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Inactivation of *rsmA* Leads to Overproduction of Extracellular Pectinases, Cellulases, and Proteases in *Erwinia carotovora* subsp. *carotovora* in the Absence of the Starvation/Cell Density-Sensing Signal, *N*-(3-Oxohexanoyl)-L-Homoserine Lactone†

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The soft-rotting bacterium, *Erwinia carotovora* subsp. *carotovora* 71, produces extracellular enzymes such as pectate lyase isozymes (Pels), cellulase (Cel), polygalacturonase (Peh), and protease (Prt). While the extracellular levels of these enzymes are extremely low when the bacterium is grown in salts-yeast extract-glycerol (SYG) medium, the enzymatic activities are highly induced in SYG medium supplemented with celery extract. By transposon (mini-Tn*5*) mutagenesis, we isolated a RsmA$^-$ mutant, AC5070, which overproduces extracellular enzymes; the basal levels of Pel, Peh, and Cel in AC5070 are higher than the induced levels in the RsmA$^+$ parent, AC5047. While Peh production is mostly constitutive in AC5070, Pel, Cel, and Prt production is still inducible with celery extract. The high basal levels of *pel-1*, *pel-3*, and *peh-1* mRNAs in AC5070 demonstrate that overproduction of the pectolytic enzymes is due to the stimulation of transcription. Using chromosomal DNA flanking mini-Tn*5* as a probe, we cloned the wild-type *rsmA*$^+$ allele, which suppresses Pel, Peh, Cel, and Prt production in both RsmA$^+$ and RsmA$^-$ strains. The RsmA$^-$ mutant, like its parent, produces *N*-(3-oxohexanoyl)-L-homoserine lactone (HSL), a starvation/cell density-sensing signal required for extracellular enzyme production. To examine the role of HSL, we constructed HSL-deficient strains by replacing *hslI*, a locus required for HSL production, with *hslI*::Tn*3*HoHo1-Spc. While the basal levels of Pel, Peh, Cel, and Prt are comparable in the RsmA$^-$ mutant and its HSL$^-$ derivative, these enzymes are barely detectable in the Hsl$^-$ derivative of the RsmA$^+$ parent strain. The Hsl$^-$ RsmA$^+$ strain fails to elicit soft rot, whereas the Hsl$^-$ RsmA$^-$ strain, like its Hsl$^+$ RsmA$^-$ parent, remains hypervirulent. These findings demonstrate that the RsmA$^-$ mutant produces extracellular enzymes and macerates plant tissue in the absence of HSL. We conclude that overproduction of extracellular enzymes in an HSL-independent manner occurs because of the inactivation of a global repressor locus, *rsmA*.

The enterobacterium *Erwinia carotovora* subsp. *carotovora* causes tissue-macerating (soft-rotting) disease in a wide variety of plants and plant organs (32, 33). Elicitation of the disease requires the action of extracellular enzymes produced by the bacterium, most importantly the pectinases, such as pectate lyase (Pel), polygalacturonase (Peh), and pectin lyase (3, 15, 16). A recent report (47) also documents reduced virulence in a cellulase (Cel)-deficient *E. carotovora* subsp. *carotovora* strain. Likewise, genetic data (44) suggest that proteases may also contribute to virulence of *E. carotovora* subsp. *carotovora.*

There is both genetic and physiological evidence that extracellular Pel, Peh, Cel, and protease (Prt) production is coregulated in *E. carotovora* subsp. *carotovora*. For example, Pel, Peh, Cel, and Prt production is simultaneously activated in the *E. carotovora* subsp. *carotovora* strains 71 and SCRI193 when the bacteria are grown in media supplemented with celery extract (27, 30). Pleiotropic mutants (i.e., AepA$^-$, AepB$^-$, and AepH$^-$ [Aep, activator of extracellular enzyme production] [8, 27, 28, 30]) in which Pel, Peh, Cel, and Prt production no longer responds to signals present in celery extract have been isolated from *E. carotovora* subsp. *carotovora* 71; the cognate genes cloned from *E. carotovora* subsp. *carotovora* 71 restore inducibility in such mutants. Pleiotropic mutants resembling the Aep$^-$ mutants of *E. carotovora* subsp. *carotovora* 71 have also been isolated from the *E. carotovora* subsp. *carotovora* strains SCRI193 and SCC3193 (23, 35, 40).

Recent studies have firmly established that the starvation/cell density (quorum)-sensing signal, *N*-(3-oxohexanoyl)-L-homoserine lactone (HSL) (20, 22), is required for the production of Pel, Peh, Cel, and Prt in the *E. carotovora* subsp. *carotovora* strains SCRI193 (1, 23) and SCC3193 (34). We subsequently determined that HSL controls extracellular enzyme production in *E. carotovora* subsp. *carotovora* 71 as well (5, 6). These observations provide additional evidence for common regulatory steps controlling extracellular enzyme production in these bacteria. However, at this juncture we do not know how HSL, plant signals, and the *aep* gene products interact to activate the expression of the genes specifying extracellular enzymes.

To gain additional insights into the regulatory systems, we isolated, by transposon mutagenesis, a RsmA$^-$ (Rsm, repressor of secondary metabolites) mutant of *E. carotovora* subsp. *carotovora* 71 derepressed for extracellular enzyme production. The production of high levels of Pel, Peh, Cel, and Prt in the RsmA$^-$ mutant occurs in the absence of HSL, which is needed for the activation of extracellular enzyme production in the RsmA$^+$ bacteria. Our findings demonstrate that inactivation of the *rsmA* locus causes the derepression of extracellular enzyme production.

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MATERIALS AND METHODS

Bacterial strains and plasmids. 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 yeast extract-glucose-calcium carbonate (YGC) agar.

Media. Recipes of cellulase detection agar, LB medium, minimal salts medium, nutrient gelatin agar, polygalacturonate-yeast extract agar, salts-yeast extract-glycerol (SYG) medium, SYG-celery extract medium, and YGC agar are described in our previous publications (2, 11, 29, 30). When required, the following antibiotics and drugs were added at the indicated concentrations (in micrograms per milliliter): ampicillin, 50; chloramphenicol, 10; kanamycin, 50; nalidixic acid, 50; spectinomycin, 50; streptomycin, 100; and tetracycline, 10. Media were solidified by the addition of 1.5% agar.

The compositions of agarose media for semiquantitative assays of enzymatic activities were as follows: Pel assay medium, 1% polygalacturonic acid (PGA), 1% yeast extract, 0.38 μM $CaCl_2$, and 100 mM Tris-HCl, pH 8.5; Peh assay medium, 1% PGA, 1% yeast extract, 2.2 mM EDTA, and 110 mM sodium acetate, pH 5.5; Cel assay medium, 0.1% carboxymethyl cellulose and 25 mM sodium phosphate, pH 7.0; and Prt assay medium, 3% gelatin and 0.4% nutrient broth. In addition, the media were supplemented with 0.8% agarose and 0.2% sodium azide. Each petri plate (100-mm diameter) contained 15 ml of assay medium.

Preparation of samples for enzyme assays and assay conditions. Growth conditions, preparation of culture supernatants, and assay conditions for Cel, Peh, Pel, and Prt were previously described (14, 30). The following substrates were used in quantitative enzymatic assays: PGA for Pel and Peh, carboxymethyl cellulose for Cel, and azocasein for Prt. For semiquantative assays, wells were made in agarose media with a number 2 cork borer and the bottoms were sealed with molten agarose (0.8% [wt/vol]). Samples (10 μl) of appropriately diluted enzyme were applied to the wells, and the plates were incubated at 28°C. After 16 to 18 h, Pel and Peh assay plates were developed with 4 N HCl, and the Cel assay plates were developed with Congo red and NaCl solutions (see Fig. 1) (2, 14). Halos around the wells due to protease activity became visible in Prt assay plates within 24 to 36 h without any further treatment (see Fig. 1).

Each result presented in tables and figures for enzymatic activities is from a single experiment. All assays were, however, repeated with consistent results, and experiments were performed three or more times.

Bioluminescence assay for HSL. HSL in spent cultures of *E. carotovora* subsp. *carotovora* strains and other bacteria were detected in the following manner. Bacterial strains were grown on a rotary shaker at 28°C in the desired media. Following 20 to 22 h of incubation, culture samples were removed, centrifuged for 30 min at 10,000 × *g* at 4°C, and filtered through membrane filters (0.22-μm pore size; MSI, Westboro, Mass.). Samples (5 μl) of this preparation or of various dilutions were placed over cells of *Escherichia coli* (VJS533 carrying pHV200I) on LB agar surfaces. The plates were incubated in the dark at room temperature, and light production after 4 to 6 h was detected visually or on X-ray films. Another assay entailed coinoculation of cells of test bacteria and of *E. coli* VJS533 carrying pHV200I on LB agar, incubation of the plates at 28°C, and visual examination of light production after 4 to 6 h of incubation.

For quantitative assays, the test bacteria were grown overnight in SYG medium or SYG-celery extract medium at 28°C. The cultures were centrifuged, and the supernatants were sterilized by filtration. Appropriate volumes of supernatants and sterile water were added to double-strength LB (with ampicillin) to yield desired concentrations of spent culture. This medium was inoculated with *E. coli* VJS533 containing pHV200I. At desired intervals, culture samples were removed to determine cell density by measurement of turbidity at 600 nm with a spectophotometer (model Du70; Beckman, Palo Alto, Calif.) and light production with a luminometer (model Opticomp1; MGM Instrument, Hamden, Conn.).

Recombinant DNA techniques. Standard procedures were used in the isolation of plasmid and chromosomal DNAs, transformation of *E. coli*, electroporation of *E. carotovora* subsp. *carotovora*, restriction digests, gel electrophoresis, electroelution of DNA fragments, DNA ligation, and Southern blot hybridization (41). Restriction and modifying enzymes were obtained from Promega Biotec (Madison, Wis.) and U.S. Biochemicals (Cleveland, Ohio). The Random primer system of U.S. Biochemicals was used for labeling of DNA.

Isolation of a RsmA$^-$ mutant by mini-Tn*5* mutagenesis. The Nal^r strain AC5047 (Table 1) was mutagenized with mini-Tn*5* with *E. coli* S17-1(lambda-Pir)/pUT::mini-Tn*5* as the donor strain. Biparental matings were carried out as described by de Lorenzo et al. (17). Transconjugants were selected on nutrient gelatin agar plates containing kanamycin and nalidixic acid. Prt-overproducing mutants, identified by the size of the halo around each colony, were screened for the levels of Pel, Peh, and Cel with agar plate assays (30).

Inactivation of the *hslI* locus by Tn*3*HoHo1::Spc mutagenesis. Since *E. carotovora* subsp. *carotovora* 71 and its derivatives are naturally resistant to ampicillin, in order to use Tn*3*HoHo1 in these bacteria, we modified it by inserting the omega fragment conferring Spc^r (37) at the *Bgl*II site (43). The HslI$^+$ plasmid, pAKC852, was mutagenized in *E. coli* HB101 carrying pTn*3*HoHo1::Spc and pSShe according to the method of Stachel et al. (43). Briefly, pAKC852 was transformed into this strain, and the Tc^r, Cm^r, and Spc^r transformants were pooled and mated with *E. coli* PolA$^-$ strain C2110. The Tc^r Spc^r transconjugants of C2110 were selected on LB agar supplemented with nalidixic acid, spectinomycin, and tetracycline and then tested for HSL production by using the qualitative LUX bioassay described above. The plasmids from Light$^-$ colonies were extracted and transformed into *E. coli* DH5α.

Nucleotide sequence determination. pAKC856 and pAKC857, two subclones of the HslI$^+$ plasmid pAKC852, were used for nucleotide sequence determination. Exonuclease III-mediated successive deletions were made by using the Erase-a-Base system (Promega Biotec). Derivatives of overlapping deletions were used for sequence analysis with the Sequenase system II of U.S. Biochemicals. The nucleotide sequence was analyzed with the PC/gene program (IntelliGenetics, Inc., Mountain View, Calif.).

Construction of bacterial strains by marker exchange recombination. pAKC870, carrying the inactivated *rsmA*, or pAKC855, carrying the inactivated *hslI*, was transferred into the desired *E. carotovora* subsp. *carotovora* 71 derivatives by triparental matings with the helper *E. coli* HB101 carrying pRK2013 (19). Transconjugants were selected for the marker used for gene inactivation and then tested for the loss of vector marker. This procedure works quite well with pRK415 (24) derivatives in *E. carotovora* subsp. *carotovora* 71. The allelic exchange was confirmed by Southern hybridization and by testing for the expected phenotype.

Isolation of total RNA and Northern blot analysis. AC5047 and AC5070 were grown to a value of ca. 200 Klett units in SYG medium at 28°C. The procedures for RNA isolation and Northern (RNA) hybridization described by Chatterjee et al. (9) and Liu et al. (26) were followed. Utilizing the sequence information (7, 26), we digested the plasmids (Table 1) with appropriate restriction endonucleases. The 314-bp *Eco*RV-*Kpn*I fragment from pAKC783, the 362-bp *Acc*I-*Hin*dIII fragment from pAKC761, and the 743-bp *Hin*dIII fragment from pAKC781 were eluted from gels, labeled with [α-^{32}P]dCTP, and used as *pel-1*, *pel-3*, and *peh-1* probes, respectively.

Plant tissue maceration. The celery petiole assay was previously described (30). The extent of tissue maceration was estimated visually.

Nucleotide sequence accession number. The nucleotide sequence of *hslI* of *E. carotovora* subsp. *carotovora* 71 has been entered into the Genbank database under the accession number L40174.

RESULTS

Isolation and characterization of a RsmA$^-$ mutant. AC5047, a Nal^r derivative of AC5006, was mutagenized with mini-Tn*5* (17), and Km^r transconjugants were screened for the overproduction of extracellular enzymes. We obtained several putative RsmA$^-$ mutants that overproduced Pel, Peh, Cel, and Prt. The levels of these enzymes in one representative RsmA$^-$ mutant, AC5070, and its RsmA$^+$ parent, AC5047, are shown in Fig. 1 (wells A and C). These strains were also grown in SYG medium and SYG-celery extract medium and quantitatively assayed for Pel, Peh, Cel, and Prt activities. AC5047 produced barely detectable levels of Pel, Peh, Cel, and Prt in SYG medium, and these enzymes were strongly induced by celery extract (Table 2). The basal levels of Pel, Peh, and Cel activities were higher in the RsmA$^-$ mutant than the induced levels of the enzymes in its parent strain. The levels of Pel, Cel, and Prt in AC5070 were further stimulated by celery extract (Table 2): the Pel and Cel levels were about threefold higher and the Prt level was about 18-fold higher in SYG-celery extract medium than in SYG medium. On the other hand, the Peh level in SYG-celery extract medium was about 82% of that in SYG medium. We should point out that the suppressive effect of celery extract on Peh production was consistently seen only with the RsmA$^-$ mutant and not with the parent strain. However, the Peh activity in the RsmA$^-$ mutant grown in the presence of celery extract was still considerably higher than the levels in the parent strain grown under similar conditions (Table 2).

Linkage between mini-Tn*5* DNA and *rsmA* in AC5070. To establish that mini-Tn*5* insertion in *rsmA* is responsible for the derepression of extracellular enzyme production, we cloned the transposon along with flanking DNA from AC5070 by cosmid cloning in pLAFR5. The Km^r Tc^r plasmid pAKC870 was transferred to AC5047, and Km^r transconjugants were selected. Among 24 Km^r transconjugants analyzed, two were Km^r Tc^r whereas the rest were Km^r Tc^s. Since the mini-Tn*5* element lacks transposase (17), the Km^r Tc^s clones probably

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristic(s)	Reference, source, or derivation
Bacteria		
Erwinia carotovora subsp. *carotovora*		
71	Wild type	48
AC5006	Lac^- mutant of 71	29
AC5047	Nal^r derivative of AC5006	Laboratory collection
AC5070	$RsmA^-$ Km^r	Generated by mini-Tn*5* mutagenesis of AC5047; this study
AC5090	$RsmA^-$ Hsl^- Km^r Spc^r	$HslI^-$ derivative of AC5070 by marker exchange with pAKC855; this study
AC5091	$RsmA^+$ Hsl^- Km^r Spc^r	$HslI^-$ derivative of AC5047 by marker exchange with pAKC855; this study
AC6000	$RsmA^-$ Km^r	$RsmA^-$ derivative of AC5047 by marker exchange with pAKC870; this study
SCRI193	Wild type	23
SCC3193	Wild type	34
E. carotovora subsp. *atroseptica* ECA12	Wild type	48
E. chrysanthemi EC16	Wild type	12
E. carotovora subsp. *betavasculorum* JCL11129	Wild type	J. E. Loper
E. amylovora		
E9	Wild type	36
E77	Wild type	R. N. Goodman
EA273	Wild type	S. V. Beer
EA246	Wild type	4
EA178	Wild type	46
E. rhapontici ER1	Wild type	Laboratory collection
Pseudomonas fluorescens Pf7-14	Wild type	10
Escherichia coli		
DH5α	φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 hsdR17 recA1 endA1 thi-1*	Bethesda Research Laboratories
HB101	*proA1 lacY hsdS20* (r_B^- m_B^-) *recA56 rpsL20*	48
C2110	$PolA^-$ Nal^r	Laboratory collection
S17-1	F^- *pro recA1* r_B^- m_B^+ RP4-2 integrated (Tc::Mu) (Km::Tn*7*[Sm^r Tp^r])	42
VJS533	*ara* Δ(*lac-proAB*) *rpsL* φ80*lacZ*ΔM15 *recA56*	21
Plasmids		
pCL1920	Spc^r Sm^r	25
pLARF5	Tc^r	24
pRK415	Tc^r	24
pBluescript KS^+	Ap^r	Stratagene
pRK2013	Mob^+ Tra^+ Km^r	19
pUT mini-Tn*5*-Km	A lambda-pir vector containing mini-Tn*5*-Km transposon	17
pTn*3*HoHo1	Tn*3-lacZ* transposon; Ap^r	43
pTn*3*HoHo1::Spc	Omega fragment (Spc^r) cloned into the *Bgl*II site in pTn*3*HoHo1; Ap^r Spc^r	This study
pHP45Ω	Source of Spc^r omega fragment	37
pSShe	Cm^r plasmid carrying the transposase gene	43
pHV200	8.8-kb *Sal*I fragment containing the *lux* operons; Ap^r	21
pHV200I	Frameshift mutation of *luxI* in pHV200; Ap^r	21a
pAKC761	*pel-3*$^+$ Ap^r	26
pAKC781	*peh-1*$^+$ Ap^r	26
pAKC783	*pel-1*$^+$ Ap^r	7, 26
pAKC851	Hsl^+ Tc^r	pLAFR5 containing *hslI*$^+$ DNA of strain 71; this study
pAKC852	Hsl^+ Tc^r	9.7-kb *Pvu*II fragment of pAKC851 containing *hslI*$^+$ cloned into pRK415; this study

Continued on following page

TABLE 1—*Continued*

Bacterial strain or plasmid	Relevant characteristic(s)	Reference, source, or derivation
pAKC855	Hsl$^-$ Spc^r Tc^r	Generated by Tn*3*HoHo1::Spc mutagenesis of pAKC852; this study
pAKC856	Ap^r	1.5-kb *Eco*RI fragment of pAKC852 cloned into pBluescript KS$^+$; this study
pAKC857	Ap^r	3.0-kb *Eco*RI-*Hin*dIII fragment of pAKC852 cloned into pBluescript KS$^+$; this study
pAKC870	RsmA$^-$ Km^r Tc^r	pLARF5 containing mini-Tn*5* and flanking chromosomal DNA from AC5070; this study
pAKC875	RsmA$^+$ Tc^r	RsmA$^+$ plasmid from genomic library of strain 71; this study

resulted from marker exchange and not secondary transposition. This was also indicated by the finding that Km^r Tc^r transconjugants produced extracellular enzymes like those from the parent strain, AC5047, whereas Km^r Tc^s derivatives, like strain AC5070, overproduced extracellular enzymes. The levels of Pel, Peh, Cel, and Prt in one such isolate, AC6000, were comparable with those in AC5070 (Fig. 1, wells C and E). However, Southern blot hybridization disclosed that mini-Tn*5* as well as *rsmA* DNAs hybridized to the same 11-kb *Cla*I fragments in AC5070 and AC6000. This was determined by hybridizing the blot first with mini-Tn*5* DNA (Fig. 2), stripping off the probe DNA, and then rehybidizing the blot with *rsmA* DNA (see below for the cloning of *rsmA*$^+$ DNA). These data indicated that bacteria carrying mini-Tn*5*::*rsmA* in the chromosome and lacking plasmid DNA were, like AC5070, derepressed for extracellular enzyme production. On the other hand, the RsmA$^+$ transconjugants carrying pAKC870 produced extracellular enzymes like those of the recipient.

Cloning of the *rsmA* locus from *E. carotovora* subsp. *carotovora* 71. An *E. carotovora* subsp. *carotovora* 71 genomic library in pLAFR5 (24, 28) was screened by in situ colony hybridization with a 400-bp DNA fragment which contained ca. 60 bp from one end of mini-Tn*5* and the *rsmA* sequences flanking it as a probe. Of 700 colonies screened, we found two that apparently carried the *rsmA* locus. Plasmids from these colonies were transferred to AC5047 and AC5070, and the levels of extracellular enzymes were determined by agar plate assays. Both the plasmids severely suppressed the levels of extracellular Pel, Peh, Cel, and Prt. Restriction analysis revealed that the plasmids had a very similar restriction pattern with the exception of the presence of an additional 5-kb *Bam*HI fragment in pAKC875 (3a). AC5070 cells carrying pAKC875 or pLAFR5 were grown in SYG medium, and the enzymatic activities in culture supernatants were determined. The data (Table 3) confirmed the results of agar plate assays in that pAKC875 suppressed Pel, Peh, Cel, and Prt levels in AC5070. As expected, high basal levels of these enzymes were produced by AC5070/pLAFR5 (Table 3). On the basis of these observations, we concluded that pAKC875 carried the wild-type *rsmA*$^+$ allele.

Transcription of the pectinase genes in the RsmA$^-$ mutant AC5070. Northern blot hybridizations were carried out to compare the levels of *pel-1*, *pel-3*, and *peh-1* transcripts in AC5070 and AC5047 grown in SYG medium. Figure 3 shows that the basal levels of *pel-1*, *pel-3*, and *peh-1* transcripts were present in the RNA samples from the RsmA$^+$ parent strain, AC5047. The levels of the transcripts were, however, markedly higher in the RsmA$^-$ mutant AC5070.

HSL production. Since HSL is required for the production of extracellular enzymes in *E. carotovora* subsp. *carotovora*, it was of interest to ascertain whether enzyme overproduction in the RsmA$^-$ mutants resulted from an overproduction of HSL. To test this possibility, we used an *E. coli*-based bioassay system that utilizes a *lux* plasmid. Greenberg and associates (21, 21a) have constructed pHV200, which carries all of the *lux* operons, and pHV200I, which is deficient for HSL synthesis because a mutation in the *luxI* gene (Table 1). Since *E. coli* VJS533 carrying pHV200 produces HSL, light production (bioluminescence) does not require the addition of this inducer. By contrast, in *E. coli* carrying pHV200I, light production occurs only when HSL is provided exogenously, and there is a linear relationship between the quantity of HSL and the degree of bioluminescence. Therefore, in our studies, we tested dilutions of spent cultures for their abilities to render the *E. coli* LuxI$^-$ strain (VJS533 carrying pHV200I) luminescent. In initial trials, we determined that bioluminescence was induced by spent cultures of *E. carotovora* subsp. *carotovora* 71 and

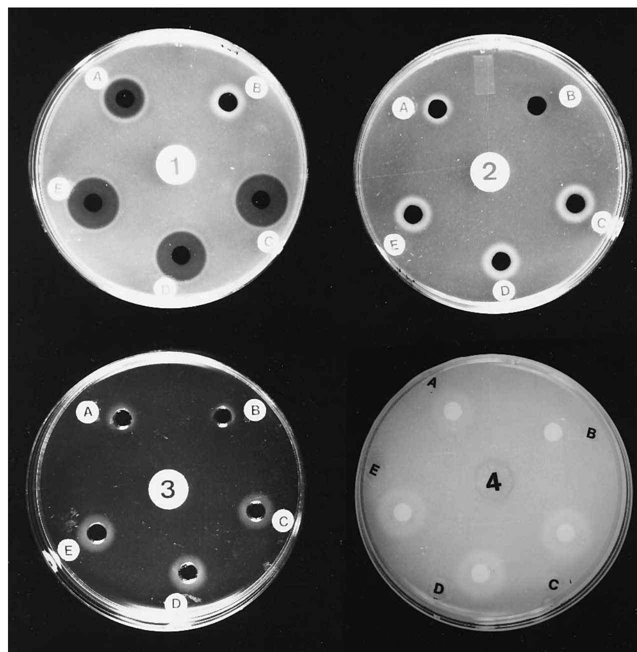

FIG. 1. Agarose plate assays for pectate lyase (plate 1), Peh (plate 2), Prt (plate 3), and Cel (plate 4) activities of *E. carotovora* subsp. *carotovora* strains. Bacteria were grown at 28°C in SYG-celery extract medium and harvested at a value of ca. 350 Klett units. See Materials and Methods for additional details. Culture supernatants were diluted in 10 mM Tris-HCl buffer, pH 7.0, to yield the final concentration of 15% (vol/vol). Portions (10 μl) of these samples were added to each well. Wells: A, AC5047 (RsmA$^+$ HSL$^+$); B, AC5091 (RsmA$^+$ HSL$^-$); C, AC5070 (RsmA$^-$ HSL$^+$); D, AC5090 (RsmA$^-$ HSL$^-$); E, AC6000 (RsmA$^-$ HSL$^+$).

TABLE 2. The levels of extracellular enzymes produced by AC5047 and its RsmA⁻ derivative AC5070

Bacterial strain	Sp act of indicated enzyme in culture supernatant in indicated medium[a]							
	Peh		Peh		Cel		Prt	
	SYG	SYG + CE[b]	SYG	SYG + CE	SYG	SYG + CE	SYG	SYG + CE
AC5047	0.4	22.4	ND[c]	73.7	ND	1.0	0.4	10.0
AC5070	61.0	196.3	434.3	356.8	4.5	11.5	5.9	109.4

[a] Specific activities are expressed as units per milligram of protein.
[b] CE, celery extract.
[c] ND, not detectable.

various other *E. carotovora* subsp. *carotovora* strains, *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *betavasculorum*, and *Erwinia chrysanthemi* but not by spent cultures of *Erwinia amylovora* E9, EA246, EA273, EA178, and E77 or *Pseudomonas fluorescens* Pf7-14. From these observations, we concluded that the soft-rotting *Erwinia* strains produced HSL or its structural analogs capable of activating the *V. fischeri lux* operons. The results of subsequent experiments shown in Fig. 4A demonstrate that the degrees of bioluminescence induced by the spent cultures of AC5070 and its parent strain, AC5047, were comparable. Therefore, we concluded that similar HSL levels were present in spent cultures of these bacteria and that HSL appeared not to be responsible for Pel, Cel, Prt, and Peh overproduction in the RsmA⁻ mutant. The latter conclusion was confirmed by construction of Hsl⁻ derivatives by site-directed mutagenesis.

Cloning of *hsl*⁺ DNA of *E. carotovora* subsp. *carotovora* 71, construction of HslI⁻ strains by marker exchange, and phenotypes of Hsl⁻ strains. By screening an *E. carotovora* subsp. *carotovora* 71 genomic library in *E. coli* HB101 carrying pHV200I, we isolated two plasmids that allowed light production. One of these plasmids, pAKC851, directed HSL production in Hsl⁻ strains of *E. amylovora* EA178 and *Erwinia rhapontici* ER1 as well as in *P. fluorescens* Pf7-14. The nucleotide sequence of the 1.8-kb *E. carotovora* subsp. *carotovora* 71 *hsl*⁺ DNA revealed the presence of a *luxI* homolog designated *hslI* (Fig. 5). The deduced *hslI* product has 98% identity with the predicted *carI* product of *E. carotovora* subsp. *carotovora* SCRI193 (45), 70% identity with the predicted *expI* product of *E. carotovora* subsp. *carotovora* SCC3193 (34), 54% identity with the predicted *eagI* product of *Enterobacter agglomerans* (45), and 26% identity with the *luxI* product of *Vibrio fischeri* (18); all of these genes have been found to specify HSL production.

pAKC852, a 9.7-kb *Pvu*II subclone of pAKC851 carrying the *hslI*⁺ DNA, was mutagenized with Tn*3*HoHo1::Spc to produce the HslI⁻ plasmid pAKC855. Insertion of Tn*3*HoHo1::Spc in *hslI* was confirmed by restriction analysis. This plasmid was used to replace chromosomal *hslI*⁺ DNA with *hslI*::Tn*3* HoHo1-Spc. Southern blot hybridizations confirmed allele exchange. AC5090, the derivative of AC5070, and AC5091, the derivative of AC5047, did not elicit light production in the *E. coli* bioassay system (Fig. 4B). The results of plate assays (Fig. 1, well B) as well as quantitative assays (3a) revealed that AC5091 produced barely detectable levels of Pel, Peh, Cel, and Prt. By contrast, AC5090, like the Hsl⁺ RsmA⁻ strain AC5070, produced high levels of these enzymes (Fig. 1, wells C and D; Table 4). Thus, while HSL deficiency in AC5047 resulted in the loss of extracellular enzyme production, in the RsmA⁻ mutant AC5070 extracellular enzyme production no longer was dependent upon the presence of HSL.

Plant tissue maceration. The tissue-macerating abilities of the RsmA⁻ mutants AC5070 and AC6000 were compared with that of the parent, AC5047, by inoculation of celery petioles (Fig. 6). The RsmA⁻ mutants caused more extensive maceration than the parent RsmA⁺ strain. In addition, the mutants were effective even at a low inoculum dosage (ca. 10^6 cells per inoculation site) which did not allow the parent to elicit tissue

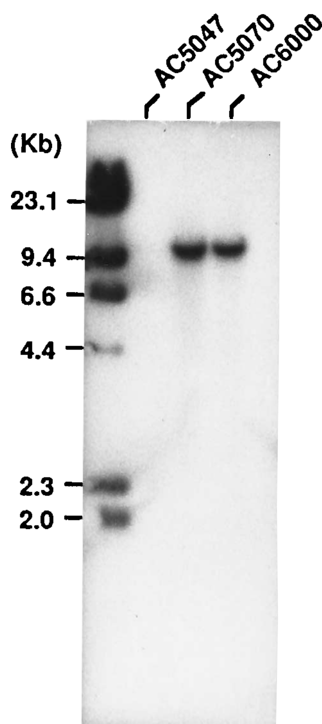

FIG. 2. Southern hybridization of *Cla*I-digested chromosomal DNAs of AC5047 and its RsmA⁻ mutants AC5070 and AC6000. The 2.2-kb *Eco*RI fragment of the mini-Tn*5* was labeled with [α-^{32}P]dATP for use as the probe. The lambda size markers are shown at the left.

TABLE 3. The effect of the *rsmA*⁺ allele on extracellular enzyme production in the RsmA⁻ mutant AC5070

Plasmid carried by AC5070	Sp act in culture supernatant of[a]:			
	Pel	Peh	Cel	Prt
pLAFR5	33.9	348.1	3.4	8.5
pAKC875	0.4	9.4	ND[b]	0.4

[a] Bacteria were grown at 28°C in SYG medium containing tetracycline and harvested at a value of ca. 250 Klett units. Specific activities are expressed as units per milligram of protein.
[b] ND, not detectable.

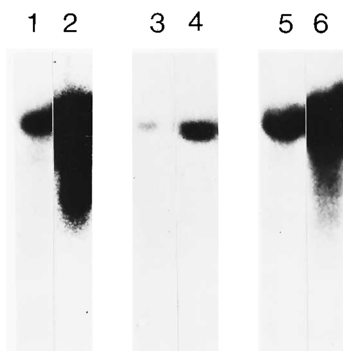

FIG. 3. Northern blots showing the levels of *pel-1*, *pel-3*, and *peh-1* mRNAs in AC5047 and its RsmA$^-$ mutant AC5070 grown in SYG medium. Each lane contained 20 μg of total RNA. *pel-1* transcripts in AC5047 (lane 1) and AC5070 (lane 2), *pel-3* transcripts in AC5047 (lane 3) and AC5070 (lane 4), and *peh-1* transcripts in AC5047 (lane 5) and AC5070 (lane 6) are shown.

maceration (3a). The tissue-macerating ability was not impaired in AC5090, the Hsl$^-$ derivative of AC5070, although HSL deficiency completely eliminated tissue maceration in the parent strain, AC5047 (Fig. 6).

DISCUSSION

In *E. carotovora* subsp. *carotovora*, activation of extracellular Pel, Peh, Cel, and Prt production is believed to be mediated by common regulatory steps. This was previously indicated by the coinduction of Pel, Peh, Cel, and Prt with celery extract and the isolation of pleiotropic mutants deficient in extracellular enzyme production (23, 30, 35). We now provide additional evidence for this hypothesis by isolating a RsmA$^-$ mutant of *E. carotovora* subsp. *carotovora* 71 that overproduces Pel, Peh, Cel, and Prt. Transcript assays show that the elevated basal levels of the pectinases are due to stimulation of transcription of the cognate genes.

Several lines of evidence establish that the derepression of extracellular enzyme production in the RsmA$^-$ mutant is due to the inactivation of a global repressor presumed to be encoded by *rsmA*. (i) A gene-knockout mutation due to mini-Tn*5* insertion led to the derepression of extracellular enzyme production. (ii) The replacement of the wild-type *rsmA*$^+$ locus by the *rsmA*::mini-Tn*5* DNA resulted in the derepression of extracellular enzyme production. By contrast, the RsmA$^+$ strain carrying *rsmA*::mini-Tn*5* DNA as a plasmid produced normal levels of the enzymes, as did its recipient. This phenotype would be expected since the wild-type repressor should exert a *trans*-dominant effect. (iii) The most convincing evidence, however, was the *trans*-dominant repressor effect exerted by the cloned wild-type *rsmA*$^+$ allele. Further studies are in progress to elucidate the structural features of *rsmA*, including its genetic relationship with the negative regulator genes *kdgR* and *pecS* of *E. chrysanthemi* (31, 38).

There is a basal level of transcription of *pel-1* and *peh-1* in the RsmA$^+$ strain AC5047 in SYG medium (Fig. 3), and yet there is little or no enzymatic activity in the culture supernatant (Table 2). We have found that almost all of the Pel and Peh activities produced in SYG medium remain cell bound, possibly due to the lack of export of the synthesized polypeptides. By contrast, when the RsmA$^+$ bacterium is grown in SYG-celery extract, most of these activities are found in the culture supernatant (Table 2 and reference 3a). Thus, enzyme synthesis and export are apparently uncoupled when the bacterium is growing in SYG but not when it is growing in the presence of celery extract. In the RsmA$^-$ mutant, most of the enzymatic activities are found in culture supernatants irrespective of growth conditions (Table 2 and reference 3a). Thus, *rsmA* may also control the enzyme export (i.e., OUT [29]) system in *E. carotovora* subsp. *carotovora* 71. This possibility is currently being investigated.

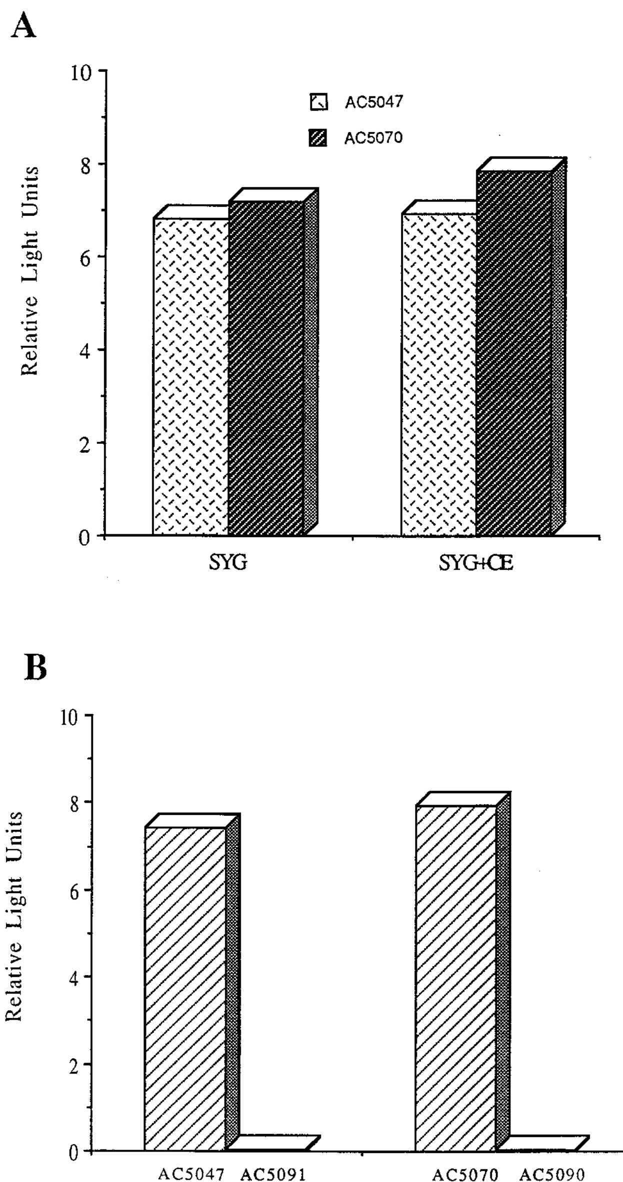

FIG. 4. Effect of spent cultures of *E. carotovora* subsp. *carotovora* strains on light production by *E. coli* VJS533 carrying the LuxI$^-$ plasmid pHV200I. The *E. carotovora* subsp. *carotovora* strains were grown in SYG or SYG-celery extract (CE) medium at 28°C. Supernatants from *E. carotovora* subsp. *carotovora* cultures and the *E. coli* cell suspension were prepared as described in Materials and Methods. For the bioluminescence assay, sterile supernatants and water were added to double-strength LB-ampicillin medium to yield the final concentration of 0.5% (vol/vol) of spent medium in a total volume of 20 ml. The media were inoculated with the *E. coli* cells 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 are expressed as the $\log_{10}$ counts per minute per milliliter of culture normalized for culture turbidity. (A) Relative light units with spent cultures of the RsmA$^-$ mutant AC5070 and its parent strain AC5047 grown in SYG medium or SYG-celery extract medium. (B) Relative light units with spent cultures of AC5047 (RsmA$^+$ Hsl$^+$), AC5091 (RsmA$^+$ Hsl$^-$), AC5070 (RsmA$^-$ Hsl$^+$), and AC5090 (RsmA$^-$ Hsl$^-$) grown in SYG-celery extract medium.

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HslI   M-LEIFDVNHTLLSETKSEELFTLRKETFKDRLNWAVQCTDGMEFDQYDN   49
CarI   M-LEIFDVNHTLLSETKSGELFTLRKETFKDRLNWAVQCTDGMEFDQYDN   49
ExpI   M-LEIFDVSYTLLSEKKSEELFTLRKETFKDRLNWAVKCINGMEFDQYDD   49
EagI   M-LEIFDVSYNDLTERRSEDLYKLRKITFKDRLDWAVNCSNDMEFDEFDN   49
LuxI   MTIMIKKSDFLAIPSEEYKGILSLRYQVFKQRLEWDLVVENNLESDEYDN   50
       * . * . .   ...    .. .**  .**.**.*..   ...* *..*.

HslI   NNTTYLFGIKDNTVICSL-RFIETKYPNMITGTFFPYFKEINIPEGNYLE   98
CarI   NNTTYLFGIKDNTVICSL-RFIETKYPNMITGTFFPYFKEINIPEGNYLE   98
ExpI   DNATYLFGVEGDQVICSS-RLIETKYPNMITGTFFPYFEKIDIPEGKYIE   98
EagI   SGTRYMLGIYDNQLVCSV-RFIDLRLPNMITHTFQHLFGDVKLPEGDYIE   98
LuxI   SNAEYIYACDDTENVSGCWRLLPTTGDYMLKSVFPELLGQQSAPKDPNIV  100
       ... *. .  ..  ...  *..      *.. .*   . . . *..  .

HslI   SSRFFVDKSRAKDILGNEYPISSMLFLSMINYSRDKGYDGIYTIVSHPML  148
CarI   SSRFFVDKSRAKDILGNEYPISSMLFLSMINYSKDKGYDGIYTIVSHPML  148
ExpI   SSRFFVDKARSKTILGNSYPVSTMFFLATVNYSKSKGYDGVYTIVSHPML  148
EagI   SSRFFVDKNRAKALLGSRYPISYVLFLSMINYARHHGHTGIYTIVSRAML  148
LuxI   ELSRFAVGKNSSKINNSASEITMKLFEAIYKHAVSQGITEYVTVTSTAIE  150
       . . *.   ... . ..   ..  .* .  ...  .* ..  *..* ..

HslI   TILKRSGWGIRVVEQGLSEKEERVYLVFLPVDDENQEALARRINRSGTFM  198
CarI   TILKRSGWGIRVVEQGLSEKEERVYLVFLPVDDENQEALARRINRSGTFM  198
ExpI   TILKRSGWKISIVEQGMSEKHERVYLLFLPVDNESQDVLVRRINHNQEFV  198
EagI   TIAKRSGWEIEVIKEGFVSENEPIYLLRLPIDCHNQHLLAKRIRDQSESN  198
LuxI   RFLKR----IKVPCHRIGDKEIHV------LGDTKSVVLSMPINEQ--FK  188
        . **    * .  . . ... ..      ..  .   * ..*.

HslI   SNELKQWPLK-GPAAIAQA   216
CarI   SNELKQWPLR-VPAAIAQA   216
ExpI   ESKLREWPLSFEPMTEPVG   217
EagI   IAALCQWPMSLT-VTPEQV   216
LuxI   KAVLN--------------   193
        . *
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FIG. 5. Alignment of the deduced amino acid sequence of HslI of *E. carotovora* subsp. *carotovora* 71 with those of CarI of *E. carotovora* subsp. *carotovora* SCRI193 (45), ExpI of *E. carotovora* subsp. *carotovora* SCC3193 (34), EagI of *E. agglomerans* (45), and LuxI of *V. fischeri* (18). Asterisks indicate identical amino acids, and single dots indicate conservative substitutions. The numbers at the right indicate amino acid positions in each protein.

Mutants phenotypically similar to AC5070 have also been isolated by ethyl methanesulfonate mutagenesis (28). Moreover, the RsmA$^+$ plasmid pAKC875 suppresses Pel, Peh, Cel, and Prt production in some such mutants (3a). Although we do not yet know whether mutations in *rsmA* are responsible for extracellular enzyme overproduction, it is apparent that the cloned *rsmA*$^+$ exerts a *trans*-dominant response in these mutants as well.

Since HSL is required for extracellular enzyme production in *E. carotovora* subsp. *carotovora* (1, 5, 23, 34), we tested the possibility that HSL levels were higher in the RsmA$^-$ mutant than in the parent strain and that this elevation in turn contributed to extracellular enzyme overproduction. Clearly, our data did not support this notion. The findings indicated that the levels of HSL were not markedly different in the parent strain and the RsmA$^-$ mutant and that HSL concentration appeared not to be a limiting factor under the cultural conditions used in this study. The construction of HSL-deficient mutants by site-directed mutagenesis allowed us to rigorously evaluate the role of HSL in RsmA$^+$ and RsmA$^-$ strains. While HSL was required for extracellular enzyme production and pathogenicity in the RsmA$^+$ bacteria, the production of Pel, Peh, Cel, and Prt in the RsmA$^-$ strain as well as its pathogenicity were not affected by HSL deficiency. The production of extracellular enzymes in an HSL-independent manner by the RsmA$^-$ mutant raised the possibility that HSL and the *rsmA* product interact in some manner to modulate the expression of genes specifying the extracellular enzymes.

TABLE 4. Levels of extracellular enzymes produced by the RsmA$^-$ mutant AC5070 and its Hsl$^-$ derivative AC5090

Bacterial strain	Sp act in culture supernatant of[a]:			
	Pel	Peh	Cel	Prt
AC5070 (Hsl$^+$ RsmA$^-$)	85.4	465.9	2.4	4.5
AC5090 (Hsl$^-$ RsmA$^-$)	74.7	472.0	2.5	5.3

[a] Bacteria were grown in SYG medium at 28°C and harvested at a value of ca. 250 Klett units. Specific activities are expressed as units per milligram of protein.

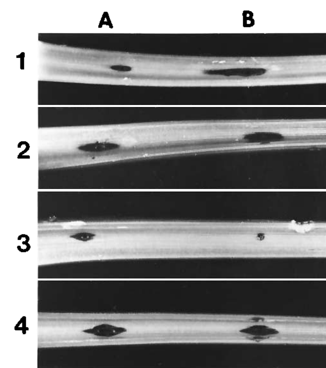

FIG. 6. Plant tissue maceration induced by *E. carotovora* subsp. *carotovora* strains. About 2×10^8 cells were injected into celery petioles at each inoculation site. Inoculated celery tissues were incubated in a moist chamber at 25°C for 24 h. (1) A, AC5047 (RsmA$^+$ Hsl$^+$); B, AC5070 (RsmA$^-$ Hsl$^+$). (2) A, AC5070 (RsmA$^-$ Hsl$^+$); B, AC6000 (RsmA$^-$ Hsl$^+$). (3) A, AC5047 (RsmA$^+$ Hsl$^+$); B, AC5091 (RsmA$^+$ Hsl$^-$). (4) A, AC5070 (RsmA$^-$ Hsl$^+$); B, AC5090 (RsmA$^-$ Hsl$^-$).

Pel-, Peh-, Cel-, and Prt-producing systems in the RsmA$^-$ mutant responded differently to celery extract. The degree of induction of Pel, Cel, and Prt with celery extract varied depending upon the enzyme. By contrast, Peh levels were slightly lower in SYG-celery extract medium than in SYG medium. These differential responses with celery extract can be reconciled by invoking the operation of Pel-specific, Prt-specific, Peh-specific, and Cel-specific regulatory systems that respond differently to plant signals but similarly to the step(s) controlled by HSL. Indeed, specific regulation of Peh has been indicated by the detection of *pehR*, a positive regulatory gene in *E. carotovora* subsp. *carotovora* SCC3193 (39). Moreover, the available evidence, albeit indirect, suggests that the genes for Pel isoenzymes, i.e., *pel-1* and *pel-3* of *E. carotovora* subsp. *carotovora* 71 (8, 26), may be regulated differently. Clearly, much additional work is needed to establish the existence of gene-specific regulatory systems and to determine how they interface with the global HSL-RsmA-Aep systems to activate extracellular enzyme production in *E. carotovora* subsp. *carotovora.*

The RsmA$^-$ mutant is hypervirulent in that it causes extensive tissue maceration at low inoculum dosage. Also, at equivalent cell concentration, the RsmA$^-$ mutant elicits disease symptoms at a faster rate than the parent strain. One explanation for these responses is that the entire population of the RsmA$^-$ mutant starts producing pectinases and other extracellular enzymes immediately after inoculation, whereas enzyme production in the RsmA$^+$ bacteria is initiated after a lag period during which HSL and plant signals interact with regulatory components to activate transcription of the genes specifying the extracellular enzymes. We are now making chromosomal *pel-gus* and *peh-gus* fusions to compare the kinetics of gene expression in planta in the RsmA$^-$ mutants and their parent strains.

In summary, we have described a mutant of *E. carotovora*

subsp. *carotovora* 71 that overproduces extracellular Pel, Peh, Cel, and Prt in an HSL-independent manner and the cloning of the cognate wild-type $rsmA^+$ locus responsible for global repression. Our ongoing studies should help resolve a number of interesting issues including the target(s) of RsmA, structural and functional relationships between RsmA and global repressors of *Erwinia* spp. and other enterobacteria, and the pathogenic potential of bacteria carrying genetically modified *rsmA*, i.e., the gene encoding a superrepressor of extracellular enzyme production.

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