsmB* RNA (lane 2), RsmC in the absence of RsmA had no such effect (lane 3).

Effects of the quorum-sensing signal, OHL, on *rsmA* and *rsmB* expression. A recent report has documented the fact that OHL deficiency results in elevated levels of *rsmA* and *rsmB* transcripts in *E. carotovora* subsp. *carotovora* strain SCC3193 (25). The data in Fig. 1D and E (lanes 1 and 2) show a similar effect of OHL on Ecc7l. We have extended these observations by examining the expression of an *rsmA-lacZ* transcriptional fusion and an *rsmB-lacZ* transcriptional fusion in OHL$^+$ and OHL$^-$ bacteria. The data in Table 2 reveal about two- to threefold-higher levels of β-galactosidase in OHL$^-$ bacteria carrying the *rsmA-lacZ* fusion than in OHL$^+$ bacteria carrying the same fusion. On the other hand, the levels of β-galactosidase in OHL$^+$ and OHL$^-$ bacteria carrying the *rsmB-lacZ* transcriptional fusion are comparable. These results suggest that *rsmA* transcription, but not *rsmB* transcription, is affected by OHL. Western blot analysis (Fig. 3) also revealed that the RsmA level is higher in Ohl$^-$ strains than in the Ohl$^+$ parent.

We next considered the possibility that the higher levels of *rsmB* RNA in OHL$^-$ bacteria than in OHL$^+$ bacteria (Fig. 1E, lanes 1 and 2) could be due to increased production of RsmA and consequent RsmA-mediated stability of *rsmB* RNA. Two lines of evidence support this hypothesis. First, the levels of *rsmB* RNA are much lower in the OHL$^-$ RsmA$^-$ double mutant than in its OHL$^-$ RsmA$^+$ parent (Fig. 1E, lanes 4 and 2). Second, the results shown in Fig. 2B demonstrate that *rsmB* RNA is more stable in RsmA$^+$ OHL$^-$ bacteria than in RsmA$^+$ OHL$^+$ bacteria. By contrast, the stability of *rsmB* RNA is not affected in RsmA$^-$ OHL$^+$ and RsmA$^-$ OHL$^-$ strains (Fig. 2C). These observations establish that the OHL effect is abrogated in the absence of RsmA and support the prediction that in OHL$^-$ bacteria the pool size of RsmA will be high, which presumably binds every available *rsmB* RNA, conferring stability.

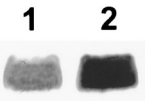


FIG. 3. Western blot analysis of RsmA produced by Ecc71 (lane 1) and AC5094 (Ohl$^-$; lane 2). Each lane contained 15 μg of total bacterial protein.

It is now fairly well established that OHL is required for extracellular protein production as well as the expression of various other traits in *E. carotovora* subsp. *carotovora* and other soft-rotting *Erwinia* spp. (8, 12, 22, 39). Since the production of both RsmA and *rsmB* RNAs is stimulated by OHL deficiency (see above and reference 25) and *rsmB* RNA is believed to sequester RsmA, at first glance one is inclined to dismiss the possibility that the Rsm regulatory system is responsible for the pleiotropic negative phenotype of OHL$^-$ bacteria. Direct evidence for this would require determining the relative levels of free RsmA and RsmA-*rsmB* RNA complex. While technologies are being developed for such analyses, we have obtained indirect evidence that excess RsmA is indeed responsible for the negative effects of OHL deficiency. We transferred a *lacp-rsmB* plasmid, pAKC1049, and pDK7 carrying *lacI*^{q} into the OHL$^-$ strain, AC5094, grew the bacteria in minimal-salts medium plus sucrose containing various levels of IPTG (isopropyl-β-D-thiogalactopyranoside), and assayed for Pel, Peh, Cel, and Prt activities. AC5094 carrying the empty vectors served as a control. Table 3 and Fig. 4A show that production of extracellular enzymes did not occur in the absence of pAKC1049 but did occur in AC5094 carrying pAKC1049. Moreover, the

TABLE 3. Levels of Pel produced by *E. carotovora* subsp. *carotovora* constructs[a]

Strain	Relevant phenotype	IPTG (mM)	Pel sp act (U/ml/A_{600} unit)
AC5094/pCL1920/pDK7	Ohl$^-$/(vectors)	0	0.04
AC5094/pAKC1049/pDK7	Ohl$^-$/(*lacp-rsmB*$_{Ecc}$)	0	0.6
AC5094/pAKC1049/pDK7	Ohl$^-$/(*lacp-rsmB*$_{Ecc}$)	0.1	1.2
AC5094/pAKC1049/pDK7	Ohl$^-$/(*lacp-rsmB*$_{Ecc}$)	1.0	1.4
AC5088/pCL1920	RsmB$^-$/(vector)	0	ND[b]
AC5088/pAKC861	RsmB$^-$/(*lacp-ohlI*)	0	ND

[a] Bacteria were grown in minimal-salts medium plus sucrose (0.5% [wt/vol]) plus drugs, with or without IPTG, at 28°C to a Klett value of ca. 200. Culture supernatants were used for enzyme assays.
[b] ND, not detectable.

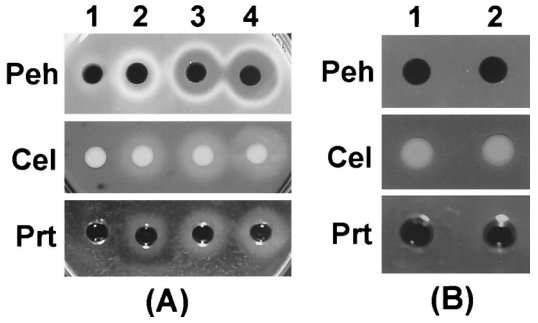


FIG. 4. (A) Agarose plate assays of Peh, Cel, and Prt activities of *E. carotovora* subsp. *carotovora* strain AC5094 (Ohl^-) carrying PDK7 and pCL1920 (vectors; column 1), AC5094 carrying pDK7 and pAKC1049 (*lacp-rsmB*; column 2), AC5094 carrying pDK7 and pAKC1049 with 0.1 mM IPTG treatment (column 3), and AC5094 carrying pDK7 and pAKC1049 with 1.0 mM IPTG treatment (column 4). (B) Agarose plate assays of Peh, Cel, and Prt activities of *E. carotovora* subsp. *carotovora* strain AC5088 ($RsmB^-$) carrying pCL1920 (vector; column 1) and AC5088 carrying pAKC861 (*lacp-ohlI*; column 2). The bacteria were grown at 28°C in minimal-salts medium plus sucrose and drugs to a Klett value of 200, and the culture supernatants were used for assays. Each well contained 10 μl of culture supernatant.

data show that increased levels of extracellular enzymes were produced with increasing concentrations of IPTG. The results of Northern analysis indicate a correlation between the levels of *rsmB* RNA and *pel-1* transcripts (Fig. 1F). Since RsmA binds *rsmB* RNA, we conclude that overproduction of *rsmB* RNA in the presence of IPTG resulted in titration of most or all available RsmA molecules, thereby relieving the inhibitory effects of RsmA on extracellular enzyme production.

As discussed above (see the introduction), several lines of evidence suggested that the quorum-sensing signal acts via the posttranscriptional regulatory system. To further test this, we reexamined the properties of an Ohl^+ $RsmB^-$ mutant. Extracellular enzyme production does not occur in this construct (Chatterjee, unpublished). We transferred the *lacp-ohlI* plasmid, pAKC861, into the mutant, where the *lac* promoter drives *ohlI* expression. The construct was grown in minimal-salts medium plus sucrose. Despite the production of high levels of *ohlI* RNA, extracellular enzymes and *pel-1* transcripts did not occur under these conditions (Fig. 1G and 4B and Table 3). Thus, the OHL effect did not manifest itself in the absence of *rsmB* RNA. We have previously documented the fact that $RsmA^-$ strains do not require OHL for enzyme production and pathogenicity (8). These observations collectively demonstrate that the OHL effect in *E. carotovora* subsp. *carotovora* is channeled via the RsmA-*rsmB* regulatory pathway. To our knowledge, this is the first example of a quorum-sensing signal acting via a posttranscriptional regulatory system. How OHL represses transcription of *rsmA* is not evident. Several lines of evidence suggest that an OHL receptor, similar to LuxR, does not directly regulate *rsmA* transcription. First, a mutant of *E. carotovora* subsp. *carotovora* strain SCC3193 deficient in a LuxR homolog, ExpR, is not affected in extracellular enzyme production and the expression of *rsmA* (4, 25). Second, sequences corresponding to the LuxR box are not found upstream of *rsmA* DNA. Therefore, it is quite likely that the OHL effect in *E. carotovora* subsp. *carotovora* is channeled via a novel regulator that controls *rsmA* transcription. We have initiated a search for such a regulator.

In the experiments described above, the total-RNA and -protein samples were obtained from bacterial cultures grown to a Klett value of 150, and β-galactosidase and extracellular-enzyme assays were performed using cultures grown to a Klett value of 200. To verify that the OHL levels were comparable under these conditions, we have estimated the levels of OHL using an *E. coli*-based bioluminescence assay system (8). Ecc71 produced 7.3×10^7 RLU at a Klett value of 150 and 7.8×10^7 RLU at a Klett value of 200, demonstrating that OHL levels were generally similar under these growth conditions.

In conclusion, we have established that higher levels of *rsmB* transcripts in $RsmA^+$ bacteria than in $RsmA^-$ bacteria are mostly due to the stability of *rsmB* RNA conferred by RsmA and not to increased transcription, as is the case with CsrA and *csrB* in *E. coli*. In addition, the regulatory effect of RsmA on RsmC may partly contribute to the levels of *rsmB* RNA. Despite structural and functional similarities between RsmA and CsrA and between *rsmB* RNA and *csrB* RNA, there are significant differences in these two systems as well. For example, a homolog of RsmC has not been detected in *E. coli*. RsmA and CsrA appear to affect different genes in vivo. While CsrA has been found to positively affect the *flhCD* operon in *E. coli* (43), no such effect of RsmA was seen in *E. carotovora* subsp. *carotovora* (Chatterjee, unpublished). *E. coli* is known not to produce OHL or its analogs, although *E. coli* responds to quorum-sensing signals produced by other bacteria (32). Thus, it is clear that OHL or similar molecules, at least in axenic culture, do not affect CsrA production. As documented here, *rsmA* expression in *E. carotovora* subsp. *carotovora* is indeed stimulated by OHL deficiency. These differences probably reflect the fact that the RsmA-*rsmB* and CsrA-*csrB* systems target different set of genes in bacteria inhabiting animals and humans and those inhabiting plants.

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