JOURNAL OF BACTERIOLOGY, Dec. 2005, p. 8026–8038 Vol. 187, No. 23
0021-9193/05/$08.00+0 doi:10.1128/JB.187.23.8026–8038.2005

Comparative Analysis of Two Classes of Quorum-Sensing Signaling Systems That Control Production of Extracellular Proteins and Secondary Metabolites in *Erwinia carotovora* Subspecies

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

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

Received 19 June 2005/Accepted 16 September 2005

In *Erwinia carotovora* subspecies, *N*-acyl homoserine lactone (AHL) controls the expression of various traits, including extracellular enzyme/protein production and pathogenicity. We report here that *E. carotovora* subspecies possess two classes of quorum-sensing signaling systems defined by the nature of the major AHL analog produced as well as structural and functional characteristics of AHL synthase (AhlI) and AHL receptor (ExpR). Class I strains represented by *E. carotovora* subsp. *atroseptica* strain Eca12 and *E. carotovora* subsp. *carotovora* strains EC153 and SCC3193 produce 3-oxo-C8-HL (*N*-3-oxooctanoyl-L-homoserine lactone) as the major AHL analog as well as low but detectable levels of 3-oxo-C6-HL (*N*-3-oxohexanoyl-L-homoserine lactone). In contrast, the members of class II (i.e., *E. carotovora* subsp. *betavasculorum* strain Ecb168 and *E. carotovora* subsp. *carotovora* strains Ecc71 and SCRI193) produce 3-oxo-C6-HL as the major analog. ExpR species of both classes activate *rsmA* (Rsm, repressor of secondary metabolites) transcription and bind *rsmA* DNA. Gel mobility shift assays with maltose-binding protein (MBP)-$ExpR_{71}$ and MBP-$ExpR_{153}$ fusion proteins show that both bind a 20-mer sequence present in *rsmA*. The two ExpR functions (i.e., *expR*-mediated activation of *rsmA* expression and ExpR binding with *rsmA* DNA) are inhibited by AHL. The AHL effects are remarkably specific in that *expR* effect of EC153, a strain belonging to class I, is counteracted by 3-oxo-C8-HL but not by 3-oxo-C6-HL. Conversely, the *expR* effect of Ecc71, a strain belonging to class II, is neutralized by 3-oxo-C6-HL but not by 3-oxo-C8-HL. The AHL responses correlated with *expR*-mediated inhibition of exoprotein and secondary metabolite production.

Extracellular proteins produced by *Erwinia carotovora* subspecies are critical to the development of soft-rotting disease of plants and plant organs (3, 8, 9, 51). Production of those extracellular proteins is controlled by quorum-sensing (QS) signals, plant signals, and an assortment of transcriptional factors and posttranscriptional regulators (1, 7, 11, 13, 15–17, 21, 22, 29, 30, 38, 39, 43, 50). Of these regulators, posttranscriptional regulation by the RsmA-RsmB RNA pair is absolutely critical in the expression of exoprotein genes. RsmA is a small RNA-binding protein that promotes decay of RNA (7, 13). *rsmB* specifies an untranslated regulatory RNA that binds RsmA and neutralizes its negative regulatory effect (29). Many of the transcription factors and QS signal, known to regulate extracellular protein production, actually act via these posttranscriptional regulators (6, 11, 15, 25, 30, 39, 40).

QS signaling systems have been found in a wide range of bacterial genera, including a variety of animal and plant pathogens. The first biological function known to be regulated in a QS signal-dependent manner is the bioluminescence in the marine bacterium *Vibrio fischeri* (20). In gram-negative bacteria, the QS signal molecules are almost exclusively *N*-acyl-homoserine lactones (AHLs). AHLs are involved in the regulation of a range of biological functions, including bacterium-microbe and bacterium-plant/animal interactions, conjugation, virulence, motility, biofilm formation, production of secreted proteins, antibiotics, extracellular polysaccharide, pigment, and other secondary metabolites (see references 18, 19, 31, 34, 42, 48, 49, 54, 56, and 57 and references cited therein).

Studies of the *lux* operon and similar AHL-controlled systems have revealed that minimally three components are required: an AHL synthase gene (a homolog of the *luxI* gene); AHL species; and an AHL receptor (a homolog of LuxR). In most instances, complexes between AHL and LuxR or LuxR-like proteins activate gene expression (31, 49, 53). However, there is burgeoning evidence for exceptions to this generalization. One well-studied example is the AHL-regulated production of capsular polysaccharide (CPS) in *Pantoea* (*Erwinia*) *stewartii* (35, 36). In this instance, EsaR, a LuxR homolog, inhibits the CPS production by repressing transcription of *rcsA* which encodes an essential coactivator of *cps* genes. The repressor activity of EsaR is relieved by AHL. In *E. carotovora*, ExpR, the putative AHL receptor of *E. carotovora* subsp. *carotovora*, activates transcription of *rsmA*, and AHL prevents this activation (12). Our findings taken together with those of von Bodman et al. (54) established that ExpR is a DNA-binding protein and that its DNA-binding property is modified by AHL. Evidence was also presented showing that RsmA overproduction is indeed responsible for inhibition of extracellular enzyme/protein and secondary metabolite production in AHL-deficient bacteria.

In subsequent studies, we noticed remarkable specificity in the effects of ExpR species on extracellular enzyme production

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

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
Erwinia carotovora subsp. *atroseptica* Ecal2	Wild type	58
Erwinia carotovora subsp. *betavasculorum* Ecb168	Wild type	J. E. Loper
Erwinia carotovora subsp. *carotovora*		
Ecc71	Wild type	58
AC5094	AhlI$^-$ derivative of Ecc71	7
AC5111	AhlI$^-$ derivative of EC153	This study
SCC3193	Wild type	43
SCRI193	Wild type	44
EC153	Wild type	Laboratory collection
Escherichia coli		
DH5α	ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169 hsdR17 recA1 endA1 thi-1*	Gibco BRL
MC4100	*araD139* Δ(*lacIPOZYA*)*U169 recA1 thi-1* Str^r	27
VJS533	*araD*(*lac-proAB*) *rpsL* ϕ80 *lacZ*ΔM15 *recA56*	20
Plasmids		
pDK6	Km^r	24
pLARF5	Tc^r	23
pMAL-c2g	Ap^r, protein expression vector	New England Biolabs
pMP220	Tc^r, promoter-probe vector	47
pCL1920	Sp^r Sm^r	26
pAKC781	*peh-1*$^+$ DNA in pBluescriptSK(+)	27
pAKC783	Ap^r, *pel-1*$^+$ DNA in pBluescriptSK(+)	27
pAKC856	Ap^r, *ahlI*$^+$ DNA in pBluescriptSK(+)	7
pAKC882	Ap^r, *rsmA* coding region in pT7-7	38
pAKC924	Ap^r, *hrpN*$^+$ in pBluescriptSK(+)	14
pAKC935	Sp^r, $expR_{3193}{}^+$ in pCL1920	This study
pAKC936	Sp^r, $expR_{71}{}^+$ in pCL1920	This study
pAKC937	Sp^r, 0.8 kb $expR_{153}{}^+$ in pCL1920	This study
pAKC1034	Ap^r, 200-bp *celV* fragment in pGEM-T Easy	30
pAKC1100	Tc^r, $rsmA_{71}$-*lacZ* in pMP220	6
pAKC1101	Tc^r, $rsmA_{71}$-*lacZ* in pMP220	This study
pAKC1102	Tc^r, $rsmA_{71}$-*lacZ* in pMP220	This study
pAKC1103	Tc^r, $rsmA_{71}$-*lacZ* in pMP220	This study
pAKC1104	Tc^r, $rsmA_{71}$-*lacZ* in pMP220	This study
pAKC1106	Tc^r, $rsmA_{153}$-*lacZ*, 0.4-kb $rsmA_{153}$ upstream DNA in pMP220	This study
pAKC1201	Km^r, $ptac\text{-}ahlI_{71}$, $ahlI_{71}$ coding region in pDK6	12
pAKC1210	Tc^r, *ahlI*$^+$ DNA of EC153 in pLARF5	Laboratory collection
pAKC1211	Ap^r Tc^r, AhlI$^-$ derivative of pAKC1210	Laboratory collection
pAKC1221	Ap^r, $expR_{153}$ coding region in pMAL-c2g	This study
pAKC1222	Km^r, $ptac\text{-}ahlI_{153}$, $ahlI_{153}$ coding region in pDK6	This study
pHV200	Ap^r, 8.8-kb *Sal*I fragment containing *lux* operon	20
pHV200I	Ap^r, frameshift mutant of *luxI* in pHV200	E. P. Greenberg
pSB401	Tc^r, fusion of *luxRI*′::*luxCDABE* on pACYC184 plasmid backbone	52

in Ecc71, which produces 3-oxo-C6-HL, and in EC153, which produces 3-oxo-C8-HL as the major AHL analog. We show that this specificity is actually conferred by AHL analogs. Comparative studies with *E. carotovora* subspecies revealed the occurrence of two QS signaling systems characterized by the nature of AHL analogs, sequences of AHL synthases, and specificity in the interactions between AHL and ExpR.

MATERIALS AND METHODS

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

The compositions of LB medium and minimal salts medium have been described in previous publications (7, 41). When required, antibiotics were supplemented as follows: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; spectinomycin, 50 μg/ml; and tetracycline, 10 μg/ml. Media were solidified using 1.5% (wt/vol) agar.

The composition of media for agarose plate assays for enzymatic activities was described by Chatterjee et al. (7).

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 (7). The enzymatic activities are indicated by halos around the wells on the assay plates.

Sequence alignment. Sequence alignment was performed using ClustalW at www.expasy.ch, and default parameters were used.

DNA techniques. Standard procedures were used in the isolation of plasmids and chromosomal DNA, gel electrophoresis, and DNA ligation (45). Restriction and modification enzymes were obtained from Promega Biotec (Madison, WI). The Prime-a-Gene DNA labeling system (Promega Biotec) was used for labeling DNA probes. Southern blot analysis was carried out under 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 by 0.1× SSC plus 0.1% [wt/vol] SDS for 30 min) as well as under low-stringency conditions (hybridization and washing conditions are the same as high-stringency conditions, except temperature was 55°C). A 500-bp BamHI-ClaI fragment from pAKC935 was used as an $expR_{3193}$ probe.

Construction of AhlI mutant of EC153. AC5111 (AhlI mutant of EC153) was constructed by marker exchange of EC153 with pAKC1211. The procedures for marker exchange have been described by Chatterjee et al. (7). Inactivation of *ahlI* in the mutant was confirmed by Northern blot analysis.

Northern and Western blot analyses. Bacterial cultures were grown at 28°C in minimal salts medium supplemented with sucrose (0.5% [wt/vol]) and appropriate antibiotics. Cells were collected while cultures reached a Klett value of ca. 150. RNA isolation and Northern blot analysis were performed as described by Liu et al. (29). The probes used were the 183-bp NdeI-SalI fragment of $rsmA_{71}$ from pAKC882, a 314-bp *Eco*RV-KpnI fragment of *pel-1* from pAKC 783, a 743-bp HindIII fragment of *peh-1* from pAKC781, a 200-bp EcoRI fragment of *celV* from pAKC1034, and a 779-bp EcoRV-SmaI fragment of *hrpN* from pAKC924. For Western blot analysis, bacterial cells were collected, suspended in 1× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (45), and boiled. The protein concentrations were determined by using the CB-X protein assay kit (Geno Technology, Inc., St. Louis, MO) according to the manufacturer's specifications. Western blot analysis of the total bacterial protein was performed as described by Muhkerjee et al. (38). The antisera raised against RsmA of Ecc71 (15) were used as probes.

Bioluminescence assays for AHL production. *Erwinia carotovora* strains were grown in minimal salts-plus-sucrose medium supplemented with or without spectinomycin to a Klett value of ca. 200. Culture supernatants and high-performance liquid chromatography (HPLC) fractions were assayed for bioluminescence using *Escherichia coli*-based bioassay systems (7). *E. coli* strain VJS533 harboring pHV200I or *E. coli* strain DH5α carrying pSB401 was used as a biosensor indicator. Relative light units (RLU) are expressed as counts per min per ml of culture. There is a linear relationship between the quantity of AHL production and the emission of bioluminescence.

Production and fractionation of AHLs. *E. carotovora* strains were 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. Each culture supernatant was extracted with an equal volume of ethyl acetate. The ethyl acetate extracts were evaporated to dryness, and the residues were dissolved in 5 ml of distilled water. The ethyl acetate extract from EC153 was 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% (vol/vol) 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. (7). Active fractions were pooled, concentrated, and rechromatographed under similar conditions for further purification.

Detection of AHLs by analytical TLC. A procedure described by Cui et al. (12) was used for the detection of AHLs by analytical thin-layer chromatography (TLC). Crude extracts were applied in volumes of 0.5 to 2.5 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]). 3-Oxo-C6-HL [*N*-(β-ketocaproyl)-DL-homoserine lactone, purchased from Sigma] and 3-oxo-C8-HL (kindly provided by Paul Williams, University of Nottingham, United Kingdom) were used as standards. The plates were dried and overlaid with the biosensor indicator bacterium *E. coli* VJS533 carrying pHV200I or DH5α carrying pSB401. The overlaid plates were incubated at 28°C for 2 h and exposed to X-ray film to record bioluminescent spots.

Mass spectrometry. All mass spectrometry (MS) 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. The inlet capillary was heated to 250°C; a 4.5-kV bias was applied to the stainless steel electrospray needle. 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 tandem mass spectrometry (MS/MS) experiments, the collision gas was argon and collision energies ranged from 20 to 40 eV.

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 electrospray source at 80 lb/in^2. The spectra acquired for each sample are an average of 150 individual scans.

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.

Expression and purification of MBP-ExpR$_{153}$ protein. A fragment containing the entire coding region of $expR_{153}$ was PCR amplified from EC153 by using primers 5′-TGTGGATCCATGTCGCAATTATTTTACAACAATG-3′ and 5′-TGTAAGCTTCTATGACTGAACCGGTCGGATGAG-3′. The fragment was digested with BamHI and HindIII and cloned into pMAL-c2g vector (New England Biolabs, Beverly, MA) to yield pAKC1221.

E. coli strain DH5α carrying pAKC1221 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. Maltose-binding protein (MBP)-ExpR$_{153}$ fusion protein was purified by amylose resin (New England Biolabs) affinity chromatography according to the protocol provided by the company. The protein concentration was determined by using an CB-X protein assay kit (Geno Technology, Inc., St. Louis, MO). Crude extracts and purified MBP-ExpR$_{153}$ were analyzed by SDS-PAGE in a 10% (wt/vol) polyacrylamide gel.

Gel mobility shift assays. The DNA fragments were generated by PCR using the primers listed as follows: $rsmA_{71}$, 5′-GCTGGATCCGGCAAGCAGGATAGAA-3′ and 5′-GCTGAATTCGATTATAAAGAGTCGGGTCTCT-3′ (corresponding to −199 to +30 from the transcriptional start site T2); and $rsmA_{153}$, 5′-TGCGAATTCTTGAATCCTGGGTTGCTGCTAAGC-3′ and 5′-TGACTGCAGAGGGTTTCGCCAACTCGACGAGTC-3′ (corresponding to −355 to +35 from the putative translational start site). The DNA fragments were purified using the Wizard SV gel and PCR clean-up system (Promega Biotec, Madison, WI) and end labeled with [α-^{32}P]dATP and Klenow fragment. Double-stranded DNA fragments containing the *expR* box or part of the *expR* box were generated by annealing oligonucleotides (5′-ATGGTGTGGTTATACCATCGTCTA-3′ plus 5′-TACCTAGACGATGGTATAACCACA-3′ or 5′ ATGGTGTGGTTATACCATCGT-3′ plus 5′-CTAGACGATGGTATAACCACA-3′). The probe DNAs were 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, and purified MBP-ExpR$_{71}$ or MBP-ExpR$_{153}$ proteins with or without competitors. The reaction mixtures were incubated at room temperature for 20 min and subjected to electrophoresis in 5% (wt/vol) polyacrylamide gels. The gels were dried and exposed to X-ray film.

DNase I protection analysis. The DNA probe was PCR amplified using 10 pmol of end-labeled primer 5′-GCTGAATTCGATTATAAAGAGTCGGGTCTCT-3′ (corresponding to +30 to +9 from the transcriptional start site T2 of $rsmA_{71}$) and 10 pmol of unlabeled primer 5′-GCTGGATCCGGCAAGCAGGATAGAA-3′ (corresponding to −199 to −178 from the transcriptional start site T2 of $rsmA_{71}$). PCR labeling of DNA probe and DNase I protection assays were carried out according to the procedures described by Liu et al. (28).

β-Galactosidase assays. Bacterial constructs were grown at 28°C in LB medium supplemented with appropriate antibiotics and AHLs as described in footnotes to the tables. The β-galactosidase assays were performed according to Miller (33).

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

RESULTS

Characterization of AHL analogs produced by *E. carotovora* subspecies. To identify the AHL species produced by different *E. carotovora* strains, ethyl acetate extracts of spent cultures were subjected to analytical TLC assays. The TLC profiles of the samples from Eca12, SCC3193, and EC153 show two spots with retention factors identical to those of synthetic 3-oxo-C6-HL and 3-oxo-C8-HL standards, respectively (Fig. 1A and B, lanes 1 to 3). The density of the spots identical to 3-oxo-C8-HL is much stronger than that of the spots identical to 3-oxo-C6-HL. In contrast, the samples from Ecb168, SCRI193, and Ecc71 show one spot with a retention factor identical to that of the synthetic 3-oxo-C6-HL standard (Fig. 1A and B, lanes 4 to 6). HPLC and the MS scan reveal that the levels of 3-oxo-C8-HL (153AHL2) are much higher (>22 fold) than

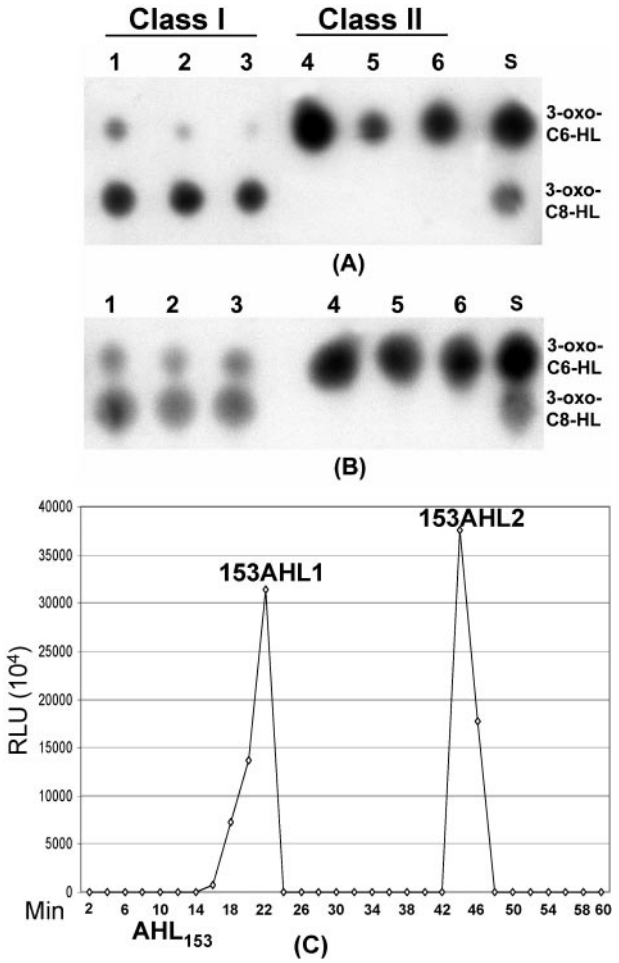

FIG. 1. (A and B) TLC analysis of AHLs. (A) *E. coli* VJS533 harboring the LuxI$^-$ plasmid pHV200I or (B) DH5α carrying pSB401 was used as a biosensor indicator. Lanes 1 to 6, crude AHL extracts of Eca12, SCC3193, EC153, Ecb168, SCRI193, and Ecc71, respectively; lane S, mixture of synthetic 3-oxo-C6-HL and 3-oxo-C8-HL (5 nmol of each loaded). (C) RLU produced by HPLC fractions of crude AHL extract from EC153 in indicator strain *E. coli* VJS533 harboring pHV200I.

that of 3-oxo-C6-HL (153AHL1) in EC153 (data not shown). The major AHL produced by Ecc71 (designated as 71AHL) has been identified as 3-oxo-C6-HL by HPLC fractionation and LCMS/MS (12). To further characterize the AHL analogs produced by EC153, the ethyl acetate extract of EC153 was fractionated by HPLC on a C_{18} reverse-phase column. The fractions were assayed for bioluminescence using *E. coli* strain VJS533 harboring pHV200I as a biosensor indicator. Two peaks corresponding to bioluminescence activity were observed: one at a retention time of about 20 min (designated as 153AHL1), which matched well with that of 71AHL, and the other one at a retention time of about 46 min (designated as 153AHL2) (Fig. 1C). The active fractions corresponding to each peak were pooled and rechromatographed for further purification. TLC profiles show that purified 153AHL1 and 153AHL2 have retention factors identical to those of synthetic 3-oxo-C6-HL and 3-oxo-C8-HL standards, respectively (data not shown).

TABLE 2. Differential effects of AHL analogs and AHL synthase of Ecc71 and EC153 on pectate lyase production in AC5094 (AhlI mutant of Ecc71) and AC5111 (AhlI mutant of EC153)

Bacterial construct[a]	Relevant characteristic[b]	AHL	Pel activity[c]
AHL analog			
AC5094	AhlI$^-$		0.07 ± 0.01
AC5094	AhlI$^-$	71AHL	0.62 ± 0.03
AC5094	AhlI$^-$	153AHL1	0.57 ± 0.02
AC5094	AhlI$^-$	153AHL2	0.19 ± 0.02
AC5111	AhlI$^-$		0.06 ± 0.01
AC5111	AhlI$^-$	71AHL	0.20 ± 0.01
AC5111	AhlI$^-$	153AHL1	0.15 ± 0.01
AC5111	AhlI$^-$	153AHL2	0.56 ± 0.02
AHL synthase			
AC5094(pDK6)	AhlI$^-$ (vector DNA)		0.08 ± 0.02
AC5094(pAKC1201)	AhlI$^-$ (*ahlI*$_{71}^+$ DNA)		0.28 ± 0.03
AC5094(pAKC1222)	AhlI$^-$ (*ahlI*$_{153}^+$ DNA)		0.24 ± 0.01
AC5111(pDK6)	AhlI$^-$ (vector DNA)		0.11 ± 0.02
AC5111(pAKC1201)	AhlI$^-$ (*ahlI*$_{71}^+$ DNA)		0.15 ± 0.01
AC5111(pAKC1222)	AhlI$^-$ (*ahlI*$_{153}^+$ DNA)		1.05 ± 0.04

[a] For the AHL analogs, bacterial cultures were started at a Klett value of ca. 25 in 3 ml of minimal salts medium plus sucrose with or without AHLs and grown at 28°C for 8 h. AHLs were added to a final concentration of 50 μM. Cultural supernatants were used for enzyme assays. For AHL synthase AhlI$^-$ mutants, bacteria were grown at 28°C in minimal salts medium plus sucrose (0.5% [wt/vol]), spectinomycin, and kanamycin to a Klett value of ca. 200, and culture supernatants were used for assays.

[b] The relevant DNAs carried by bacteria are given in parentheses.

[c] Expressed as A_{235}/A_{600} per 30 min. Values are means ± standard deviations of three repetitions.

Analysis of 153AHL1 by LCMS/MS (data not shown) yielded a peak with retention time, parent ion, and fragment ion spectrum the same as those for standard 3-oxo-C6-HL. These results confirm that 153AHL1 is 3-oxo-C6-HL.

153AHL2 was analyzed by direct infusion MS and MS/MS (data not shown), producing the same fragment ion spectrum as that seen with standard 3-oxo-C8-HL. This verifies that 153AHL2 is 3-oxo-C8-HL.

Based upon the AHL analogs produced, the tested *E. carotovora* strains can be classified into two classes: class I strains produce 3-oxo-C8-HL as the major AHL analog and 3-oxo-C6-HL as a minor component, and class II strains produce 3-oxo-C6-HL as the major AHL analog. *E. carotovora* subsp. *carotovora* strains EC153 and Ecc71 were selected as the representatives of those two classes for further studies.

Having identified the AHL analogs present in Ecc71 and EC153, we tested the effects of these AHL analogs on exoenzyme production in AhlI-deficient strains of Ecc71 (AC5094) and EC153 (AC5111). The data in Table 2 and Fig. 2A show that 71AHL and 153AHL1 were much more effective in restoring enzyme production in the AhlI$^-$ strain of Ecc71 than in the EC153AhlI mutant. On the other hand, 153AHL2 was effective in restoring enzyme production in the EC153AhlI mutant but had little effect in the Ecc71AhlI mutant. These observations demonstrate that 71AHL and 153AHL1 are structurally and functionally different from 153AHL2.

Similarity in AHL synthase sequences. Alignment results (Fig. 3) revealed that AhlI of *E. carotovora* subsp. *carotovora* strains Ecc71 (accession no. L40174), SCC1 (accession no. AY507108), *E. carotovora* subsp. *atroseptica* strain SCRI1043 (accession no. CAG73025), CarI of *E. carotovora* subsp. *carotovora* strain GS101 (accession no. X74299), and EcbI (10) of *E. carotovora* subsp. *betavasculorum* strain Ecb168 (accession no. AF001050)

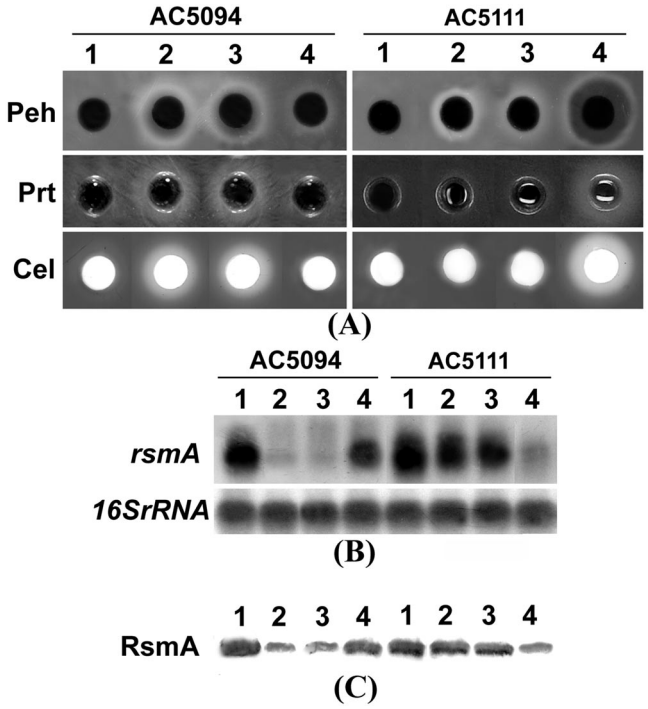

FIG. 2. (A) Agarose plate assays for Peh, Prt, and Cel activities of ExpR$^+$ AhlI$^-$ derivatives of Ecc71 (AC5094) and EC153 (AC5111). Thirty microliters of culture supernatant was applied in each well. (B and C) Northern blot and Western blot analyses of *rsmA* or RsmA of AC5094 and AC5111. Each lane contained 10 μg of total RNA for Northern blot analysis and 10 μg of total protein for Western blot analysis. Lanes: 1, in the absence of AHL (i.e., same volume of water was added); 2, in the presence of 71AHL; 3, in the presence of 153AHL1; and 4, in the presence of 153AHL2. (Three hundred microliters of 1 mM AHLs was added to 6 ml of culture to yield a final concentration of 50 μM.) Bacteria were inoculated in minimal-salts medium plus sucrose (0.5% [wt/vol]) supplemented with or without AHL. Total RNAs and proteins were extracted after 5 h of incubation at 28°C, and culture supernatants were collected for exoenzyme assays after 8 h of incubation.

share 93 to 99% identity, but these strains show ca. 70% identity with AhlI proteins of *E. carotovora* subsp. *carotovora* strains EC153 (accession no. DQ093124), SCC3193 (accession no. X80475), and *E. carotovora* subsp. *atroseptica* strain CFBP6272 (accession no. AJ580600). On the other hand, EC153, SCC3193, and CFBP6272 share 97 to 99% identity in the AhlI sequences. These data suggested that *E. carotovora* subsp. *carotovora* strains EC153 and SCC3193 as well as *E. carotovora* subsp. *atroseptica* strain CFBP6272 belong to one class, and *E. carotovora* subsp. *carotovora* strains Ecc71, SCC1, GS101, *E. carotovora* subsp. *atroseptica* strain SCRI1043 and *E. carotovora* subsp. *betavasculorum* strain Ecb168 belong to another class. We should note that strains belonging to those two classes also differ in the nature of AHL analogs produced (detailed above and in references 2, 4, and 52) and ExpR sequences (see below).

To further prove that the different AHL analogs produced by class I and class II strains are due to specificity in actions of AHL synthases, we introduced $ahlI_{71}{}^+$ plasmid pAKC1201 and $ahlI_{153}{}^+$ plasmid pAKC1222 into the AhlI-deficient mutants of Ecc71 (AC5094) and EC153 (AC5111). TLC assays (Fig. 4A) of ethyl acetate extracts of spent cultures of these constructs revealed that (i) $ahlI_{71}{}^+$ plasmid directed the production of 3-oxo-C6-HL in AC5094 and AC5111 (lane 2) and (ii) $ahlI_{153}{}^+$ plasmid specified the production of 3-oxo-C6-HL and 3-oxo-C8-HL in both AC5094 and AC5111 (lane 3). Thus, the type of AHL produced was determined by AHL synthases and not by these *Erwinia* hosts or biosynthetic intermediates produced by these bacteria.

Differential effects of $ahlI_{71}$ and $ahlI_{153}$ on restoration of exoenzyme production in AhlI-deficient mutants of Ecc71 and EC153. The data in Fig. 4B and Table 2 show that either $ahlI_{71}$ or $ahlI_{153}$ restores exoenzyme production in AC5094, the AhlI mutant of Ecc71. In contrast, $ahlI_{71}$ has very little effect on the exoenzyme production in the AhlI mutant of EC153, AC5111. As expected, exoenzyme production is restored in this mutant by $ahlI_{153}$. Moreover, transcript levels of exoenzyme genes *pel-1*, *peh-1*, and *celV* as well as *hrpN*, a gene that encodes harpin, are restored by both $ahlI_{71}$ and $ahlI_{153}$ in AC5094 but restored by only $ahlI_{153}$ in AC5111 (Fig. 4C).

Physical evidence for two classes of *expR*. Southern blot hybridization of EcoRI-digested chromosomal DNAs of *E. carotovora* strains Eca12, Ecb168, Ecc71, SCRI193, EC153, and SCC3193 with $expR_{3193}$ of *E. carotovora* subsp. *carotovora* strain SCC3193 revealed: (i) *expR* genes occurred in all strains examined and (ii) relatively weak hybridization bands with Ecb168, Ecc71, and SCRI193 occurred only under low-stringency hybridization and washing conditions, whereas strong hybridization bands with Eca12, SCC3193, and EC153 occurred under high-stringency conditions. Comparative analysis of $expR_{71}$ of Ecc71 and $expR_{153}$ of EC153 with sequences of *expR* genes available in GenBank revealed sequence divergence. The ExpR proteins of *E. carotovora* subsp. *carotovora* strains EC153 (accession no. AY894424) and SCC3193 (accession no. X80475) and *E. carotovora* subsp. *atroseptica* strain CFBP6272 (accession no. AJ580600) share more than 95% identity with each other. In contrast, Ecc71 (accession no. AY894425) and *E. carotovora* subsp. *betavasculorum* strain Ecb168 (accession no. AF001050) share ca. 60% identity with ExpR proteins of *E. carotovora* subsp. *carotovora* strains EC153 and SCC3193 and *E. carotovora* subsp. *atroseptica* strain CFBP6272, whereas as reported previously (12) Ecc71 and Ecb168 share 90% identity with each other. These data suggest the occurrence of two classes of ExpR: class I is represented by ExpR of EC153, SCC3193, and CFBP6272, and class II is represented by ExpR of Ecc71 and Ecb168. Based upon the results of Southern blot analysis, *E. carotovora* subsp. *carotovora* strain SCRI193 was also considered to possess an ExpR belonging to class II and *E. carotovora* subsp. *atroseptica* strain Eca12 to possess ExpR belonging to class I.

Effects of $ExpR_{71}$ and $ExpR_{153}$ on expression of *rsmA-lacZ* fusions in *E. coli*. To confirm that both classes of ExpR species work well as transcriptional activators in the absence of AHL, transcriptional fusion plasmids pAKC1100 ($rsmA_{71}$-*lacZ*) and pAKC1106 ($rsmA_{153}$-*lacZ*) were transferred into *E. coli* strain MC4100 carrying pCL1920, pAKC936 ($expR_{71}$ DNA), or pAKC937 ($expR_{153}$ DNA). β-Galactosidase assay results established that the transcription of $rsmA_{71}$ and $rsmA_{153}$ was activated by both $expR_{71}$ and $expR_{153}$ plasmids, although $expR_{153}$ was consistently more effective than $expR_{71}$ (Table 3).

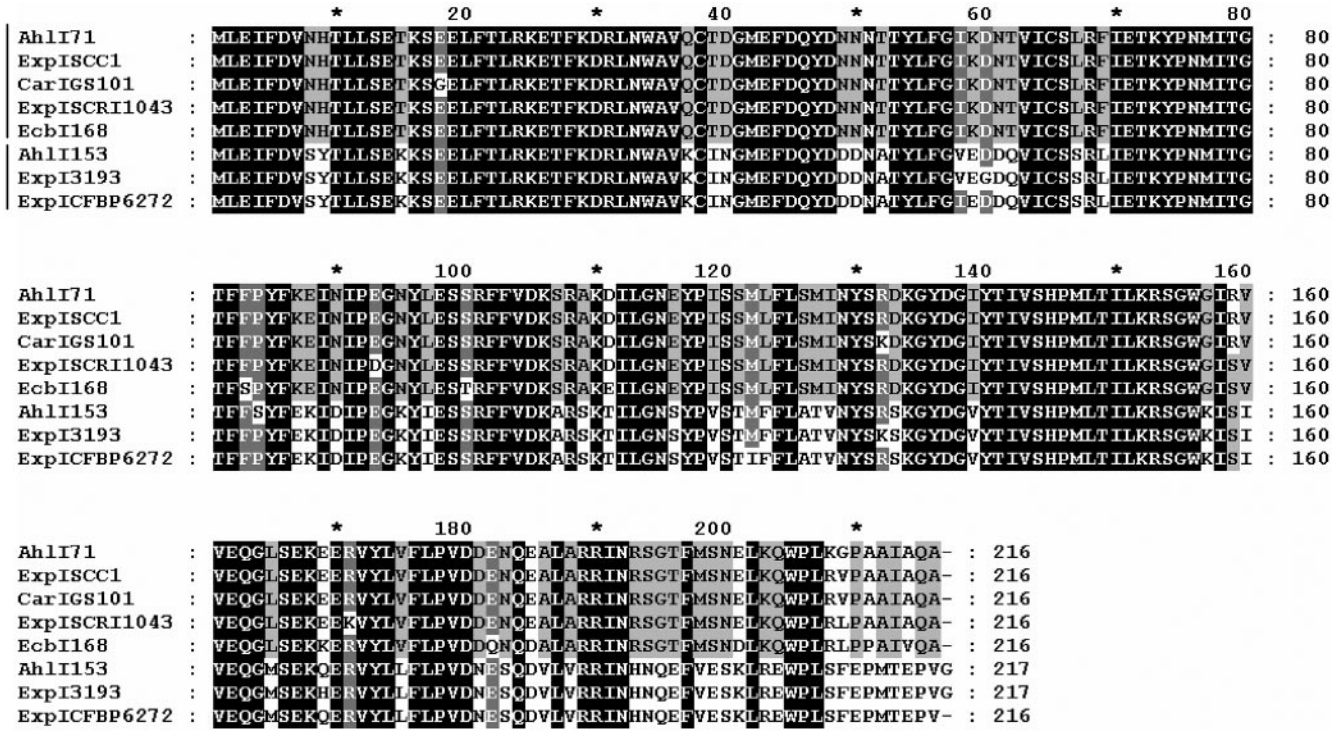

FIG. 3. Alignment of the deduced AhlI amino acid sequence of *E. carotovora* subsp. carotovora strains Ecc71 (AhlI71), SCC1 (ExpISCC1); GS101 (CarIGS101), EC153 (AhlI153), and SCC3193 (ExpI3193), *E. carotovora* subsp. *atroseptica* strains SCRI1043 (ExpISCRI1043) and CFBP6272 (ExpICFBP6272), and *E. carotovora* subsp. betavasculorum strain Ecb168 (EcbI168). Numbers on the right refer to the positions of the amino acid residues. Black-shaded areas indicate identical amino acids in all strains, and gray-shaded areas indicate identical amino acids in five or more strains at any position.

ExpR$_{71}$ and ExpR$_{153}$ bind *rsmAs* of Ecc71 and EC153. Our previous results (12) demonstrated that purified MBP-ExpR$_{71}$ protein binds upstream DNA of *rsmA* of Ecc71. The β-galactosidase assay results (see above) show that both ExpR$_{71}$ and ExpR$_{153}$ activate the expression of *rsmA*$_{71}$-*lacZ* and *rsmA*$_{153}$-*lacZ* in the absence of AHL. These results strongly suggested that ExpR$_{153}$, like ExpR$_{71}$, binds *rsmA* DNAs. To test this possibility, we studied the interaction of purified MBP-ExpR$_{71}$ and MBP-ExpR$_{153}$ proteins with DNA segments containing promoter regions of *rsmA*$_{71}$ and *rsmA*$_{153}$ by gel mobility shift assays. For this, we first overexpressed ExpR$_{153}$ as an MBP-ExpR$_{153}$ fusion protein using the construct pAKC1221, in which the coding region of *expR*$_{153}$ is controlled by *ptac* promoter in the vector pMAL-c2g. The apparent molecular mass of the overexpressed protein (ca. 79 kDa) matched the mass of 28.7 kDa of the polypeptide deduced from the *expR*$_{153}$ sequence plus the mass of 50.84 kDa of MBP2-β-gal α fragment made from the pMAL-c2g. MBP-ExpR$_{153}$ was purified by using amylose resin affinity chromatography and used for gel mobility shift assay. Bandshift assay results (Fig. 5A) reveal that (i) both ExpR$_{71}$ and ExpR$_{153}$ bind the *rsmA*$_{71}$ and *rsmA*$_{153}$ DNA segments in a protein concentration-dependent manner; (ii) the binding affinity of ExpR$_{153}$ is greater than that of ExpR$_{71}$: for ExpR$_{71}$, 900 nM of protein is required to completely shift the band, whereas only 120 nM of ExpR$_{153}$ can affect complete band shift; and (iii) the excess of cold *rsmA*$_{71}$ and *rsmA*$_{153}$ DNA abolishes the shifted band, indicating that the bindings are specific.

Identification of sequences to which ExpR$_{71}$ and ExpR$_{153}$ bind. DNase I protection assays were performed to define the MBP-ExpR$_{71}$-*rsmA* and MBP-ExpR$_{153}$-*rsmA* binding sites. The lower strand of the *rsmA* upstream DNA fragment containing nucleotides (nt) −199 to +30 from the transcriptional start site T2 was specifically labeled with [γ-^{32}P]dATP and then incubated in the presence of various concentrations of purified MBP-ExpR$_{71}$ or MBP-ExpR$_{153}$. These MBP-ExpR$_{71}$-*rsmA* and MBP-ExpR$_{153}$-*rsmA* complexes were subjected to partial DNase I digestion and separated on 8% (wt/vol) polyacrylamide sequencing gels. The assay results (Fig. 6) revealed that a single 20-bp region spanning nt −57 to −38 from the transcriptional start site T2 was specifically protected by both MBP-ExpR$_{71}$ and MBP-ExpR$_{153}$. This 20-bp sequence is designated as the *expR* box (Fig. 7).

We have identified two *rsmA* transcriptional start sites by primer extension (data not shown). The first one (T1) is located 128 nt upstream from the putative translational start site as previously reported (13), and the second one (T2) is located 46 nt upstream from the putative translational start site (Fig. 7A). The 20-mer MBP-ExpR-*rsmA* binding site (*expR* box) is 2 bases before the putative −35 sequences of the second transcriptional start site (T2). To confirm the binding of MBP-ExpR$_{71}$ and MBP-ExpR$_{153}$ to *expR* box sequence, synthetic [α-^{32}P]dATP-labeled 20-mer *expR* box was used in gel mobility shift assays. The results presented in Fig. 5B and C revealed that both MBP-ExpR$_{71}$ and MBP-ExpR$_{153}$ bind the target DNA containing the *expR* box in a protein concentration-dependent manner, although the affinity of MBP-ExpR$_{153}$ for

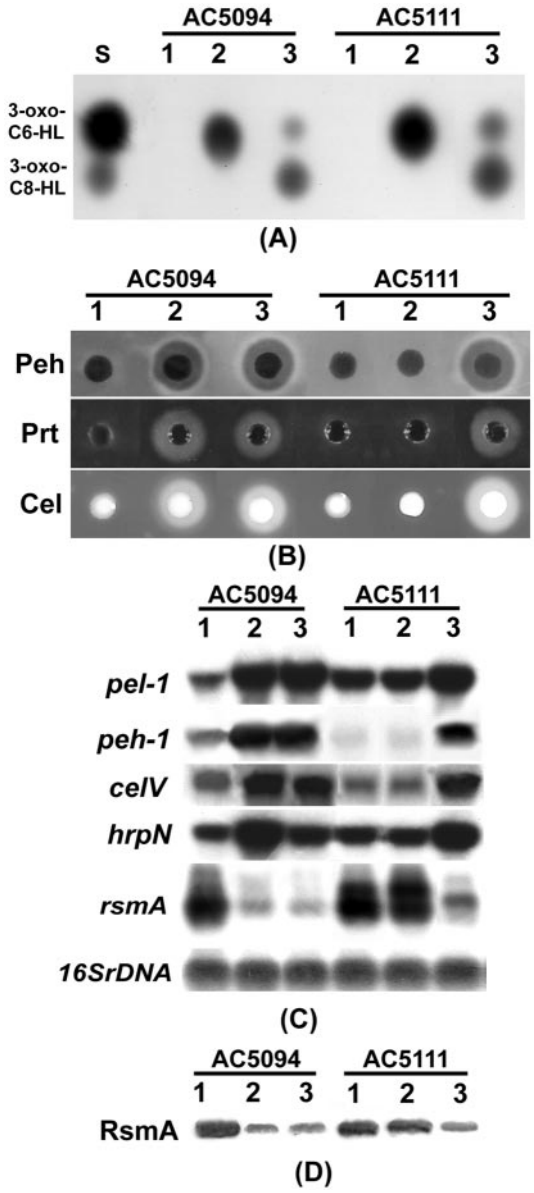

FIG. 4. (A) TLC analysis of AHLs. *E. coli* VJS533 harboring pHV200I is used as a biosensor indicator. Lanes: S, mixture of synthetic 3-oxo-C6-HL and 3-oxo-C8-HL (5 nmol of each loaded); 1 to 3, crude AHL extracts of AC5094 (AhlI mutant of Ecc71) and AC5111 (AhlI mutant of EC153) carrying pDK6 (vector), pAKC1201 ($ahlI_{71}$), and pAKC1222 ($ahlI_{153}$), respectively. (B) Agarose plate assays for Peh, Prt, and Cel activities. (C) Northern blot analysis of exoenzyme genes, *hrpN* and *rsmA*. (D) Western blot analysis of RsmA. For exoenzyme assays, 30 μl of culture supernatant was applied to each well. For Northern blot analysis, each lane contained 10 μg of total RNA, and for Western blot analysis, each lane contained 10 μg of total protein. Equal loading of RNA was checked by hybridization of the blot with a probe corresponding to 16S rRNA (rDNA). Lanes 1 to 3, AC5094 and AC5111 carrying pDK6, pAKC1201, and pAKC1222, respectively.

binding of the probe is greater than that of MBP-$ExpR_{71}$. The shifted band is abolished by addition of excess of cold annealed oligonucleotides, indicating that the bindings are specific. In contrast, a probe generated by annealing oligonucleotides containing part of the *expR* box (17-mer) does not bind MBP-$ExpR_{71}$ or MBP-$ExpR_{153}$ (data not shown). These results indicate that the 20-mer *expR* box DNA is essential for ExpR-*rsmA* binding.

TABLE 3. Expression of $rsmA_{71}$-*lacZ* and $rsmA_{153}$-*lacZ* fusions in *E. coli* in the presence of $expR_{71}$ or $expR_{153}$

Bacterial construct[a]	Relevant characteristic[b]	β-Galactosidase activity (Miller units)[c]
MC4100(pCL1920, pAKC1100)	Vector + $rsmA_{71}$-*lacZ*	620 ± 13
MC4100(pAKC936, pAKC1100)	$expR_{71}$ + $rsmA_{71}$-*lacZ*	1,905 ± 24
MC4100(pAKC937, pAKC1100)	$expR_{153}$ + $rsmA_{71}$-*lacZ*	7,465 ± 27
MC4100(pCL1920, pAKC1106)	Vector + $rsmA_{153}$-*lacZ*	715 ± 16
MC4100(pAKC936, pAKC1106)	$expR_{71}$ + $rsmA_{153}$-*lacZ*	2,222 ± 48
MC4100(pAKC937, pAKC1106)	$expR_{153}$ + $rsmA_{153}$-*lacZ*	7,761 ± 32

[a] Bacteria were grown at 28°C in LB agar supplemented with spectinomycin and tetracycline to a Klett value of ca. 250, and the whole cultures were used for the assay.
[b] The relevant genes carried by bacteria are given.
[c] Values are means ± standard deviations of three repetitions.

To further prove that the ExpR binds the *expR* box and activates transcription of *rsmA*, several PCR-amplified *rsmA* upstream DNA segments were cloned into the promoter probe vector, pMP220. The expression of these *rsmA-lacZ* fusions in MC4100 in the presence or absence of $expR_{71}^+$ and $expR_{153}^+$ DNAs was compared by assaying for β-galactosidase (Fig. 7B). In MC4100 carrying pAKC1100, pAKC1101, or pAKC1103, β-galactosidase levels were stimulated in the presence of $expR_{71}^+$ (pAKC936) or $expR_{153}^+$ (pAKC937) since all three fusions contain the 20-mer *expR* box sequences. pAKC1100 and pAKC1101 contain both transcriptional start sites (T1 and T2), whereas pAKC1103 contains only T2. $expR_{71}^+$ and $expR_{153}^+$ DNAs failed to stimulate the expression of *lacZ* in pAKC1102, which contains the *expR* box but lacks the transcriptional start site T2. pAKC1104, which does not contain the *expR* box, produced very low levels of β-galactosidase in the presence or absence of $expR_{71}^+$ or $expR_{153}^+$ DNAs. These results, particularly the observation that $expR_{71}$ and $expR_{153}$ stimulate the expression of pAKC1103, which contains the *expR* box and T2, strongly suggest that ExpR-activated *rsmA* transcription is initiated from the T2 start site.

Specificity of 3-oxo-C6-HL and 3-oxo-C8-HL on neutralization $ExpR_{71}$ and $ExpR_{153}$ effects. The data presented above demonstrate that both $ExpR_{71}$ and $ExpR_{153}$ bind $rsmA_{71}$ and $rsmA_{153}$ and activate the expression of $rsmA_{71}$-*lacZ* and $rsmA_{153}$-*lacZ* fusions. However, exogenous addition of 3-oxo-C6-HL (71AHL and 153AHL1) restored extracellular enzyme production in the AhlI mutant of Ecc71 but not in the EC153 AHL-deficient strain. In contrast, 3-oxo-C8-HL (153AHL2) restored exoenzyme production in the EC153 AHL-deficient strain but not in the AhlI mutant of Ecc71 (Fig. 2A; Table 2). To resolve this apparent paradox, we invoked the possibility that the two classes of ExpR species respond differently to specific AHL species. To test this hypothesis, we examined the effects of 71AHL, 153AHL1, and 153AHL2 on *expR*-mediated activation of *rsmA* expression in *E. coli* strain MC4100. The results of β-galactosidase assay in Table 4 revealed that in the presence of 71AHL or 153AHL1, expression of $rsmA_{71}$-*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), whereas 153AHL2 was totally

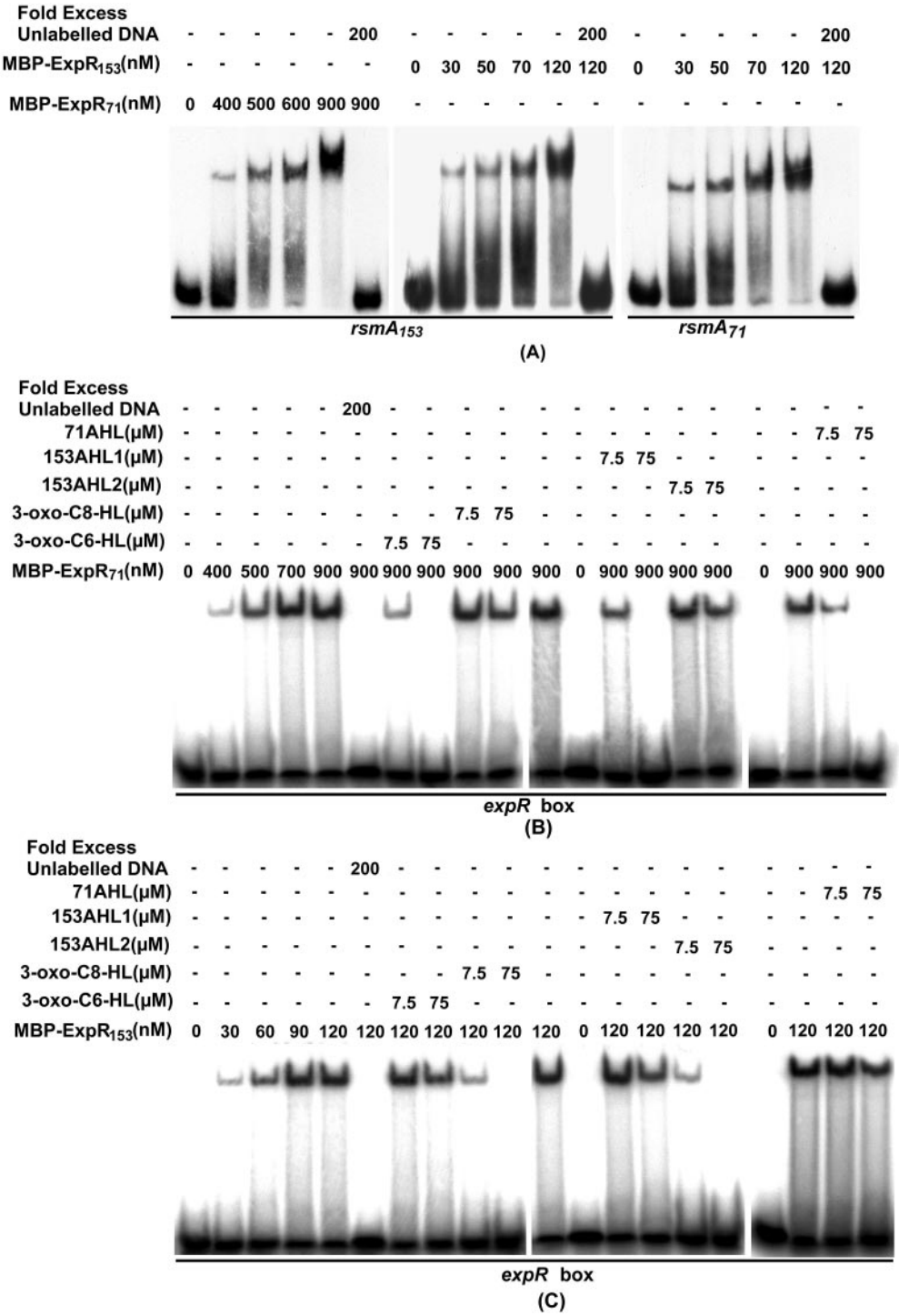

FIG. 5. Interactions of purified MBP-ExpR$_{153}$ and MBP-ExpR$_{71}$ proteins with (A) *rsmA*$_{71}$ and *rsmA*$_{153}$ DNAs and (B and C) *expR* box DNA. DNA fragments were end labeled with [α-^{32}P]dATP. Two nanograms of probe DNAs was added in each reaction. The amounts of proteins, unlabeled DNAs, and AHLs used in each reaction are indicated at the top of each figure.

ineffective in neutralizing the action of ExpR$_{71}$: i.e., the β-galactosidase level remained high. In contrast, expression of *rsmA*$_{71}$-*lacZ* in MC4100 carrying pAKC1100 and pAKC937 (*expR*$_{153}^{+}$ plasmid) was much reduced in the presence of 153AHL2, while 71AHL and 153AHL1 had little or no effect on expression of *rsmA*$_{71}$-lacZ in MC4100 carrying pAKC1100 and pAKC937 (Table 4). These results demonstrate that in *E. coli*, ExpR$_{71}$ and ExpR$_{153}$ stimulate the expression of *rsmA-lacZ* in the absence of AHL and the effects are neutralized in an AHL-specific manner. To eliminate the possibility of arti-

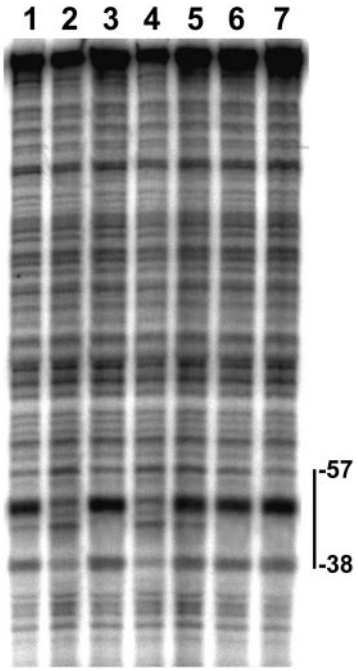

FIG. 6. DNase I protection analysis of the *rsmA* promoter DNA fragment by MBP-$ExpR_{71}$ and MBP-$ExpR_{153}$. A black bar indicates the nucleotide positions related to the transcriptional start site T2, which are protected from DNase I digestion by MBP-$ExpR_{71}$ and MBP-$ExpR_{153}$. In lane 2, no protein was added; lanes 1 and 3 contained 120 ng and 360 ng of MBP-$ExpR_{153}$; and lanes 4 to 7 contained 400 ng, 900 ng, 1,500 ng, and 1,800 ng of MBP-$ExpR_{71}$, respectively.

facts in *E. coli* studies, we examined the expression of *rsmA* in $ExpR^+$ and $AhlI^-$ strains of Ecc71 (AC5094) and EC153 (AC5111) in the presence of 71AHL, 153AHL1, or 153AHL2. The data in Fig. 2B and C (lane 1) show high levels of *rsmA* transcripts and RsmA protein are produced in both strains in the absence of AHL. However, in the presence of 71AHL or 153AHL1, *rsmA* transcript and RsmA protein levels were significantly reduced in the Ecc71 $ExpR^+$ $AhlI^-$ strain (AC5094; Fig. 2B and C, lanes 2 and 3). The 71AHL and 153AHL1 samples were only slightly effective in the EC153 $ExpR^+$ $AhlI^-$ strain (AC5111; Fig. 2B and C, lanes 2 and 3). Conversely, 153AHL2 was marginally effective in the Ecc71 $ExpR^+$ $AhlI^-$ strain (Fig. 2B and C, lane 4) but was most effective in the EC153 $ExpR^+$ $AhlI^-$ strain (Fig. 2B and C, lane 4). These results taken together with the effects of $ahlI^+$ DNAs in AhlI mutants of Ecc71 and EC153 (see above) demonstrate that (i) AHL neutralizes the effects of ExpR and (ii) neutralization of ExpR is AHL specific.

Differential effects of 3-oxo-C6-HL and 3-oxo-C8-HL on prevention of $ExpR_{71}$-$rsmA_{71}$ and $ExpR_{153}$-$rsmA_{71}$ bindings. Having established the specificity of 3-oxo-C6-HL and 3-oxo-C8-HL on neutralization of $ExpR_{71}$ and $ExpR_{153}$ effects, we examined the effects of those two AHL species on the interactions of MBP-$ExpR_{71}$ and MBP-$ExpR_{153}$ with the *expR* box DNA. Our results (Fig. 5B and C) show that (i) synthetic 3-oxo-C6-HL partially prevents the binding of MBP-$ExpR_{71}$ with the *expR* box DNA at a concentration of 7.5 μM and completely prevents the MBP-$ExpR_{71}$-*expR* box DNA binding at higher concentration (75 μM). (ii) Synthetic 3-oxo-C6-HL has no effect on MBP-$ExpR_{153}$-*expR* box DNA binding at low concentration (7.5 μM). A 75 μM concentration of 3-oxo-C6-HL has only a slight effect on MBP-$ExpR_{153}$-*expR* box DNA binding. (iii) In contrast, synthetic 3-oxo-C8-HL at a concentration of 7.5 μM partially prevents MBP-$ExpR_{153}$-*expR* box DNA binding but has no effect on MBP-$ExpR_{71}$-*expR* box DNA binding. Higher concentration (75 μM) of 3-oxo-C8-HL completely abolished the MBP-$ExpR_{153}$-*expR* box DNA binding but it only slightly affected the MBP-$ExpR_{71}$-*expR* box DNA interaction. (iv) Purified 71AHL and 153AHL1 behave exactly as synthetic 3-oxo-C6-HL and fractionated 153AHL2 like synthetic 3-oxo-C8-HL.

DISCUSSION

In this study, we have demonstrated that several members of *E. carotovora* subspecies, which are otherwise closely related, possess two classes of structurally and functionally distinct ExpR species. Strains containing these two classes of ExpR also differ in the sequences of AHL synthases, profiles of AHL analogs, and specificity in their interaction with ExpR species. The data shown here strongly suggest that AHL biosynthetic specificity mainly resides with AHL synthases and is not due to precursor availability or the lack of it. For example, the AhlI mutant of Ecc71 carrying the *ahlI* gene of class I strain EC153 produces 3-oxo-C8-HL and 3-oxo-C6-HL, AHL analogs similar to those produced by the EC153 wild type. Moreover, the AhlI mutant of EC153 carrying $ahlI_{71}$ produces only 3-oxo-C6-HL as the major AHL analog (Fig. 4A) and not both 3-oxo-C8-AHL and 3-oxo-C6-HL. These findings support the observations of Brader et al. (4) that the acyl-chain-length specificity of AHL depends on AHL synthases.

Class I strains produce 3-oxo-C8-HL as the major AHL analog and 3-oxo-C6-HL as a minor component. In this case, the major and minor designations are used to describe their relative abundance. The class II strains produce 3-oxo-C6-HL as the main and the only detectable AHL. However, the AHL profile, especially the composition of minor AHL components, may be variable, depending upon bacterial strains and growth conditions. This is supported by two recent reports documenting that soft-rotting *Erwinia carotovora* subspecies produce, in relatively low concentrations, an assortment of AHL species (see for example references 46 and 52). Brader et al. (4) found that *E. carotovora* subsp. *carotovora* strain SCC3193 produces 3-oxo-C8-HL as the main AHL species in LB medium or in planta. Our results, however, reveal that class I strains including SCC3193 produce relatively low but readily detectable levels of 3-oxo-C6-HL in addition to 3-oxo-C8-HL. This apparent contradiction may be attributed to differences in culture conditions and the use of different biosensors to identify the AHL analogs.

Amino acid residues of AHL synthases responsible for the AHL biosynthetic specificity have been previously identified by Chakrabarti and Sowdhamini (5). Comparison of AhlI sequences between class I and class II strains in light of their findings allows predictions of amino acid residues responsible for specificity in AHL synthesis. Specific residues of 3-oxo-C6-HL-producing synthases, such as Phe69, Tyr96, Phe102, Lys106, Pro118, Ile119, Val121, Leu123, Leu125, Ile142, Ile150, and Leu151, are present except for Val121 in class II *E. carotovora* strains, which produce only 3-oxo-C6-HL. On the other hand,

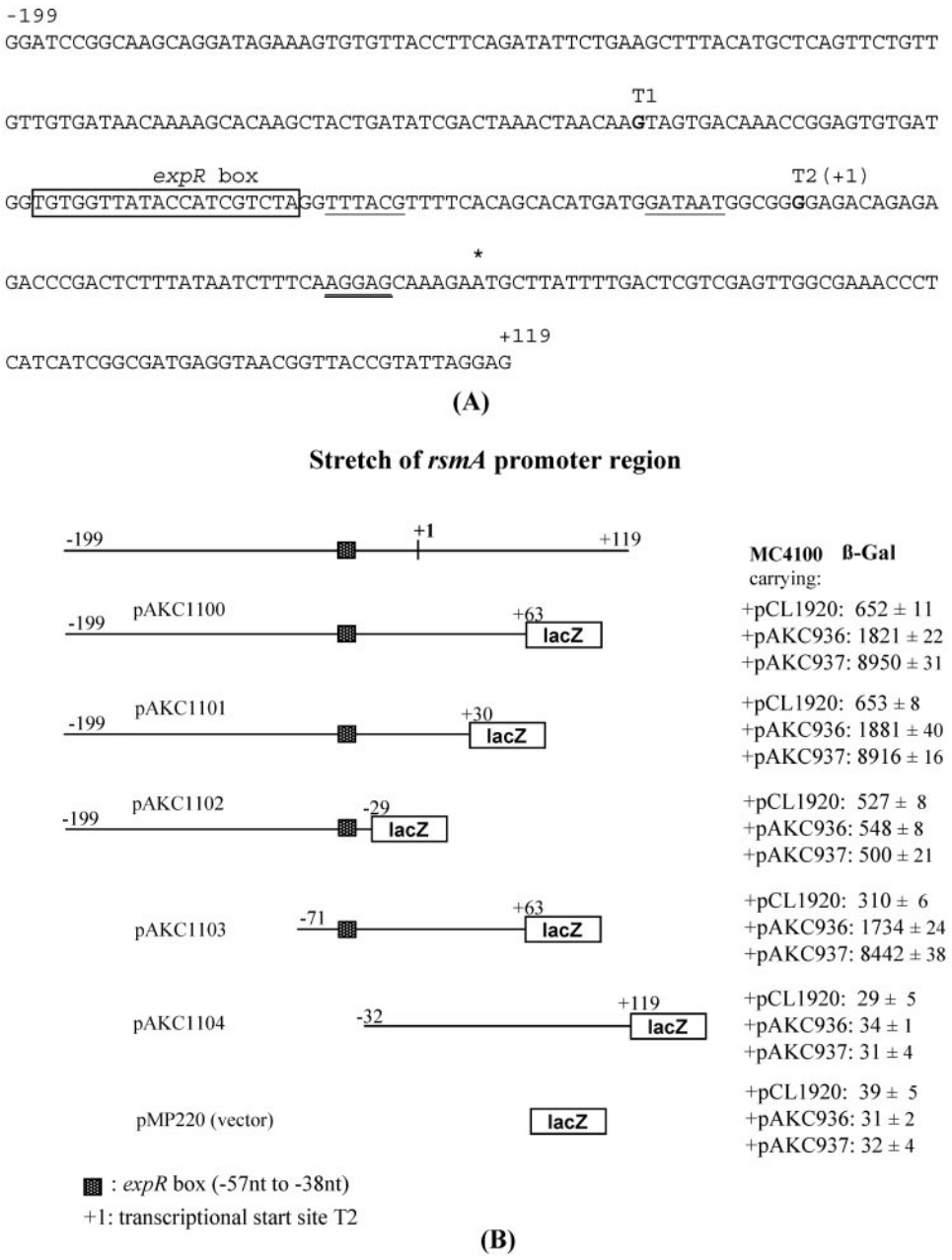

FIG. 7. (A) Nucleotide sequence of upstream region of $rsmA_{71}$. Two transcriptional start sites are indicated as T1 and T2 (+1), respectively. The *expR* box is marked. The putative −10 and −35 sequences are underlined, The Shine-Dalgarno site (SD) is double underlined, and the translational start site is indicated by an asterisk. (B) Expression of *rsmA-lacZ* fusions in *E. coli* strain MC4100 in the absence (pCL1920) or presence (pAKC936 and pAKC937) of the $expR_{71}^{+}$ and $expR_{153}^{+}$ DNAs. The numbers given for each construct refer to the bases from the transcriptional start site T2. *E. coli* contructs were grown at 28°C in LB agar plus drugs to a Klett value of ca. 300 for β-galactosidase assays. The values shown are the means ± standard deviations of three repetitions.

only Tyr96, Phe102, Lys106, Pro118, Leu125, Ile150, and Leu151 are present in class I *E. carotovora* strains, which produce 3-oxo-C8-HL and relatively low levels of 3-oxo-C6-HL, implying those seven residues determine the biosynthetic specificity of 3-oxo-C6-HL synthase. Among 12 of the possible 3-oxo-C8-HL synthase-specific residues (5), only 5 residues (Leu69, Thr111, Leu125, Ala126, and Thr127) are present in class I *E. carotovora* strains. Brader et al. (4) by mutational analysis identified the residue Met127 in $ExpI_{SCC1}$ (AHL synthase of *E. carotovora* subsp. *carotovora* strain SCC1, which produces 3-oxo-C6-HL) as critical for the determination of the substrate chain length. Introduction of mutagenized $expI_{SCC1}$ in which Met127 and Phe69 were replaced with Thr127 and Leu69, respectively, led to the production of 3-oxo-C8-HL. The AhlI sequence alignment (Fig. 3) shows that AHL synthases of class II strains possess Met127 and Phe69, whereas AHL synthases of all class I strains contain Thr127 and Leu69. These observations taken together with the results of Brader et

TABLE 4. Effects of $expR_{71}$ or $expR_{153}$ plasmids on the expression of $rsmA_{71}$-*lacZ* fusion in *E. coli* in the presence of 71AHL (3-oxo-C6-HL), 153AHL1 (3-oxo-C6-HL), or 153AHL2 (3-oxo-C8-HL)

Bacterial construct[a]	Relevant characteristic[b]	AHL	β-Galactosidase activity (Miller units)[c]
MC4100(pCL1920, pAKC1100)	Vector + $rsmA_{71}$-*lacZ*		548 ± 10
	Vector + $rsmA_{71}$-*lacZ*	+71AHL	535 ± 14
	Vector + $rsmA_{71}$-*lacZ*	+153AHL1	524 ± 15
	Vector + $rsmA_{71}$-*lacZ*	+153AHL2	534 ± 10
MC4100(pAKC936, pAKC1100)	$expR_{71}$ + $rsmA_{71}$-*lacZ*		1,801 ± 20
	$expR_{71}$ + $rsmA_{71}$-*lacZ*	+71AHL	584 ± 10
	$expR_{71}$ + $rsmA_{71}$-*lacZ*	+153AHL1	574 ± 15
	$expR_{71}$ + $rsmA_{71}$-*lacZ*	+153AHL2	1,922 ± 31
MC4100(pAKC937, pAKC1100)	$expR_{153}$ + $rsmA_{71}$-*lacZ*		6,250 ± 60
	$expR_{153}$ + $rsmA_{71}$-*lacZ*	+71AHL	5,406 ± 25
	$expR_{153}$ + $rsmA_{71}$-*lacZ*	+153AHL1	4,682 ± 21
	$expR_{153}$ + $rsmA_{71}$-*lacZ*	+153AHL2	1,790 ± 10

[a] Bacteria were grown at 28°C in LB agar supplemented with spectinomycin and tetracycline to a Klett value of ca. 100 and divided into four flasks. Three flasks were used for adding 71AHL, 153AHL1, or 153AHL2 (to a final concentration of 50 μM), respectively, and the other one was used as control (added water). After an additional 3 h of incubation at 28°C, cultures were used for assay.

[b] The relevant characteristics of the genes carried by bacteria are given.

[c] Values are means ± standard deviations of three repetitions.

al. (4) strongly suggest that Met127 and Phe69 are critical for producing 3-oxo-C6-HL and Thr127 and Leu69 are essential for making both 3-oxo-C6-HL and 3-oxo-C8-HL.

Sequence analysis disclosed that, like the LuxR family of proteins, ExpR possesses N-terminal (AHL binding) and C-terminal (helix-turn-helix [HTH] DNA binding) domains. It is significant that the C-terminal HTH DNA binding domains in both classes of ExpR are almost identical, suggesting that these may interact with similar, if not the same, DNA targets. Gel mobility shift assay data confirm that, in the absence of AHL, both $ExpR_{71}$ and $ExpR_{153}$ bind $rsmA_{71}$ and $rsmA_{153}$. Actually, sequence alignment of upstream DNAs of $rsmA_{71}$ and $rsmA_{153}$ demonstrates that the 20-mer *expR* box which is identified by DNase I protection upstream of $rsmA_{71}$ also occurs upstream of $rsmA_{153}$ with a 2-base difference in the 5′ end of the box. The gel mobility shift assay results show that the binding affinities of $ExpR_{153}$ with $rsmA_{71}$ and $rsmA_{153}$ are greater than those of $ExpR_{71}$. The expression of $rsmA_{71}$-*lacZ* and $rsmA_{153}$-*lacZ* is also higher with $ExpR_{153}$ than with $ExpR_{71}$. Although sequence comparisons of $ExpR_{71}$ and $ExpR_{153}$ reveal high homology between the HTH motifs of those two ExpRs, there are differences in six residues between them. Whether these residues are responsible for the observed differences awaits clarification.

N-terminal AHL binding domains, as opposed to the C-terminal HTH domains, are significantly different between the ExpRs of two classes. These differences are potentially significant in the context of specificity of interactions with AHL species. The evidence provided in this report demonstrates that $ExpR_{153}$ preferentially interacts with one of the AHL species (153AHL2; 3-oxo-C8-HL) it produces but poorly interacts with the other AHL species, which is also produced by Ecc71 (153AHL1 or 71AHL; 3-oxo-C6-HL). $ExpR_{71}$, on the other hand, interacts only with 71AHL (3-oxo-C6-HL) but not with 3-oxo-C8-HL. Specific interactions of $ExpR_{153}$ and $ExpR_{71}$ with 153AHL2 and 71AHL, respectively, lead to the inactivation of transcriptional activity of these ExpR species. This conclusion is supported by the following data: (i) 3-oxo-C6-HL prevents $ExpR_{71}$-*rsmA* binding, whereas 3-oxo-C8-HL inhibits $ExpR_{153}$-*rsmA* binding (Fig. 5); (ii) 3-oxo-C6-HL or 3-oxo-C8-HL specifically neutralizes the effects of $expR_{71}$ or $expR_{153}$ on expression of *rsmA-lacZ* fusion in *E. coli* (Table 4); (iii) $ahlI_{71}$ or $ahlI_{153}$ as well as exogenous additions of purified 3-oxo-C6-HL or 3-oxo-C8-HL specifically reduces the levels of *rsmA* transcript and RsmA protein (Fig. 2B and C and Fig. 4D).

Consistent with the effects of specific AHL analogs on the ExpR-*rsmA* binding and the ExpR-mediated activation of *rsmA* transcription is our finding that most of the pleiotropic effects of *expR* products in homologous system (i.e., $expR_{71}$ in class II strains and $expR_{153}$ in class I strains) are neutralized by AHL analogs produced in respective bacterial cells. In contrast, ExpR molecules are transcriptionally proficient in heterologous systems, activating RsmA production and concomitantly inhibiting exoprotein production (data not shown). In Ecc71 or class II strains, $ExpR_{153}$ would not interact with the 3-oxo-C6-HL these bacteria produce and ExpR consequently is predicted to occur predominantly as free $ExpR_{153}$. Likewise, in class I strains, $ExpR_{71}$ most likely does not interact with 3-oxo-C8-HL and should mostly occur as free $ExpR_{71}$.

It is now apparent that several *E. carotovora* subspecies use two AHL signaling systems to control gene expression. One comprising ExpR/AHL controls extracellular protein and secondary metabolite production. In this case, the primary target of regulation is *rsmA*, which specifies a global RNA regulator. The other, consisting of CarR/AHL, controls antibiotic production (32, 55). In this instance, AHL-CarR complex activates transcription of the *car* genes required for antibiotic biosynthesis. Thus, these bacteria possess two regulatory systems, both requiring AHL, to regulate gene expression. In the absence or in the presence of a low concentration of AHL, bacteria activate *rsmA* transcription, leading to the inhibition of secreted proteins and secondary metabolites, including antibiotics. As the AHL pool size increases, ExpR is inactivated, *rsmA* transcription is reduced, and expression of genes for exoproteins and secondary metabolites commences. Concomitantly, the AHL-CarR complex is assembled, leading to the activation of transcription of antibiotic biosynthetic genes. In this manner, *E. carotovora* subspecies uses AHL for global control as well as gene/operon-specific regulation.

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 J. E. Schoelz for critically reviewing the manuscript, P. Williams for providing 3-oxo-C8-HL, J. E. Loper for providing the strain Ecb168, E. T. Palva for providing the strain SCC3193, G. P. C. Salmond for providing the strain SCRI193, 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. **Bainton, N. J., P. Stead, S. R. Chhabra, B. W. Bycroft, G. P. C. Salmond, G. S. Stewart, and P. Williams.** 1992. *N*-(3-oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*. Biochem. J. **288:**997–1004.
3. **Barras, F., F. Van Gijsegem, and A. K. Chatterjee.** 1994. Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. Annu. Rev. Phytopathol. **32:**201–234.
4. **Brader, G., S. Sjöblom, H. Hyytiäinen, K. Sims-Huopaniemi, and E. T. Palva.** 2005. Altering substrate chain length specificity of an acylhomoserine lactone synthase in bacterial communication. J. Biol. Chem. **280:**10403–10409.
5. **Chakrabarti, S., and R. Sowdhamini.** 2003. Functional sites and evolutionary connections of acylhomoserine lactone synthases. Protein Eng. **16:**271–278.
6. **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.
7. **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.
8. **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.
9. **Collmer, A., and N. T. Keen.** 1986. The role of pectic enzymes in plant pathogenesis. Annu. Rev. Phytopathol. **24:**383–409.
10. **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.
11. **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.
12. **Cui, Y., A. Chatterjee, H. Hasegawa, V. Dixit, N. Leigh, and A. K. Chatterjee.** 2005. ExpR, a LuxR homolog of *Erwinia carotovora* subsp. *carotovora*, activates transcription of *rsmA*, which specifies a global regulatory RNA-binding protein. J. Bacteriol. **187:**4792–4803.
13. **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.
14. **Cui, Y., L. Madi, A. Mukherjee, C. K. Dumenyo, and A. K. Chatterjee.** 1996. The $RsmA^-$ mutants of *Erwinia carotovora* subsp. *carotovora* strain Ecc71 overexpress $hrpN_{Ecc}$ and elicit a hypersensitive reaction-like response in tobacco leaves. Mol. Plant-Microbe Interact. **9:**565–573.
15. **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.
16. **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.
17. **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.
18. **Fuqua, C., M. R. Parsek, and E. P. Greenberg.** 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. Annu. Rev. Genet. **35:**439–468.
19. **González, J. E., and M. M. Marketon.** 2003. Quorum sensing in nitrogen-fixing rhizobia. Microbiol. Mol. Biol. Rev. **67:**574–592.
20. **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.
21. **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.
22. **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.
23. **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.
24. **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.
25. **Köiv, 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.
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.** 1997. Activation of the *Erwinia carotovora* subsp. *carotovora* pectin lyase structural gene *pnlA*: a role for *rdgB*. Microbiology **143:**705–712.
29. **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.
30. **Liu, Y., G. 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.
31. **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.
32. **McGowan, S. J., M. Sebaihia, S. O'Leary, K. R. Hardie, P. Williams, G. S. Stewart, B. W. Bycroft, and G. P. Salmond.** 1997. Analysis of the carbapenem gene cluster of *Erwinia carotovora*: definition of the antibiotic biosynthetic genes and evidence for a novel beta-lactam resistance mechanism. Mol. Microbiol. **26:**545–556.
33. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, 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. 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-Microbe Interact. **4:**239–246.
42. **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. ASM Press, Washington, D.C.
43. **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.
44. **Salmond, G. P. C., J. C. D. Hinton, D. R. Gill, and M. C. M. Perombelon.** 1986. Transposon mutagenesis of *Erwinia* using phage λ vectors. Mol. Gen. Genet. **203:**524–528.
45. **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.
46. **Smadja, B., X. Latour, D. Faure, S. Chevalier, Y. Dessaux, and N. Orange.** 2004. Involvement of *N*-acylhomoserine lactones throughout plant infection

by *Erwinia carotovora* subsp. *atroseptica* (*Pectobacterium atrosepticum*). Mol. Plant-Microbe Interact. **17:**1269–1278.
47. **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.
48. **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.
49. **Taga, M. E., and B. L. Bassler.** 2003. Chemical communication among bacteria. Proc. Natl. Acad. Sci. USA **100:**14549–14554.
50. **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.
51. **Thomson, N. R., J. D. Thomas, and G. P. C. Salmond.** 1999. Virulence determinants in the bacterial phytopathogen *Erwinia*. Methods Microbiol. **29:**347–426.
52. **Toth, I. K., J. A. Newton, L. J. Hyman, A. K. Lees, M. Daykin, C. Ortori, P. Williams, and R. G. Fray.** 2004. Potato plants genetically modified to produce *N*-acylhomoserine lactones increase susceptibility to soft rot erwiniae. Mol. Plant-Microbe Interact. **17:**880–887.
53. **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.
54. **von Bodman, S. B., W. D. Bauer, and D. L. Coplin.** 2003. Quorum sensing in plant-pathogenic bacteria. Annu. Rev. Phytopathol. **41:**455–482.
55. **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.
56. **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 Leeuwenhoek Int. J. Gen. Mol. Microbiol. **81:**223–231.
57. **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.
58. **Zink, R. T., R. J. Kemble, and A. K. Chatterjee.** 1984. Transposon Tn*5* mutagenesis in *Erwinia carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica*. J. Bacteriol. **157:**809–814.