JOURNAL OF BACTERIOLOGY, July 2005, p. 4792–4803 Vol. 187, No. 14
0021-9193/05/$08.00+0 doi:10.1128/JB.187.14.4792–4803.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

ExpR, a LuxR Homolog of *Erwinia carotovora* subsp. *carotovora*, Activates Transcription of *rsmA*, Which Specifies a Global Regulatory RNA-Binding Protein

Yaya Cui,[1] Asita Chatterjee,[1]* Hiroaki Hasegawa,[1] Vaishali Dixit,[1] Nathan Leigh,[2] and Arun K. Chatterjee[1]

Department of Plant Microbiology and Pathology[1] *and Department of Chemistry,*[2] *University of Missouri, Columbia, Missouri 65211*

Received 24 January 2005/Accepted 18 April 2005

***N*-acyl homoserine lactone (AHL) is required by *Erwinia carotovora* subspecies for the expression of various traits, including extracellular enzyme and protein production and pathogenicity. Previous studies with *E. carotovora* subsp. *carotovora* have shown that AHL deficiency causes the production of high levels of RsmA, an RNA binding protein that functions as a global negative regulator of extracellular enzymes and proteins and secondary metabolites (Rsm, regulator of secondary metabolites). We document here that ExpR, a putative AHL receptor belonging to the LuxR family of regulators, activates RsmA production. In the absence of AHL, an ExpR$^+$ *E. carotovora* subsp. *carotovora* strain compared to its ExpR$^-$ mutant, produces higher levels of *rsmA* RNA and better expresses an *rsmA-lacZ* transcriptional fusion. Moreover, the expression of the *rsmA-lacZ* fusion in *Escherichia coli* is much higher in the presence of *expR$_{71}$* (the *expR* gene of *E. carotovora* subsp. *carotovora* strain Ecc71) than in its absence. We also show that purified preparation of MBP-ExpR$_{71}$ binds (MBP, maltose binding protein) *rsmA* DNA. By contrast, MBP-ExpR$_{71}$ does not bind *ahlI* (gene for AHL synthase), *pel-1* (gene for pectate lyase), or *rsmB* (gene for regulatory RNA that binds RsmA), nor does ExpR$_{71}$ activate expression of these genes. These observations strongly suggest transcriptional activation of *rsmA* resulting from a direct and specific interaction between ExpR$_{71}$ and the *rsmA* promoter. Several lines of evidence establish that *N*-3-oxohexanoyl-L-homoserine lactone (3-oxo-C6-HL), the major AHL analog produced by *E. carotovora* subsp. *carotovora* strain Ecc71, inhibits ExpR$_{71}$-mediated activation of *rsmA* expression. These findings for the first time establish that the *expR* effect in *E. carotovora* subsp. *carotovora* is channeled via RsmA, a posttranscriptional regulator of *E. carotovora* subspecies, and AHL neutralizes this ExpR effect.**

Erwinia carotovora subspecies cause soft-rotting or tissue macerating disease in many plants and plant organs. Their capacity to cause rotting depends heavily upon their ability to produce secreted proteins and enzymes (2, 6). These bacteria utilize Type I, Type II, and Type III secretion systems to translocate proteins into the milieu. Proteases (Prt) are secreted by the Type I system, pectate lyase (Pel), polygalacturonase (Peh), and cellulase (Cel) are exported via the Type II system, and Harpin and other putative effectors are secreted by the Type III system (18, 48). Of the effectors and enzymes secreted by these bacteria, especially significant in the context of plant tissue maceration are the pectinases such as Pel, Peh, and pectin lyase (Pnl). These enzymes acting in concert with Prt and Cel cause degradation of plant cell wall components triggering cell separation and cell death. Harpins are required for the elicitation of the hypersensitive response and also for symptom production in *Arabidopsis thaliana* (38, 46). Aside from extracellular proteins, many of these bacteria produce antibiotics and other secondary metabolites (5).

Extracellular proteins are coregulated in *E. carotovora* subsp. *carotovora* and most likely in other *E. carotovora* subspecies by plant signals, quorum sensing signals (QSS), and an assortment of transcriptional factors and posttranscriptional regulators (4, 12, 13, 17, 21, 28, 45, 55). While many of the regulatory steps await identification and characterization, several generalizations are possible. As an example, posttranscriptional regulation by the RsmA-RsmB pair is of paramount importance in the expression of exoprotein genes and their secretion pathways. RsmA is a small RNA-binding protein that promotes decay of RNA (4, 9). *rsmB* specifies an untranslated regulatory RNA that binds RsmA and neutralizes its negative regulatory effect (28). Many of the transcriptional factors, known to regulate extracellular protein production, actually act via these posttranscriptional regulators (8, 10, 24, 29, 39, 40).

N-acyl homoserine lactones (AHLs) function as cell density (quorum) sensing signals (reviewed in references 14, 30, 52, 54, 59, and 61). Since its discovery in the marine bacterium *Vibrio fischeri*, where the signal is responsible for the expression of *lux* genes controlling light production, such signals have now been detected in many bacteria. These molecules control diverse phenotypes, including bacteria-microbe and bacteria-plant/animal interactions, conjugation, production of secreted proteins, extracellular polysaccharides, antibiotics, pigments, and other secondary metabolites (see 11, 43, 44, 54, 59, 61, 62, 63 and references cited therein). Extensive studies of the *lux* genes and other AHL-regulated genes have demonstrated that signaling systems minimally comprise biosynthetic precursors (acyl-ACP [acyl-carrier protein] and SAM [S-adenosyl-L-methionine]); LuxI or similar proteins that function as AHL syn-

* Corresponding author. Mailing address: Department of Plant Microbiology and Pathology, University of Missouri, Columbia, MO 65211. Phone: (573) 882-1892. Fax: (573) 882-0588. E-mail: chatterjeeas@missouri.edu.

thases; and LuxR and LuxR-like protein receptors of AHL that bind target DNAs and activate transcription (30, 54, 59). Many homologs of LuxR are now known that function as transcriptional activators upon AHL-binding (see, for example, 42, 57, and 58). While most QSS systems conform to this pattern, there are instances in which LuxR-like proteins are known to bind DNA in their native state (i.e., in the absence of AHL) and modulate transcription. One noteworthy example is the AHL-regulated production of capsular polysaccharide (CPS) in *Pantoea* (*Erwinia*) *stewartii* subsp. *stewartii* (35, 36). In this instance, EsaR, a LuxR homolog, governs the expression of the *cps* locus. EsaR directly represses the transcription of the *rcsA* gene which encodes an essential coactivator for RcsA/RcsB–mediated transcription of *cps* genes. The repressor activity of EsaR is compromised by AHL binding, thereby relieving repression of the *cps* genes.

In *E. carotovora*, AHL controls extracellular enzymes, antibiotic production, and pathogenicity (20, 21, 44, 45, 53). Two different transcriptional regulators that also function as AHL receptors are believed to operate in *E. carotovora* subsp. *carotovora*. One such regulator, CarR, a LuxR-like transcriptional factor, controls the production of the antibiotic carbapenem. Binding of AHL to CarR imparts specificity onto this transcriptional factor. It also is known that CarR occurs as dimers and that AHL-binding converts the dimers to higher order multimers. The CarR-AHL complex binds DNA and activates the transcription of the *carA-H* operon required for the biosynthesis of the antibiotic (20, 32, 60). A similar regulatory mechanism most likely controls antibiotic production in *E. carotovora* subsp. *betavasculorum* (7). It should be noted that neither the CarR nor the CarR-AHL complex control exoenzyme or AHL production (61).

The LuxR homologs, ExpR and RexR, are the other AHL receptors of *E. carotovora* subsp. *carotovora* (1, 15). The initial studies with ExpR of *E. carotovora* subsp. *carotovora* strain SCC3193 ($ExpR_{3193}$) did not establish a clear role of the AHL receptor in exoprotein production. For example, inactivation of *expR* had very little effect on extracellular pectate lyase production or AHL synthesis. However, multiple copies of *expR* had some inhibitory effect on extracellular enzyme production. von Bodman et al. (58) have documented that $ExpR_{3193}$ is a DNA-binding protein and that its DNA-binding property is inhibited by AHL. Thus, ExpR, like other members of the LuxR family of regulators, is believed to function as a transcriptional factor, although the identity of the target(s) of ExpR in *E. carotovora* subsp. *carotovora* has until now remained obscure. In this report we document that (i) ExpR activates expression of *rsmA*; (ii) ExpR specifically binds *rsmA* DNA; and (iii) AHL prevents ExpR-*rsmA* binding and ExpR-mediated activation of *rsmA* transcription. Our findings also explain how AHL deficiency causes RsmA overproduction responsible for the inhibition of exoprotein and secondary metabolite production in *E. carotovora* subsp. *carotovora*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids are described in Table 1. All the wild-type strains were maintained on Luria-Bertani (LB) agar. The strains carrying antibiotic markers were maintained on LB agar containing appropriate antibiotics.

The composition of LB, minimal salts, and minimal salts plus celery extract media have been described in previous publications (4, 41). When required, antibiotics were supplemented as follows: ampicillin (Ap), 100 μg/ml; kanamycin (Km), 50 μg/ml; spectinomycin (Sp), 50 μg/ml; and tetracycline (Tc), 10 μg/ml. Media were solidified using 1.5% (wt/vol) agar.

The media compositions for agarose plate enzymatic activity assays have been described by Chatterjee et al. (4).

Extracellular enzyme assays. The extracellular pectate lyase (Pel), polygalacturonase (Peh), protease (Prt), and cellulase (Cel) activities in the culture supernatants were tested according to procedures published previously (4). Bacterial cultures were grown at 28°C in minimal salts medium with sucrose or sucrose plus celery extract. Supernatants were collected by centrifugation (10,000 rpm, 10 min; Beckman J2-21 centrifuge) and used for assays. Halos around the wells on the assay plates indicate the enzymatic activities.

Nucleotide sequence determination of $expR_{71}$ and alignment of ExpRs. The nucleotide sequence of $expR_{71}$ was determined from pAKC851 using primers based on the nucleotide sequence of the *ahlI* gene of Ecc71, which overlapped with $expR_{71}$. Sequence alignment was performed using ClustalW at www.expasy.ch, and default parameters were used. Domain search was performed using rpsblast at www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi.

DNA techniques. Standard procedures were used in the isolation of plasmids and chromosomal DNA, gel electrophoresis, and DNA ligation (49). Restriction and modification enzymes were obtained from Promega Biotec (Madison, WI). Prime-a-Gene DNA labeling system (Promega Biotec) was used for labeling DNA probes. Southern blot analysis was carried out at high-stringency conditions (hybridization at 65°C in 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5× Denhardt's, 0.5% [wt/vol] sodium dodecyl sulfate [SDS], and 100 μg/ml denatured salmon sperm DNA; washing at 65°C with 2× SSC for 30 min, 1× SSC plus 0.1% [wt/vol] SDS for 30 min followed with 0.1× SSC plus 0.1% [wt/vol] SDS for 30 min) as well as at low-stringency conditions (hybridization and washing conditions are the same as high-stringency conditions except the temperature was at 55°C). For $expR_{3193}$ probe, a 500-bp BamHI-ClaI fragment from pAKC935 was used.

Construction of plasmids and mutant strains used. A fragment containing the entire open reading frame (ORF) of $expR_{3193}$ was PCR amplified from SCC3193 using the primers 5′-TGTAAGCTTAGGTTACTATGGATATACGTAT-3′ and 5′-TGTGGATCCAGGTTGCGAGAGTGGCCACTG-3′ and cloned in the BamHI-HindIII site of pCL1920 to yield pAKC935. The $expR_{71}$ fragment was amplified from Ecc71 using the primers designed based on Ecc71 *expR* nucleotide sequences 5′-TGCAAGCTTGCCCTTAGCATTGTCGTTCGATTG-3′ and 5′-TGCGGATCCCGCTATTGCACAGGCTTGATGAGC-3′. The PCR product was digested with BamHI-HindIII and cloned in a BamHI-HindIII site of pCL1920 to yield pAKC936. The plasmids pAKC1204, pAKC1205, and pAKC1206 were obtained from genomic libraries of *E. carotovora* subsp. *carotovora* strain SCC3193, *E. carotovora* subsp. *atroseptica* strain Eca12 and *E. carotovora* subsp. *carotovora* strain EC153 and by in situ colony hybridization using *rsmA* probe of Ecc71. AC5098 and AC5099 were constructed by marker exchange of AC5006 and AC5091 with pAKC1212, respectively. The procedures for marker exchange have been described in Chatterjee et al. (4). Inactivation of *expR* in mutants was confirmed by Northern blot analysis.

Bioluminescence assay for AHL estimation. Culture supernatants and high-performance liquid chromatography (HPLC) fractions were assayed for bioluminescence using an *E. coli* based bioassay system as described by Chatterjee et al. (4). *E. coli* strain VJS533 harboring pHV200I was used as a biosensor indicator. There is a linear relationship between the quantity of AHL present in the samples and the emission of bioluminescence. Relative light unit (RLU) is expressed as counts per minute per milliliter of culture.

Production and fractionation of AHLs. Ecc71 was grown in 2.5 liters of minimal salts medium supplemented with sucrose (0.5%, wt/vol) at 28°C to a Klett value of ca. 200. The culture supernatant was collected by centrifugation at 10,000 rpm for 10 min (Beckman J2-21 centrifuge) and extracted with an equal volume of ethyl acetate. The ethyl acetate extract was evaporated to dryness, and the residue was dissolved in 5 ml of distilled water and subjected to HPLC according to the method of Morin et al. (37) modified as follows. The residue in distilled water was loaded on a C-18 reverse-phase column (Jupiter 5U C-18 300A; 250 by 4.6 mm; Phenomenex). The column was eluted with a linear gradient from 0 to 50% methanol in water over 60 min at a flow rate of 1 ml/min. Detection was by UV light at 210 nm. The eluted fractions were assayed for bioluminescence activity according to Chatterjee et al. (4). Active fractions (71AHL) were pooled, concentrated, and rechromatographed under similar conditions for further purification.

Detection of AHLs by analytical thin-layer chromatography (TLC). A modification of the procedure described by Holden et al. (19) was used for the detection of AHLs by analytical TLC. Crude ethyl acetate extract or the HPLC

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
Erwinia carotovora subsp. *atroseptica*		
Eca12	Wild type	64
Erwinia carotovora subsp. *carotovora*		
Ecc71	Wild type	64
AC5006	Lac$^-$ derivative of Ecc71	41
AC5091	AhlI$^-$ derivative of AC5006	4
AC5094	AhlI$^-$ derivative of Ecc71	4
AC5098	ExpR$^-$ derivative of AC5006	This study
AC5099	AhlI$^-$ ExpR$^-$ derivative of AC5006	This study
SCC3193	Wild type	45
EC153	Wild type	Laboratory collection
Escherichia coli		
DH5α	*supE*44 Δ*lacU169* φ80*lacZ*Δ*M15 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Gibco BRL
MC4100	*araD139* Δ*(lacIPOZYA)U169 recA*$^+$ *thi* Str^r	27
VJS533	*ara* Δ(*lac-proAB*) *rpsL* φ*80lacZ*Δ*M15 recA56*	16
Plasmids		
pDK6	Km^r	23
pLARF5	Tc^r	22
pMAL-c2g	Ap^r, protein expression vector	New England Biolabs
pMP220	Tc^r, promoter-probe vector	51
pCL1920	Sp^r, Sm^r	26
pAKC781	Ap^r, *peh-1*$^+$ DNA in pBluescriptSK(+)	27
pAKC783	Ap^r, *pel-1*$^+$ DNA in pBluescriptSK(+)	27
pAKC851	Tc^r, *ahlI*$^+$ and *expR*$^+$ DNA in pLARF5	4
pAKC856	Ap^r, *ahlI*$^+$ DNA in pBluescriptSK(+)	4
pAKC875	Tc^r, *rsmA*$^+$ DNA in pLARF5	4
pAKC882	Ap^r, *rsmA* coding region in pT7-7	38
pAKC935	Sp^r, $expR_{3193}{}^+$ DNA of SCC3193 in pCL1920	This study
pAKC936	Sp^r, $expR_{71}{}^+$ DNA in pCL1920	This study
pAKC1002	Tc^r, *rsmB-lacZ* in pMP220	28
pAKC1034	Ap^r, 200-bp *celV* fragment in pGEM-T Easy	29
pAKC1100	Tc^r, *rsmA-lacZ* in pMP220	3
pAKC1201	Km^r, *ptac-ahlI* in pDK6	This study
pAKC1202	Tc^r, *ahlI-lacZ* in pMP220	This study
pAKC1203	Tc^r, *pel-lacZ* in pMP220	This study
pAKC1204	Tc^r, *rsmA*$^+$ DNA of SCC3193 in pLARF5	This study
pAKC1205	Tc^r, *rsmA*$^+$ DNA of Eca12 in pLARF5	This study
pAKC1206	Tc^r, *rsmA*$^+$ DNA of EC153 in pLARF5	This study
pAKC1212	Km^r, Tc^r, ExpR$^-$ derivative of pAKC851	This study
pAKC1220	Ap^r, $expR_{71}$ coding region in pMAL-c2g	This study
pHV200	Ap^r, 8.8-kb SalI fragment containing the *lux* operon	16
pHV2001	Ap^r, frameshift mutant of *luxI* in pHV200	E. P. Greenberg

fraction was applied in volumes of 1 to 5 μl to a C-18 reverse-phase TLC plate (150 μm adsorbent layer thickness; Sigma-Aldrich, St. Louis, MO), and the chromatogram was developed with methanol:water (60:40, vol/vol). *N*-3-oxohexanoyl-L-homoserine lactone (3-oxo-C6-HL) [*N*-(β-ketocaproyl)-DL-homoserine lactone; purchased from Sigma] and *N*-3-oxooctanoyl-L-homoserine lactone (3-oxo-C8-HL, kindly provided by Paul Williams, University of Nottingham, United Kingdom) were used as standards. The plate was dried and overlaid with the indicator bacterium *E. coli* VJS533 carrying pHV200I. The biosensor overlay was prepared as follows: the indicator bacterium was grown in LB medium containing ampicillin at 28°C to a Klett value of ca. 200 and was then mixed with an equal volume of warm LB medium containing 1.5% (wt/vol) agar. The overlaid plate was incubated for 3 h at 28°C and exposed to X-ray film to record bioluminescent spots.

Mass spectrometry. All mass spectrometry experiments were performed on a Thermo-Finnigan TSQ7000 triple-quadrupole mass spectrometer with the API2 source and Performance Pack (ThermoFinnigan, San Jose, CA) using electrospray ionization (ESI). The heated inlet capillary was maintained at 250°C; the voltage on the stainless steel electrospray needle was 4.5 kV. All other voltages were optimized to maximize ion transmission and minimize unwanted fragmentation and were determined during the regular tuning and calibration of the instrument. For mass spectrometry/mass spectrometry (MS/MS) experiments, argon was used as the collision gas, and collision energies ranged from 20 to 40eV.

For MS and MS/MS experiments, samples were infused at a rate of 10 μl/min using a syringe pump (Harvard Apparatus, Holliston, MA). Nitrogen sheath gas was provided to the ESI source at 80 lb/in^2. The spectra acquired for each sample are an average of 150 individual scans or spectra.

The mass spectrometer is connected to an integrated Thermo-Finnigan liquid chromatography (LC) system consisting of a P4000 quaternary LC pump and SCM1000 vacuum degasser, an AS3000 autosampler, and a UV6000LP diode-array detector. This system was used for all LCMS and LCMS/MS experiments.

Northern and Western blot analyses. Bacterial cultures were grown at 28°C in minimal salts medium supplemented with sucrose (0.5%, wt/vol), sucrose, and celery extract or LB medium with appropriate antibiotics as described in figure

legends. Cells were collected while cultures reached a Klett value of ca. 150 or 200. RNA isolation and Northern blot analysis were performed as described in Liu et al. (28). The probes used were the 183-bp NdeI-SalI fragment of *rsmA* from pAKC882, 314-bp *Eco*RV-KpnI fragment of *pel-1* from pAKC783, 743-bp HindIII fragment of *peh-1* from pAKC781, 386-bp DraI-EcoRI fragment of *ahlI* from pAKC856, and 200-bp EcoRI fragment of *celV* from pAKC1034. For Western blot analysis, bacterial cells were collected, suspended in 1× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (49), and boiled. To determine total bacterial protein concentrations, samples were precipitated with trichloroacetic acid. The protein concentrations were determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) according to the manufacturer's specifications. Western blot analysis of the total bacterial protein was performed as described in Mukherjee et al. (38). The antiserum raised against RsmA of Ecc71 (10) was used as probe.

Expression and purification of MBP-ExpR$_{71}$ protein. A fragment containing the entire coding region of *expR$_{71}$* was PCR amplified from pAKC851 by using primers 5′-TGTGGATCCATGTCGCCATTATTCTCCAGTAGC-3′ and 5′-TGTAAGCTTCTATTGCACAGGCTTGATGAGCTG-3′. The fragment was digested with BamHI and HindIII and cloned into pMAL-c2g vector (New England Biolabs, Beverly, MA) to yield pAKC1220.

E. coli strain DH5α carrying pAKC1220 was grown in LB medium supplemented with glucose (0.2%, wt/vol) and ampicillin at 37°C. When the culture reached an A_{600} value of 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to yield a final concentration of 1 mM. Three hours after IPTG addition, bacterial cells were collected by centrifugation. MBP-ExpR$_{71}$ fusion protein was purified by amylose resin (New England Biolabs) affinity chromatography according to the protocol provided by the company. Protein concentration was determined by using a CB-X Protein Assay kit (Geno Technology, Inc., St. Louis, MO). Crude extracts and purified MBP-ExpR$_{71}$ were analyzed on SDS-PAGE in a 10% (wt/vol) polyacrylamide gel.

Gel mobility shift assays. The DNA fragments were amplified from Ecc71 by PCR using primers listed as follows: *rsmA*, 5′-GCGGATCCGGCAAGCAGGATAGAA-3′ and 5′-GATTATAAAGAGTCGGGTCTCTCTGT-3′ (corresponding to −245 to −16 from the putative translational start site); *pel-1*, 5′-TGAGGATCCATGTTTCATCCGCAATACATTTAAC-3′ and 5′-TGATCTAGATATTTCATTATCACTGTCTCCTTG-3′ (corresponding to −131 to +8 from the putative translational start site); *ahlI*, 5′-TGCTCTAGATTCTGAGGGTAAATAGCTCTTCTG-3′ and 5′-TGCAGATCTGAGTAATATAAAAATCCGGAATCG-3′ (corresponding to −220 to +63 from the putative translational start site); and *rsmB* (nontranslatable RNA regulator), 5′-CGAAGCTTAAGTTAGTAACCGGTTACAGTG-3′ and 5′-TGTGAGAGATCTCTTACATTCTC-3′ (corresponding to −180 to +41 from the transcriptional start site). The DNA fragments were purified using Wizard SV Gel and PCR Clean-Up System (Promega Biotec, Madison, WI) and end labeled with [α-^{32}P]dATP and Klenow fragment. Protein-DNA interaction was assayed in 20 μl of binding buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, and 5% [wt/vol] glycerol) containing 1 μg of salmon sperm DNA, 2 μg of bovine serum albumin (BSA), and purified MBP-ExpR$_{71}$ protein with or without competitors. The reaction mixtures were incubated at room temperature for 20 min and subjected to electrophoresis in 5% (wt/vol) polyacrylamide (acryl:bis-acyl, 29:1) gels. The gels were dried and exposed to X-ray film.

β-Galactosidase assays. Bacterial constructs were grown at 28°C in minimal salts plus sucrose or LB media supplemented with appropriate antibiotics and purified AHL from Ecc71 (71AHL) as described in figure legends or footnotes to tables. The β-galactosidase assays were performed according to Miller (33).

Cell densities of bacterial cultures used in Northern blot and Western blot analyses as well as for exoenzyme and β-galactosidase assays are represented as Klett values. Klett values of 150, 200, and 250 are approximately equal to 1.6, 2.3, and 2.8 absorbance at A_{600}.

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

Nucleotide sequence accession number. The sequence of the *expR* gene of Ecc71 has been deposited in the GenBank databases under accession number L40174.

RESULTS

Occurrence of *expR* in *E. carotovora* subsp. *carotovora* strain Ecc71. To examine the occurrence of an *expR* homolog in *E. carotovora* subsp. *carotovora* strain Ecc71, Southern blot analysis of EcoRI-digested Ecc71 chromosomal DNA and a previously isolated AhlI$^+$ plasmid, pAKC851, using *expR$_{3193}$* DNA as the probe, was performed under high-stringency conditions as well as low-stringency conditions. Hybridization bands with weak signals were detected with a chromosomal DNA fragment of Ecc71 and pAKC851 under low-stringency conditions, but no signal was detected under high-stringency conditions (data not shown). To estimate the extent of sequence divergence, we sequenced Ecc71 *expR* (*expR$_{71}$*; accession no. AY894425) and compared sequences of *expR$_{71}$* with sequences of *expR* genes available in databases. Alignment of ExpR amino acid sequences (unpublished data) revealed that Ecc71 and *E. carotovora* subsp. *betavasculorum* strain Ecb168 (accession no. AF001050) share 93% identity, but both strains show ca. 60% identity with ExpR proteins of *E. carotovora* subsp. *carotovora* strains EC153 (accession no. AY894424), SCC3193 (accession no. X80475), and *E. carotovora* subsp. *atroseptica* strain CFBP6272 (accession no. AJ580600). Interestingly, significant differences in the ExpR sequences are present only in the N-terminal domain, the putative AHL binding region (residues 11 to 160). By contrast, all these ExpR homologs share high homology in the putative HTH domain located in the C terminal (residues 175 to 232).

Characterization of AHLs produced by Ecc71. We prepared ethyl acetate extracts of spent cultures of Ecc71 fractionated by HPLC on a C-18 reverse phase column. The fractions were assayed for bioluminescence as described in the Materials and Methods section. A single peak (designated as 71AHL) showing bioluminescence activity was observed in ethyl acetate extracts of Ecc71 with a retention time of about 20 min (Fig. 1A). TLC analysis of this active fraction as well as ethyl acetate extract showed one major spot which had a retardation factor identical to that of 3-oxo-C6-HL (Fig. 1B). To confirm the identity of this AHL, active fractions containing 71AHL were analyzed by LCMS and LCMS/MS (Fig. 1C), and the results were compared to standard 3-oxo-C6-HL. The retention time, parent ions observed, and product ion spectrum for 71AHL matched those for the standard.

Having established the chemical nature of the AHL fraction from Ecc71, we tested the purified preparation for biological activity, i.e., for the restoration of exoenzyme production in an AhlI$^-$ mutant of Ecc71. The data in Fig. 1D show that 71AHL restored Pel, Peh, Cel, and Prt production in an AhlI$^-$ strain of Ecc71, AC5094.

Previous studies (3, 24) in *E. carotovora* subsp. *carotovora* strains Ecc71 and SCC3193 documented that the expression of *rsmA* is higher in the AHL-deficient mutants than in the parents. To test if the effects of AHL deficiency on *rsmA* expression could be neutralized by adding the fractionated 71AHL, we compared the levels of *rsmA* transcript and RsmA protein in AC5094 (AhlI$^-$ mutant of Ecc71) in the absence or in the presence of 71AHL. The Northern blot analysis (Fig. 1E) and Western blot analysis (Fig. 1F) results reveal that the levels of *rsmA* transcript and RsmA produced by AC5094 were much lower in the presence of 71AHL than in the absence of 71AHL.

Purification of MBP-ExpR$_{71}$. To overexpress and purify ExpR$_{71}$ for DNA binding studies, we initially tried to express ExpR$_{71}$ as ExpR$_{71}$-6His fusion protein, but our attempts were hampered by protein insolubility. We therefore attempted to express ExpR$_{71}$ as MBP-ExpR$_{71}$ fusion protein. For this, the

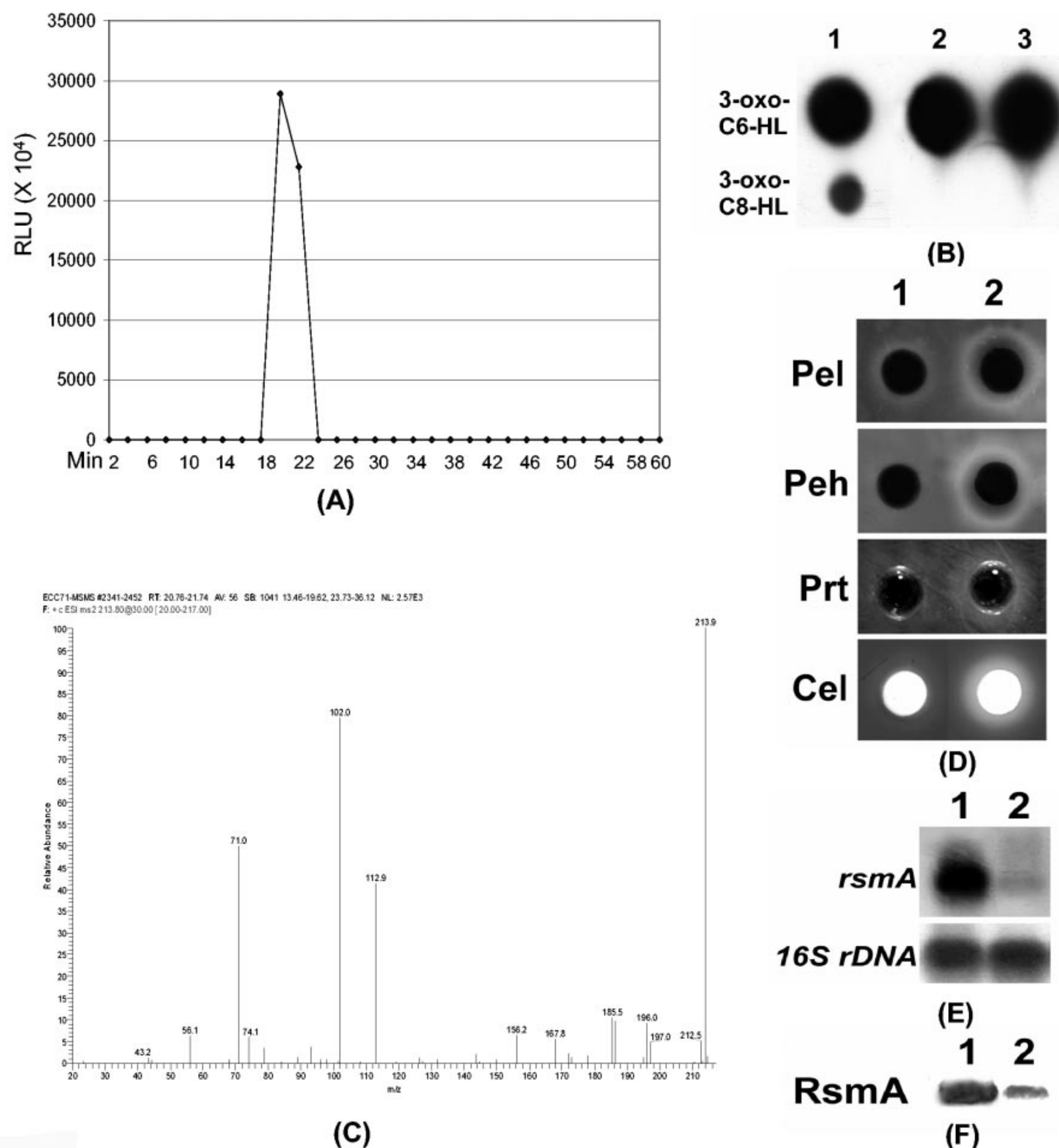

FIG. 1. (A) RLUs produced by HPLC fractions of crude AHL extract from Ecc71 in *E. coli* strain VJS533 harboring pHV200I. (B) TLC analysis of AHLs. Lane 1, mixture of synthetic 3-oxo-C6-HL and 3-oxo-C8-HL; lane 2, crude AHL extract of Ecc71; and lane 3, HPLC purified 71AHL (ca. 7 nmol). (C) LCMS/MS scan for HPLC-fractionated 71AHL. (D) Agarose plate assays for Pel, Peh, Prt, and Cel activities of AC5094 ($ExpR^+$ $AhlI^-$). Thirty microliters of culture supernatant was applied in each well. (E and F) Northern blot analysis and Western blot analysis of *rsmA* or RsmA of AC5094. Each lane contained 10 μg of total RNA for Northern blot analysis, and 10 μg of total protein for Western blot analysis. (D, E, and F) Lane 1, in the absence of AHL (i.e., same volume of water was added); lane 2, in the presence of 71AHL (300 μl of 1 mM 71AHL added to a 6-ml culture to yield a final concentration of 50 μM). Bacteria were inoculated in minimal salts plus sucrose (0.5%, wt/vol) medium supplemented with or without 71AHL. Total RNAs and proteins were extracted after 5 h incubation at 28°C, and after 8 h incubation culture supernatants were collected by centrifugation (10,000 rpm, 10 min) for exoenzyme assays.

coding region of $expR_{71}$ was PCR amplified and cloned into the pMAL-c2g vector to yield pAKC1220. After IPTG induction, a protein of ca. 78 kDa was overproduced by *E. coli* strain DH5α carrying pAKC1220 (Fig. 2, lane 4). The apparent molecular mass of ca. 78 kDa matched well with the mass of 27.95 kDa of the polypeptide deduced from the $expR_{71}$ sequence plus the mass of 50.84 kDa of MBP2-β-galactosidase α fragment made from the pMAL-c2g vector (Fig. 2, lane 2), indicating that the overproduced protein is MBP-$ExpR_{71}$ fusion protein. The MBP-$ExpR_{71}$ protein was purified by amylose resin affinity chromatography, which yielded a preparation of above 90% purity judged by the SDS-PAGE analysis (Fig. 2, lane 5).

Gel mobility shift assays with MBP-$ExpR_{71}$. Gel mobility shift assays were carried out to determine interaction of purified MBP-$ExpR_{71}$ protein with DNA segments containing promoter regions of *ahlI*, *pel-1*, *rsmA*, and *rsmB*. α-^{32}P-labeled

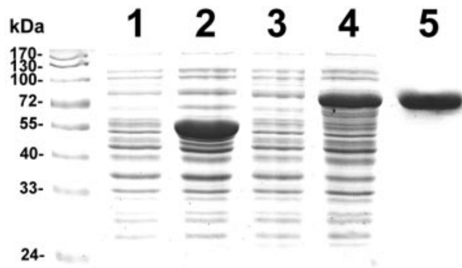

FIG. 2. Overexpression and purification of MBP-ExpR$_{71}$ protein. Lanes 1 and 2, lysates of *E. coli* DH5α carrying the vector (pMAL-c2g) without or with 1 mM IPTG; lanes 3 and 4, lysates of DH5α carrying pAKC1220 (*expR$_{71}$* in pMAL-c2g) without or with 1 mM IPTG; and lane 5, purified MBP-ExpR$_{71}$. Lanes 1 to 4 contained 5 μg of total bacterial proteins, and lane 5 contained 2 μg of purified MBP-ExpR$_{71}$. Samples were analyzed by SDS-PAGE in a 10% (wt/vol) polyacrylamide gel.

DNA fragments of *ahlI*, *pel-1*, *rsmA*, and *rsmB* and MBP-ExpR$_{71}$ protein were incubated in the DNA binding buffer and resolved on a nondenaturing 5% polyacrylamide gel. Results in Fig. 3A reveal that MBP-ExpR$_{71}$ binds the *rsmA* DNA segment. The extent of MBP-ExpR$_{71}$-*rsmA* band shift is proportional to the concentration of protein added, and the excess of cold *rsmA* DNA abolishes the retarded band, indicating that the binding is specific. Amylose resin affinity chromatography purified MBP2-β-galactosidase α fragment protein from IPTG induced cells of DH5α carrying pMAL-c2g vector did not bind the *rsmA* probe, indicating that the binding is between ExpR$_{71}$ protein and *rsmA* DNA (Fig. 3A). MBP-ExpR$_{71}$ does not bind the DNA fragments of *ahlI*, *pel-1*, and *rsmB* in the presence or in the absence of 3-oxo-C6-HL (Fig. 3B).

Effects of 3-oxo-C6-HL on MBP-ExpR$_{71}$-*rsmA* binding. We tested the effects of 71AHL (3-oxo-C6-HL) on the binding of *rsmA* and ExpR$_{71}$-MBP by adding the fractionated 71AHL or commercially available 3-oxo-C6-HL to the binding reactions. Figure 3A shows that 71AHL or synthetic 3-oxo-C6-HL prevented the MBP-ExpR$_{71}$-*rsmA* binding.

ExpR activates transcription of *rsmA*. Previous studies (3, 24) in Ecc71 and SCC3193 have demonstrated that under conditions of AHL deficiency, *rsmA* transcript levels are high, but the basis for this effect was not known. Gel mobility shift assay results demonstrated that MBP-ExpR$_{71}$ binds the promoter region of *rsmA* and that the binding is prevented by 71AHL (3-oxo-C6-HL). We therefore considered the possibility that ExpR in the absence of AHL could activate *rsmA* expression. We present below several lines of evidence that support this hypothesis.

(i) Effects of *expR$_{71}$* on expression of *rsmA-lacZ* fusion in *E. coli*. Results of *rsmA*-ExpR$_{71}$ binding assays strongly suggested

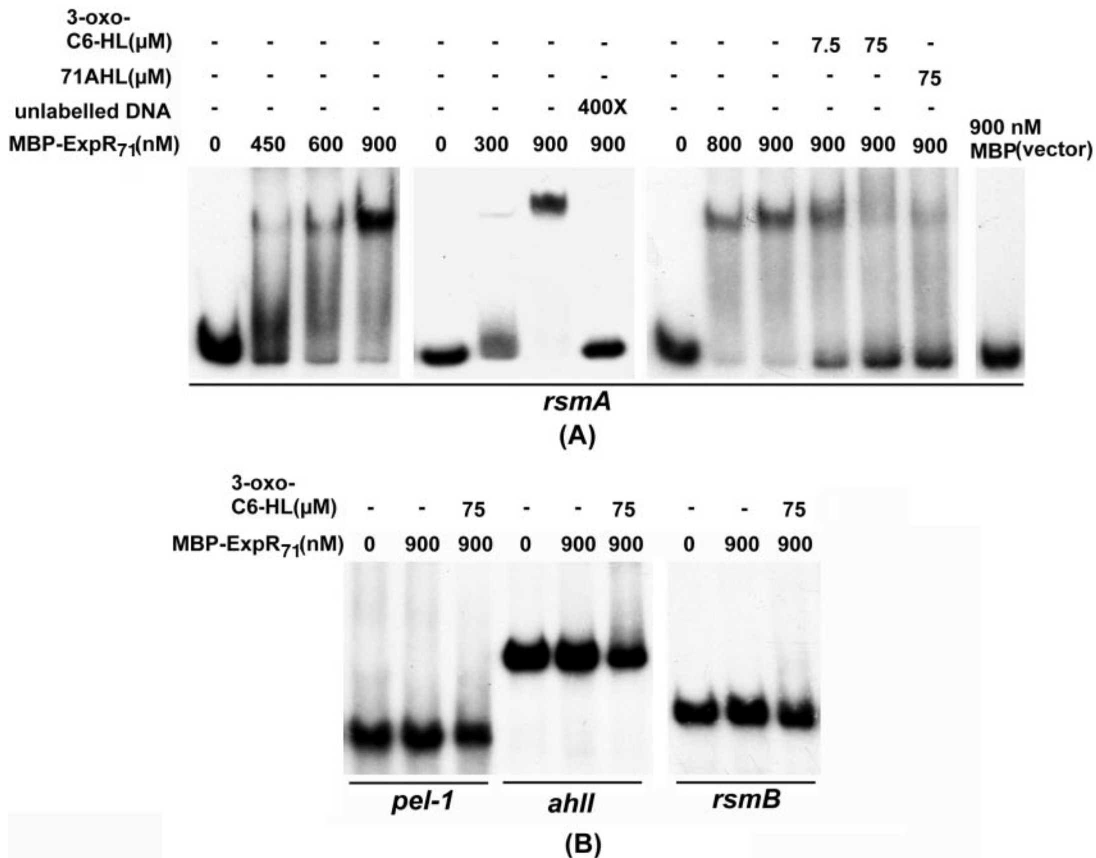

FIG. 3. Gel mobility shift assays for binding of purified MBP-ExpR$_{71}$ protein to (A) *rsmA* and (B) *pel-1*, *ahlI*, and *rsmB* DNAs. DNA fragments were end-labeled with [α-^{32}P]dATP. Two nanograms of probe DNAs was added in each reaction. The amounts of proteins, unlabeled DNA, and 71AHL or synthetic 3-oxo-C6-HL used in each reaction are indicated on the top of each figure.

TABLE 2. Expression of *rsmA-lacZ*, *pel-lacZ*, *ahlI-lacZ*, and *rsmB-lacZ* fusions in *E. coli* in the presence of $expR_{71}$

Bacterial construct[a]	Relevant characteristic[b]	β-Galactosidase activity[c]
MC4100(pCL1920+pMP220)	vectors	49 ± 3
MC4100(pAKC936+pMP220)	$expR_{71}$ + vector	52 ± 3
MC4100(pCL1920+pAKC1100)	vector + *rsmA-lacZ*	620 ± 13
MC4100(pAKC936+pAKC1100)	$expR_{71}$ + *rsmA-lacZ*	1,905 ± 17
MC4100(pCL1920+pAKC1202)	vector + *ahlI-lacZ*	262 ± 18
MC4100(pAKC936+pAKC1202)	$expR_{71}$ + *ahlI-lacZ*	269 ± 14
MC4100(pCL1920+pAKC1203)	vector + *pel-lacZ*	173 ± 7
MC4100(pAKC936+pAKC1203)	$expR_{71}$ + *pel-lacZ*	187 ± 6
MC4100(pCL1920+pAKC1002)	vector + *rsmB-lacZ*	943 ± 27
MC4100(pAKC936+pAKC1002)	$expR_{71}$ + *rsmB-lacZ*	961 ± 34

[a] Bacteria were grown at 28°C in LB supplemented with spectinomycin and tetracycline to a Klett value of ca 250, and the cultures were used for assay.
[b] The relevant characteristics of the genes carried by bacteria are given.
[c] Expressed as Miller units.

a direct effect of ExpR on *rsmA* transcription. To further examine this possibility, we used *E. coli* strains as surrogate hosts. We argued that the activation of *rsmA* expression in the presence of $expR_{71}$ but no activation in its absence would provide strong evidence that ExpR acts in the absence of any other *Erwinia*-specific factor. We transferred the *rsmA-lacZ* transcriptional fusion plasmid, pAKC1100, into *E. coli* strain MC4100 carrying vector (pCL1920) or $expR_{71}$ (pAKC936). β-Galactosidase assay data (Table 2) revealed that the expression of *rsmA-lacZ* in MC4100 was higher in the presence of $expR_{71}$ (ca. threefold) than in MC4100 carrying *rsmA-lacZ* fusion (pAKC1100) and vector, i.e., in the absence of $expR_{71}$. β-Galactosidase assay results of MC4100 carrying *ahlI-lacZ*, *pel-lacZ*, and *rsmB-lacZ* fusions with vector or $expR_{71}$ revealed that $expR_{71}$ had no effect on the expression of these fusions (Table 2).

Those data revealed that the transcription of Ecc71 *rsmA* was activated by the $expR_{71}$ plasmid in *E. coli*. To determine the effects of $expR_{71}$ DNA on the expression of *rsmA* genes from other *Erwinia carotovora* strains, we transferred *rsmA* plasmids of *E. carotovora* subsp. *carotovora* strains Ecc71, SCC3193, and EC153 as well as *E. carotovora* subsp. *atroseptica* strain Eca12 into *E. coli* strain DH5α carrying vector or $expR_{71}$ plasmid. The results of Northern blot analysis (Fig. 4A) and Western blot analysis (Fig. 4B) demonstrate that the production of *rsmA* RNAs and RsmA proteins was activated in the presence of the $expR_{71}$ gene in *E. coli* DH5α.

(ii) AHL neutralizes $expR_{71}$ effects on expression of *rsmA-lacZ* in *E. coli*. The data presented above demonstrated that expression of the *rsmA-lacZ* fusion is stimulated in the presence of $expR_{71}$ in *E. coli*, and the MBP-ExpR$_{71}$-*rsmA* binding is prevented by adding 71AHL (3-oxo-C6-HL). Therefore, it was of interest to test the effects of 71AHL on ExpR-mediated activation of *rsmA* expression in *E. coli* strain MC4100. The results of the β-galactosidase assay in Table 3 revealed that in the presence of 71AHL (3-oxo-C6-HL), expression of *rsmA-lacZ* in MC4100 carrying pAKC1100 and pAKC936 ($expR_{71}^{+}$ plasmid) was reduced to the basal level (i.e., the level in MC4100 carrying pAKC1100 and pCL1920). In addition, we tested expression of the *rsmA-lacZ* fusion in MC4100 carrying pAKC936 in the presence or absence of $ahlI^{+}$ plasmid pAKC1201. MC4100 carrying pAKC1201 produced AHL, and the levels of this metabolite were comparable to that in Ecc71 (data not shown). The results of β-galactosidase activity (Table 3) indicated that *rsmA* expression was higher in MC4100 carrying pAKC936 in the absence of pAKC1201 than in the presence of pAKC1201. These results demonstrate that in *E. coli*, $expR_{71}$ stimulates the expression of *rsmA-lacZ* in the absence of AHL and the effects are neutralized by AHL.

(iii) Effects of $expR_{71}$ on *rsmA* expression in *E. carotovora* subsp. *carotovora*. We compared the expression of *rsmA* in AC5006, a $LacZ^{-}$ derivative of Ecc71 and its mutants, $AhlI^{-}$ $ExpR^{+}$ (AC5091), $AhlI^{+}$ $ExpR^{-}$ (AC5098), and $AhlI^{-}$ $ExpR^{-}$ (AC5099). The data in Fig. 5A and B reveal that *rsmA* RNA and RsmA protein were overproduced by the $AhlI^{-}$ $ExpR^{+}$ mutant (lane 3) compared to the $AhlI^{+}$ $ExpR^{+}$ parent (lane 1). The levels of *rsmA* transcripts and RsmA protein were lower in the $AhlI^{-}$ $ExpR^{-}$ mutant than that in the $AhlI^{-}$ $ExpR^{+}$ strain (lanes 3 and 4). Furthermore, the β-galactosidase assay data (Fig. 5C) revealed that (i) expression of an *rsmA-lacZ* fusion also was higher in the $AhlI^{-}$ $ExpR^{+}$ mutant than in the parent,

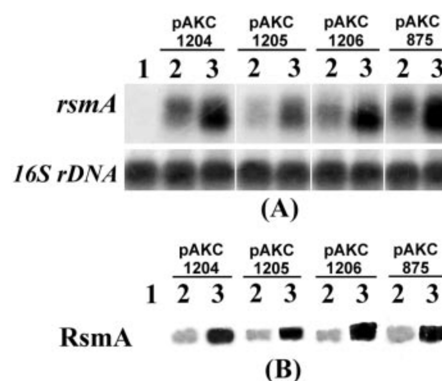

FIG. 4. (A) Northern blot analysis and (B) Western blot analysis of *E. coli* strain DH5α carrying pCL1920 or pAKC936 ($expR_{71}$) with *rsmA* plasmids from SCC3193 (pAKC1204), Eca12 (pAKC1205), EC153 (pAKC1206), and Ecc71 (pAKC875). Total RNAs and proteins were extracted from bacteria grown at 28°C in LB medium supplemented with spectinomycin and tetracycline to a Klett value of ca. 150. For Northern blot analysis, each lane contained 10 μg of total RNA, and for Western blot analysis, each lane contained 5 μg of total bacterial protein. Equal loading of RNA was checked by hybridization of the blot with a probe corresponding to 16S rRNA (rDNA). Lane 1, DH5α carrying pLARF5 plus pCL1920 (cloning vectors); 2, DH5α carrying pCL1920 and $rsmA^{+}$ plasmids; and 3, DH5α carrying $expR_{71}^{+}$ (pAKC936) and $rsmA^{+}$ plasmids.

TABLE 3. Effects of $expR_{71}$ plasmid on the expression of *rsmA-lacZ* fusion in *E. coli* in the presence of 3-oxo-C6-HL or *ahlI* plasmid

Bacterial construct	Relevant characteristic[c]	AHL	β-galactosidase activity[d]
MC4100(pCL1920+pAKC1100)[a]	vector + *rsmA-lacZ*		548 ± 18
MC4100(pCL1920+pAKC1100)[a]	vector + *rsmA-lacZ*	+71AHL	535 ± 19
MC4100(pAKC936+pAKC1100)[a]	$expR_{71}$ + *rsmA-lacZ*		1,801 ± 27
MC4100(pAKC936+pAKC1100)[a]	$expR_{71}$ + *rsmA-lacZ*	+71AHL	584 ± 20
MC4100(pCL1920+pAKC1100+pDK6)[b]	vector + *rsmA-lacZ* + vector		624 ± 14
MC4100(pCL1920+pAKC1100+pAKC1201)[b]	vector + *rsmA-lacZ* + *ahlI*		601 ± 27
MC4100(pAKC936+pAKC1100+pDK6)[b]	$expR_{71}$ + *rsmA-lacZ* + vector		1,942 ± 28
MC4100(pAKC936+pAKC1100+pAKC1201)[b]	$expR_{71}$ + *rsmA-lacZ* + *ahlI*		669 ± 19

[a] Bacteria were grown at 28°C in LB supplemented with spectinomycin and tetracycline to a Klett value of ca 100 and divided into two flasks. 71AHL (to a final concentration of 50 μM) was added to one, and the other was used as control (added water). After an additional 3 h of incubation at 28°C, cultures were used for assay.
[b] Bacteria were grown at 28°C in LB supplemented with spectinomycin, tetracycline, and kanamycin to a Klett value of ca 250. Cultures were used for assay.
[c] The relevant characteristics of the genes carried by bacteria are given.
[d] Expressed as Miller units.

and (ii) expression of the *rsmA-lacZ* in the $AhlI^-$ $ExpR^-$ strain was lower than that in the $AhlI^-$ $ExpR^+$ strain.

Effects of $ExpR_{71}$ deficiency on exoenzyme production in the absence of AHL. Based upon the observations (i) that AHL is required for exoenzyme production in Ecc71; (ii) that RsmA negatively affects exoenzyme production; and (iii) that $ExpR_{71}$ activates expression of *rsmA* in the absence of AHL, it was predicted that $AhlI^-$ $ExpR_{71}{}^-$ strain would produce higher levels of exoenzymes than the $AhlI^-$ $ExpR^+$ strain. To test this prediction, we grew *E. carotovora* subsp. *carotovora* strains in minimal salts medium supplemented with sucrose and celery extract and assayed for exoenzyme production and transcripts levels of several exoenzyme genes. Figure 6 shows that the levels of Pel, Peh, Prt, and Cel as well as *pel-1*, *peh-1*, and *celV* transcripts in $AhlI^-$ $ExpR^-$ strains (column 2) were higher than that in $AhlI^-$ $ExpR^+$ strains (column 1).

DISCUSSION

In this report we present two significant findings: (i) ExpR activates transcription of *rsmA*, which specifies an RNA-binding protein known to inhibit exoprotein and secondary metabolite production; and (ii) AHL inhibits this ExpR function, including ExpR binding its target DNA. Findings with various LuxR homologs have established that in a vast majority of cases protein-AHL complexes activate transcription of target genes or operons. In the absence of AHL, the N-terminal domains of LuxR family proteins inhibit DNA binding by the C-terminal DNA binding domains (34). However, this inhibition is relieved upon binding of AHLs to LuxR homologs. Thus, activator functions of many LuxR proteins depend upon their interaction with the cognate signals. There are several instances, including ExpR (this report), where this model of LuxR action does not apply. A well-studied example is that of EsaR. Studies by von Bodman and her associates (35, 36, 58) have demonstrated that EsaR, a LuxR homolog produced by *Pantoea stewartii*, acts as a repressor by binding the targeted promoter (*lux* box) in an AHL-independent manner. The EsaR action is neutralized by its interaction with AHL. Another example comprises SmaR, a LuxR homolog of *Serratia* sp. strain ATCC39006. In this case genetic evidence suggests that SmaR is a repressor of the *carA-H* operon (50). Furthermore, QscR in *Pseudomonas aeruginosa* functions as a repressor of various virulence genes (25). ExpR of *E. carotovora* subspecies

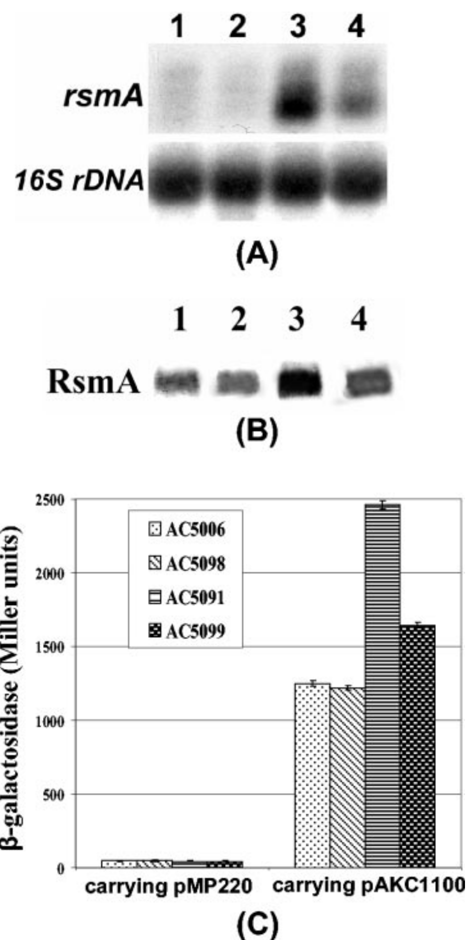

FIG. 5. (A) Northern blot analysis of *rsmA* and (B) Western blot analysis of RsmA of *E. carotovora* subsp. *carotovora* strains. Lane 1, AC5006 ($AhlI^+$ $ExpR^+$); 2, AC5098 ($AhlI^+$ $ExpR^-$); 3, AC5091 ($AhlI^-$ $ExpR^+$); and 4, AC5099 ($AhlI^-$ $ExpR^-$). Total RNAs and proteins were extracted from bacteria grown at 28°C in minimal salts plus sucrose (0.5%, wt/vol) and celery extract medium to a Klett value of ca. 200. Each lane contained 10 μg of total RNA for Northern blot analysis and 10 μg of total protein for Western blot analysis. Equal loading of RNA was checked by hybridization of the blot with a probe corresponding to 16S rRNA (rDNA). (C) β-Galactosidase assays of AC5006, AC5098, AC5091, and AC5099 carrying pMP220 (vector) or pAKC1100 (*rsmA-lacZ* fusion). Bacterial constructs were grown at 28°C in minimal salts medium plus sucrose and tetracycline to a Klett value of ca. 200 and harvested for the assays. Bars represent standard errors.

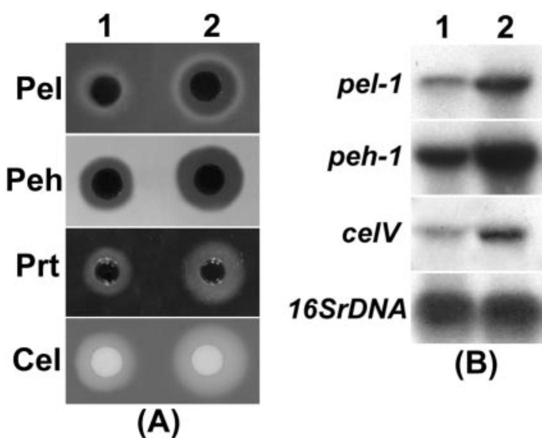

FIG. 6. (A) Agarose plate assays for Pel, Peh, Prt, and Cel activities of AC5091 (AhlI$^-$ ExpR$^+$, lane 1) and AC5099 (AhlI$^-$ ExpR$^-$, lane 2). Fifty microliters of culture supernatant was applied in each well. (B) Northern blot analysis of *pel-1*, *peh-1*, and *celV* of AC5091 (1) and AC5099 (2). Each lane contained 10 μg of total RNA. Equal loading of RNA was checked by hybridization of the blot with a probe corresponding to 16S rRNA (rDNA). Bacteria were grown in minimal salts plus sucrose (0.5%, wt/vol) and celery extract medium at 28°C to a Klett value of ca. 200 for total RNAs extraction. Culture supernatants were used for exoenzyme assays.

now adds to the growing list of QSS systems where the AHL receptors function as transcriptional regulators in the absence of the signal.

The effect of ExpR on *rsmA* is established by the levels of *rsmA* transcripts, expression of a *rsmA-lacZ* fusion, the levels of RsmA protein, and DNA binding studies. Comparative analysis of AhlI$^-$ ExpR$^+$ and AhlI$^-$ ExpR$^-$ strains of Ecc71 demonstrates an activator function of ExpR$_{71}$ (Fig. 5). It should be noted that the levels of *rsmA* RNA and RsmA protein as well as the expression of the *rsmA-lacZ* fusion were very similar in the AhlI$^+$ ExpR$^-$ and AhlI$^+$ ExpR$^+$ parent strain (Fig. 5). We attribute these responses to neutralization of ExpR action by AHL in the parent strain to the extent that it behaves phenotypically as an ExpR-deficient strain. Likewise, studies with *E. coli* strains, apparently lacking *ahlI* genes as well as an AHL producing system, strongly suggest activation of *rsmA* expression by ExpR. We have documented a higher level of expression of an *rsmA-lacZ* fusion in *E. coli* MC4100 in the presence of *expR$_{71}$*$^+$ DNA than in its absence. Moreover, in *E. coli* DH5α, ExpR$_{71}$ activates expression of *rsmA* genes from several other *E. carotovora* subsp. *carotovora* strains, including EC153, SCC3193, and *E. carotovora* subsp. *atroseptica* strain Eca12 (Fig. 4). Gel mobility shift assays demonstrate that ExpR specifically binds *rsmA* DNA (Fig. 3). This observation, taken along with transcript assay data and expression of an *rsmA-lacZ* fusion, establishes that *rsmA* overexpression in the absence of AHL is due to activation of *rsmA* promoter by ExpR.

The DNA-binding properties of the LuxR family of proteins are known to be modified by interaction with small molecules such as AHL (56). The results of gel mobility shift data (Fig. 3) clearly demonstrate inhibition of ExpR binding to *rsmA* DNA in the presence of 3-oxo-C6-HL produced by *E. carotovora* subsp. *carotovora* strain Ecc71. A previous study of von Bodman et al. (58) has revealed that EsaR, a LuxR homolog of *Pantoea stewartii* subsp. *stewartii*, binds *lux* box sequences. This binding is neutralized by 3-oxo-C6-HL as indicated by in vivo β-galactosidase assays using an artificial *35LB10-lacZ* promoter fusion containing *lux* box sequences. Interestingly, the addition of the AHL did not promote EsaR-DNA complex dissociation in their DNA mobility shift assay. However, they also reported that AHL interacts specifically with EsaR protein and induces structural changes that may neutralize its DNA binding affinity (36). In *Serratia* sp. strain ATCC 39006, BHL [*N*-(butanoyl)-L-homoserine lactone] inhibits DNA binding ability of SmaR, thereby relieving SmaR-mediated repression of the *carA-H* operon (50). We also document here a marked reduction in the expression of an *rsmA-lacZ* fusion in *E. coli* MC4100 in the presence of the *expR$_{71}$* gene and AHL. We attribute this reduction to the loss of activator function of ExpR due to the formation of ExpR-AHL complex. Another line of evidence for a negative effect of AHL comes from studies done with the Ahl$^+$ and Ahl$^-$ strains of Ecc71. *rsmA* RNA and RsmA protein levels were higher in AhlI$^-$ strains than in AhlI$^+$ bacteria (Fig. 5A and B). The expression of an *rsmA-lacZ* fusion was higher in an AhlI$^-$ strain than in the AhlI$^+$ parent (Fig. 5C). Moreover, an AhlI$^-$ ExpR$^-$ double mutant produced reduced levels of *rsmA* RNA and RsmA protein compared to the levels in an AhlI$^-$ ExpR$^+$ strain (Fig. 5A and B). These data collectively point to a conditional effect of ExpR in that it manifests under conditions of AHL deficiency or AHL limitation. We should recall that AHL deficiency has been reported to result in high levels of *rsmA* transcripts in several *E. carotovora* subsp. *carotovora* strains (3, 24). Our data on the activation of *rsmA* transcription in the presence of ExpR$_{71}$ but in the absence of AHL now explain the basis for this finding.

Studies with *E. chrysanthemi* ExpR (ExpR$_{Ech}$) have demonstrated that it binds to the promoter regions of the five major *pel* genes and *expI*. The ExpR$_{Ech}$-DNA band shift profiles changed in the presence of AHL (42, 47). There also is some evidence suggesting activation of the promoters of some of these genes by ExpR$_{Ech}$. Our data with *E. carotovora* subsp. *carotovora* present a very different picture regarding the action of ExpR$_{Ecc}$. First, it activates expression of *rsmA* but has no direct effect on *pel-1* or *ahlI*. Second, ExpR$_{Ecc}$ specifically binds *rsmA* DNA but not the DNA fragments of *pel-1* or *ahlI*. Third, ExpR$_{Ecc}$ activates *rsmA* transcription and binds *rsmA* DNA in the absence of AHL. In fact, AHL quite effectively inhibits these ExpR$_{Ecc}$ actions. Based upon these observations we conclude that the modus operandi of the quorum-sensing systems are fundamentally different in two groups of soft-rotting bacteria, namely *E. carotovora* subspecies and *E. chrysanthemi*. We have initiated studies of cross-species effects of ExpR proteins and their chimeric derivatives to better understand the molecular bases for the observed differences.

After taking into consideration the data presented here and the literature on ExpR and bacteria deficient in AHL or ExpR, we conclude that the primary quorum-sensing signaling system in *E. carotovora* subsp. *carotovora* comprises three major components: AHL, ExpR, and RsmA. As depicted in Fig. 7, ExpR and ExpR-AHL complex modulate the levels of RsmA which, in turn, is responsible for the expression of an array of genes known to be expressed in a growth phase or cell density de-

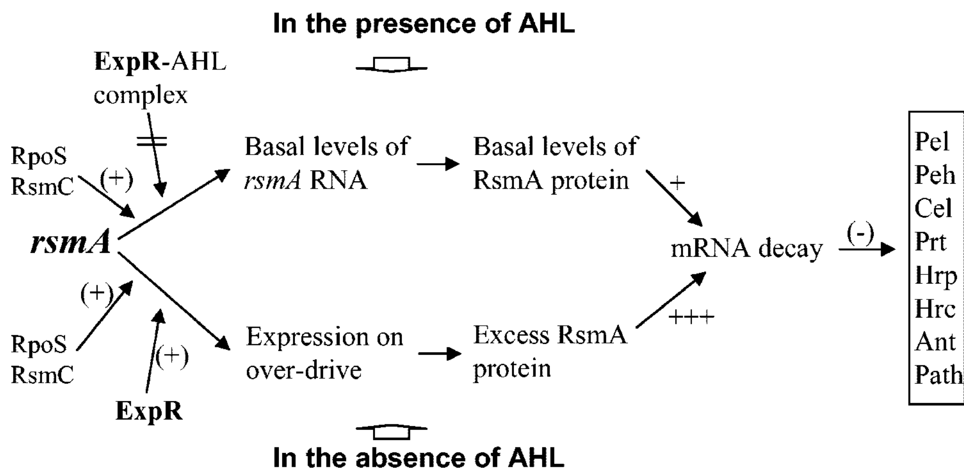

FIG. 7. A speculative model depicting the regulatory events in the expression of *rsmA* in *E. carotovora* subsp. *carotovora*. The basal level of *rsmA* expression (upper pathway) is controlled by several factors, including RpoS and RsmC. Increased pool of free ExpR but not ExpR-AHL complex (lower pathway) causes overproduction of RsmA, which in turn suppresses exoprotein and antibiotic production and pathogenicity by controlling mRNA stability.

pendent manner. In several *E. carotovora* subspecies, as in *P. stewartii* (36), AHL production is constitutive and not autoregulated (unpublished data). At low cell density, AHL concentration in the niche remains low (4, 31, 39), which cannot support high levels of target (exoprotein) gene expression. As the cell population increases, AHL concentration also rises, promoting activation of exoprotein gene expression. In the absence of AHL, free ExpR, the activator of *rsmA* transcription, is the dominant species. Under these conditions *rsmA* transcription is in overdrive (lower pathway, Fig. 7), causing a marked increase in the RsmA pool, ensuring complete or near-complete inhibition of exoprotein and secondary metabolite production. On the other hand, in the presence of high levels of AHL (upper pathway, Fig. 7), most, if not all, ExpR exists as ExpR-AHL complex, lacking this activator function. Thus, the main physiological function of ExpR appears to activate *rsmA* expression in the absence of AHL and AHL modulates this activation. This model explains the basis for the pleiotropic effects of AHL deficiency and the requirement of AHL for the expression of genes for exoproteins and secondary metabolites.

We now also understand the basis for the apparent paradox presented by the ExpR-deficient bacteria in that this deficiency did not yield a recognizable change in bacterial phenotype. There is a substantial basal level of RsmA in AHL^+ and ExpR-deficient bacteria, and this basal level of RsmA could be responsible for the lack of a recognizable phenotype in ExpR-deficient *E. carotovora* subsp. *carotovora* mutants. In this context we should recognize that RsmA pool size is determined by several other regulators, including RsmC (10), RpoS (40), and *rsmB* RNA (28), the latter controlled by regulators such as GacA (8) and KdgR (29).

ACKNOWLEDGMENTS

Our work was supported by the National Science Foundation (grants MCB-9728505) and the Food for the 21st Century program of the University of Missouri.

We thank Judy Wall for critically reviewing the manuscript, P. Williams for providing 3-oxo-C8-HL, T. Palva for providing strain SCC3193, and S. B. von Bodman for helping us with ExpR overexpression and purification.

REFERENCES

1. **Andersson, R. A., A. R. B. Eriksson, R. Heikinheimo, A. Mäe, M. Pirhonen, V. Kõiv, H. Hyytiäinen, A. Tuikkala, and E. T. Palva.** 2000. Quorum sensing in the plant pathogen *Erwinia carotovora* subsp. *carotovora*: the role of *expR* (Ecc). Mol. Plant-Microbe Interact. **13:**384–393.
2. **Barras, F., F. Van Gijsegem, and A. K. Chatterjee.** 1994. Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. Annu. Rev. Phytopathol. **32:**201–234.
3. **Chatterjee, A., Y. Cui, and A. K. Chatterjee.** 2002. 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. J. Bacteriol. **184:**4089–4095.
4. **Chatterjee, A., Y. Cui, Y. Liu, C. K. Dumenyo, and A. K. Chatterjee.** 1995. 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. Appl. Environ. Microbiol. **61:**1959–1967.
5. **Chatterjee, A. K., C. K. Dumenyo, Y. Liu, and A. Chatterjee.** 2000. *Erwinia*: genetics of pathogenicity factors, p. 236–260. *In* J. Lederberg (ed.), Encyclopedia of microbiology, 2nd ed., vol. 2. Academic Press, New York, N.Y.
6. **Collmer, A., and N. T. Keen.** 1986. The role of pectic enzymes in plant pathogenesis. Annu. Rev. Phytopathol. **24:**383–409.
7. **Costa, J. M., and J. E. Loper.** 1997. EcbI and EcbR: homologs of LuxI and LuxR affecting antibiotic and exoenzyme production by *Erwinia carotovora* subsp. *betavasculorum*. Can. J. Microbiol. **43:**1164–1171.
8. **Cui, Y., A. Chatterjee, and A. K. Chatterjee.** 2001. Effects of the two-component system comprising GacA and GacS of *Erwinia carotovora* subsp. *carotovora* on the production of global regulatory *rsmB* RNA, extracellular enzymes, and $harpin_{Ecc}$. Mol. Plant-Microbe Interact. **14:**516–526.
9. **Cui, Y., A. Chatterjee, Y. Liu, C. K. Dumenyo, and A. K. Chatterjee.** 1995. Identification of a global repressor gene, rsmA, of *Erwinia carotovora* subsp. *carotovora* that controls extracellular enzymes, *N*-(3-oxohexanoyl)-L-homoserine lactone, and pathogenicity in soft-rotting *Erwinia* spp. J. Bacteriol. **177:**5108–5115.
10. **Cui, Y., A. Mukherjee, C. K. Dumenyo, Y. Liu, and A. K. Chatterjee.** 1999. *rsmC* of the soft-rotting bacterium *Erwinia carotovora* subsp. *carotovora* negatively controls extracellular enzyme and $Harpin_{Ecc}$ production and virulence by modulating the levels of regulatory RNA (*rsmB*) and RNA binding protein (RsmA). J. Bacteriol. **181:**6042–6052.
11. **de Kievit, T. R., and B. H. Iglewski.** 2000. Bacterial quorum sensing in pathogenic relationships. Infect. Immun. **68:**4839–4849.
12. **Eriksson, A. R., R. A. Andersson, M. Pirhonen, and E. T. Palva.** 1998. Two-component regulators involved in the global control of virulence in *Erwinia carotovora* subsp. *carotovora*. Mol. Plant-Microbe Interact. **11:**743–752.
13. **Frederick, R. D., J. Chiu, J. L. Bennetzen, and A. K. HandA**. 1997. Identification of a pathogenicity locus, rpfA, in *Erwinia carotovora* subsp. *carotovora* that encodes a two-component sensor-regulator protein. Mol. Plant-Microbe Interact. **10:**407–415.

14. **Fuqua, C., M. R. Parsek, and E. P. Greenberg.** 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoerine lactone quorum sensing. Annu. Rev. Genet. **35:**439–468.
15. **Gray, K. M., and J. R. Garey.** 2001. The evolution of bacterial LuxI and LuxR quorum sensing regulators. Microbiology **147:**2379–2387.
16. **Gray, K. M., and E. P. Greenberg.** 1992. Physical and functional maps of the luminescence gene cluster in an autoinducer-deficient *Vibrio fischeri* strain isolated from a squid light organ. J. Bacteriol. **174:**4384–4390.
17. **Harris, S. J., Y. L. Shih, S. D. Bentley, and G. P. C. Salmond.** 1998. The *hexA* gene of *Erwinia carotovora* encodes a LysR homologue and regulates motility and the expression of multiple virulence determinants. Mol. Microbiol. **28:** 705–717.
18. **He, S. Y.** 1998. Type III protein secretion systems in plant and animal pathogenic bacteria. Annu. Rev. Phytopathol. **36:**363–392.
19. **Holden, M. T. G., S. R. Chabra, R. de Nys, P. Stead, N. J. Bainton, P. J. Hill, M. Manefield, N. Kumar, M. Labatte, D. England, S. Rice, M. Givskov, G. P. C. Salmond, G. S. A. B. Stewart, B. W. Bycroft, S. Kjelleberg, and P. Williams.** 1999. Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other gram-negative bacteria. Mol. Microbiol. **33:**1254–1266.
20. **Holden, M. T. G., S. J. McGowan, B. W. Bycroft, G. S. A. B. Stewart, P. Williams, and G. P. C. Salmond.** 1998. Cryptic carbapenem antibiotic production genes are widespread in *Erwinia carotovora*-facile *trans* activation by the *carR* transcriptional regulator. Microbiology **144:**1495–1508.
21. **Jones, S., B. Yu, N. J. Bainton, M. Birdsall, B. W. Bycroft, S. R. Chhabra, A. J. R. Cox, P. Golby, P. J. Reeves, S. Stephens, M. K. Winson, G. P. C. Salmond, G. S. A. B. Stewart, and P. Williams.** 1993. The *lux* autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. EMBO J. **12:**2477–2482.
22. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene **70:**191–197.
23. **Kleiner, D., W. Paul, and M. J. Merrick.** 1988. Construction of multicopy expression vectors for regulated overproduction of proteins in *Klebsiella pneumoniae* and other enteric bacteria. J. Gen. Microbiol. **134:**1779–1784.
24. **Kõiv, V., and A. Mäe.** 2001. Quorum sensing controls the synthesis of virulence factors by modulating *rsmA* gene expression in *Erwinia carotovora* subsp. *carotovora*. Mol. Genet. Genomics **265:**287–292.
25. **Ledgham, F., I. Ventre, C. Soscia, M. Foglino, J. N. Sturgis, and A. Lazdunski.** 2003. Interactions of the quorum sensing regulator QscR: interaction with itself and the other regulators of *Pseudomonas aeruginosa* LasR and RhlR. Mol. Microbiol. **48:**199–210.
26. **Lerner, C. G., and M. Inouye.** 1990. Low copy number plasmids for regulated low-level expression of cloned genes in *Escherichia coli* with blue/white insert screening capability. Nucleic Acids Res. **18:**4631.
27. **Liu, Y., A. Chatterjee, and A. K. Chatterjee.** 1994. Nucleotide sequence and expression of a novel pectate lyase gene (*pel-3*) and a closely endopolygalacturonase gene (*peh-1*) of *Erwinia carotovora* subsp. *carotovora* 71. Appl. Environ. Microbiol. **60:**2545–2552.
28. **Liu, Y., Y. Cui, A. Mukherjee, and A. K. Chatterjee.** 1998. Characterization of a novel RNA regulator of *Erwinia carotovora* ssp. *carotovora* that controls production of extracellular enzymes and secondary metabolites. Mol. Microbiol. **29:**219–234.
29. **Liu, Y., G.-Q. Jiang, Y. Cui, A. Mukherjee, W.-L. Ma, and A. K. Chatterjee.** 1999. $kdgR_{Ecc}$ negatively regulates genes for pectinases, cellulase, protease, harpin_{Ecc}, and a global RNA regulator in *Erwinia carotovora* subsp. *carotovora*. J. Bacteriol. **181:**2411–2422.
30. **Loh, J., E. A. Pierson, L. S. Pierson III, G. Stacey, and A. K. Chatterjee.** 2002. Quorum sensing in plant associated bacteria. Curr. Opin. Plant Biol. **5:**285–290.
31. **Mäe, A., M. Montesano, V. Koiv, and E. T. Palva.** 2001. Transgenic plants producing the bacterial pheromone *N*-acyl-homoserine lactone exhibit enhanced resistance to the bacterial phytopathogen *Erwinia carotovora*. Mol. Plant-Microbe Interact. **14:**1035–1042.
32. **McGowan, S., M. Sebaihia, S. Jones, B. Yu, N. Bainton, P. Chan, B. Bycroft, G. S. A. B. Stewart, P. Williams, and G. P. C. Salmond.** 1995. Carbapenem antibiotic production in *Erwinia carotovora* regulated by CarR, a homologue to the LuxR transcriptional activator. Microbiology **141:**541–550.
33. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **Miller, M. B., and B. L. Bassler.** 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol. **55:**165–199.
35. **Minogue, T. D., A. L. Carlier, M. D. Koutsoudis, and S. B. von Bodman.** 2005. The cell density-dependent expression of stewartan exopolysaccharide in *Pantoea stewartii* ssp. *stewartii* is a function of EsaR-mediated repression of the *rcsA* gene. Mol. Microbiol. **56:**189–203.
36. **Minogue, T. D., M. Wehland-von Trebra, F. Bernhard, and S. B. von Bodman.** 2002. The autoregulatory role of EsaR, a quorum sensing regulator in *Pantoea stewartii* subsp. *stewartii*: evidence for a repressor function. Mol. Microbiol. **44:**1625–1635.
37. **Morin, D., B. Grasland, K. Vallée-Réhel, C. Dufau, and D. Haras.** 2003. On-line high-performace liquid chromatography-mass spectrometric detection and quantification of *N*-acylhomoserine lactones, quorum sensing signal molecules, in the presence of biological matrices. J. Chromatogr. A **1002:** 79–92.
38. **Mukherjee, A., Y. Cui, Y. Liu, and A. K. Chatterjee.** 1997. Molecular characterization and expression of the *Erwinia carotovora* $hrpN_{Ecc}$ gene, which encodes an elicitor of the hypersensitive reaction. Mol. Plant-Microbe Interact. **10:**462–471.
39. **Mukherjee, A., Y. Cui, W. Ma, Y. Liu, and A. K. Chatterjee.** 2000. *hexA* of *Erwinia carotovora* ssp. *carotovora* strain Ecc71 negatively regulates production of RpoS and *rsmB* RNA, a global regulator of extracellular proteins, plant virulence and the quorum-sensing signal, *N*-(3-oxohexanoyl)-L-homoserine lactone. Environ Microbiol. **2:**203–215.
40. **Mukherjee, A., Y. Cui, W.-L. Ma, Y. Liu, A. Ishihama, A. Eisenstark, and A. K. Chatterjee.** 1998. RpoS (Sigma-S) controls expression of *rsmA*, a global regulator of secondary metabolites, harpin, and extracellular proteins in *Erwinia carotovora*. J. Bacteriol. **180:**3629–3634.
41. **Murata, H., J. L. McEvoy, A. Chatterjee, A. Collmer, and A. K. Chatterjee.** 1991. Molecular cloning of an *aepA* gene that activates production of extracellular pectolytic, cellulolytic, and proteolytic enzymes in *Erwinia carotovora* subsp. *carotovora*. Mol. Plant-Mircobe Interact. **4:**239–246.
42. **Nasser, W., M. L. Bouillant, G. P. C. Salmond, and S. Reverchon.** 1998. Characterization of the *Erwinia chrysanthemi expI-expR* locus directing the synthesis of two *N*-acyl-homoserine lactone signal molecules. Mol. Microbiol. **29:**1391–1405.
43. **Pemberton, C. L., N. A. Whitehead, M. Sebaihia, K. S. Bell, L. J. Hyman, S. J. Harris, A. J. Matlin, N. D. Robson, P. R. J. Birch, J. P. Carr, I. K. Toth, and G. P. C. Salmond.** 2005. Novel quorum-sensing-controlled genes in *Erwinia carotovora subsp. carotovora*: identification of a fungal elicitor homologue in a soft-rotting bacterium. Mol. Plant-Microbe Interact. **18:**343–353.
44. **Pierson, L. S., III, D. W. Wood, and S. B. von Bodman.** 1999. Quorum sensing in plant-associated bacteria, p. 101–116. *In* G. M. Dunney and S. C. Winans (ed.), Cell-cell signaling. American Society for Microbiology Press, Washington, D.C.
45. **Pirhonen, M., D. Flego, R. Heikinheimo, and E. T. Palva.** 1993. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. EMBO J. **12:**2467–2476.
46. **Rantakari, A., O. Virtaharju, S. Vähämiko, S. Taira, E. T. Palva, H. Saarilahti, and M. Romantschuk.** 2001. Type III secretion contributes to the pathogenesis of the soft-rot pathogen *Erwinia carotovora*: partial characterization of the *hrp* gene cluster. Mol. Plant-Microbe Interact. **14:**962–968.
47. **Reverchon, S., M. L. Bouillant, G. Salmond, and W. Nasser.** 1998. Integration of the quorum-sensing system in the regulatory networks controlling virulence factor synthesis in *Erwinia chrysanthemi*. Mol. Microbiol. **29:**1407–1418.
48. **Salmond, G. P. C.** 1994. Secretion of extracellular virulence factors by plant pathogenic bacteria. Annu. Rev. Phytopathol. **32:**181–200.
49. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
50. **Slater, H., M. Crow, L. Everson, and G. P. C. Salmond.** 2003. Phosphate availability regulates biosynthesis of two antibiotics, prodigiosin and carbapenem, in *Serratia* via both quorum-sensing-dependent and -independent pathways. Mol. Microbiol. **47:**303–320.
51. **Spaink, H. P., R. J. H. Okker, C. A. Wijffelman, E. Pees, and B. J. J. Lugtenberg.** 1987. Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. Plant Mol. Biol. **9:**27–39.
52. **Swift, S., A. V. Karlyshev, L. Fish, E. L. Durant, M. K. Winson, S. R. Chhabra, P. Williams, S. Macintyre, and G. S. A. B. Stewart.** 1997. Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*-identification of the *luxRI* homologs *ahyRI* and *asaRI* and their cognate *N*-acylhomoserine lactone signal molecules. J. Bacteriol. **179:**5271–5281.
53. **Swift, S., J. P. Throup, P. Williams, G. P. C. Salmond, and G. S. A. B. Stewart.** 1996. Quorum sensing: a population-density component in the determination of bacterial phenotype. Trends Biochem. Sci. **21:**214–219.
54. **Taga, M. E., and B. L. Bassler.** 2003. Chemical communication among bacteria. Proc. Natl. Acad. Sci. USA **100:**14549–14554.
55. **Thomson, N. R., A. Cox, B. W. Bycroft, G. S. Stewart, P. Williams, and G. P. C. Salmond.** 1997. The *rap* and *hor* proteins of *Erwinia*, *Serratia* and *Yersinia*: a novel subgroup in a growing superfamily of proteins regulating diverse physiological processes in bacterial pathogens. Mol. Microbiol. **26:** 531–544.
56. **Throup, J. P., M. Camara, G. S. Briggs, M. K. Winson, S. R. Chhabra, B. W. Bycroft, P. Williams, and G. S. A. B. Stewart.** 1995. Characterization of the *yenl/yenr* locus from *Yersinia enterocolitica* mediating the synthesis of two *N*-acylhomoserine lactone signal molecules. Mol. Microbiol. **17:**345–356.
57. **Urbanowski, M. L., C. P. Lostroh, and E. P. Greenberg.** 2004. Reversible acyl-homoserine lactone binding to purified *Vibrio fischeri* LuxR protein. J. Bacteriol. **186:**631–637.
58. **von Bodman, S. B., J. K. Ball, M. A. Faini, C. M. Herrera, T. D. Minogue, M. L. Urbanowski, and A. M. Stevens.** 2003. The quorum sensing negative

regulators EsaR and ExpR(Ecc), homologues within the LuxR family, retain the ability to function as activators of transcription. J. Bacteriol. **185:**7001–7007.

59. **von Bodman, S. B., W. D. Bauer, and D. L. Coplin.** 2003. Quorum sensing in plant-pathogenic bacteria. Annu. Rev. Phytopathol. **41:**455–482.
60. **Welch, M., D. E. Todd, N. A. Whitehead, S. J. Mcgowan, B. W. Bycroft, and G. P. C. Salmond.** 2000. *N*-acyl homoserine lactone binding to the CarR receptor determines quorum-sensing specificity in *Erwinia*. EMBO J. **19:**631–641.
61. **Whitehead, N. A., J. T. Byers, P. Commander, M. J. Corbett, S. J. Coulthurst, L. Everson, A. K. P. Harris, C. L. Pemberton, N. J. L. Simpson, H. Slater, D. S. Smith, M. Welch, N. Williamson, and G. P. C. Salmond.** 2002. The regulation of virulence in phytopathogenic *Erwinia* species: quorum sensing, antibiotics and ecological considerations. Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol. **81:**223–231.
62. **Withers, H., S. Swift, and P. Williams.** 2001. Quorum sensing as an integral component of gene regulatory networks in gram-negative bacteria. Curr. Opin. Microbiol. **4:**186–193.
63. **Wood, D. W., F. Gong, M. M. Daykin, P. Williams, and L. S. Pierson III.** 1997. *N*-acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30–84 in the wheat rhizosphere. J. Bacteriol. **179:**7663–7670.
64. **Zink, R. T., R. J. Kemble, and A. K. Chatterjee.** 1984. Transposon Tn*5* mutagenesis in *Erwinia carotovora* subsp. *carotovora* and *Erwinia carotovora* subsp. *atroseptica*. J. Bacteriol. **157:**809–814.