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Capsular Polysaccharide Biosynthesis and Pathogenicity in *Erwinia stewartii* Require Induction by an *N*-Acylhomoserine Lactone Autoinducer

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***N*-Acylhomoserine lactone (acyl-HSL)-mediated gene expression, also called autoinduction, is conserved among diverse gram-negative bacteria. In the paradigm *Vibrio fischeri* system, bioluminescence is autoinducible, and the *lux* operon requires the transcriptional activator LuxR and the acyl-HSL autoinducer for expression. The production of the acyl-HSL signal molecule is conferred by the *luxI* gene, and *luxR* encodes the transcriptional regulator. We show here that *Erwinia stewartii*, the etiological agent of Stewart's wilt of sweet corn, synthesizes an acyl-HSL. Mass spectral analysis identified the signal molecule as *N*-(-3-oxohexanoyl)-L-homoserine lactone, which is identical to the *V. fischeri* autoinducer. We have cloned and sequenced the gene that confers acyl-HSL biosynthesis, called *esaI*, and the linked gene, *esaR*, that encodes a gene regulator. The two genes are convergently transcribed and show an unusual overlap of 31 bp at their 3′ ends. Sequence analysis indicates that EsaI and EsaR are homologs of LuxI and LuxR, respectively. EsaR can repress its own expression but seems not to regulate the expression of *esaI*. The untranslated 5′ region of *esaR* contains an inverted repeat with similarity to the *lux* box-like elements located in the promoter regions of other gene systems regulated by autoinduction. However, unlike the other systems, in which the inverted repeats are located upstream of the −35 promoter elements, the *esaR*-associated repeat overlaps a putative −10 element. We mutagenized the *esaI* gene in *E. stewartii* by gene replacement. The mutant no longer produced detectable levels of the acyl-HSL signal, leading to a concomitant loss of extracellular polysaccharide capsule production and pathogenicity. Both phenotypes were restored by complementation with *esaI* or by exogenous addition of the acyl-HSL.**

Certain gram-negative bacteria produce signals in the form of *N*-acyl-substituted homoserine lactones (acyl-HSLs), also called autoinducers. These messengers enable the bacterial population to monitor its own density (quorum sensing) and to trigger a coordinate and unified response at the population level (quorum response) when presented with opportunities such as the potential to parasitize a host (for recent reviews, see references 5, 24, 34, and 51). For instance, the bioluminescent marine bacterium *Vibrio fischeri* colonizes the light organs of certain marine animals (reviewed in reference 43). The *lux* genes, which encode the enzymes for bioluminescence, are expressed only when the autoinducer signal, *N*-(-3-oxohexanoyl)-L-homoserine lactone (3-oxohexanoyl HSL) accumulates to a critical external concentration (18, 36). This occurs when the cells reach a high population density, and accumulation of the 3-oxohexanoyl HSL signal is believed to be one mechanism by which the bacteria sense that they are in the light organ. A single gene product encoded by *luxI* (21) is responsible for 3-oxohexanoyl HSL biosynthesis. Experimental evidence suggests that this autoinducer interacts with a cognate transcriptional regulator, LuxR, and that this signal-activator combination promotes the expression of the *lux* structural genes (1, 11, 22, 26).

Pathogenic bacteria also use acyl-HSL-mediated autoinduction to control the expression of virulence functions (24, 51). For instance, the human pathogen *Pseudomonas aeruginosa* secretes a number of extracellular factors required for invasion and infectivity. Expression of the genes encoding several of these traits is controlled by acyl-HSL-mediated activation (31, 38, 39). Similarly, *Erwinia carotovora*, which causes soft rot diseases on many plant species, produces an array of cell wall-degrading enzymes that enable the pathogen to colonize and live on plant tissue. The genes encoding these exoenzymes are also expressed coordinately in an acyl-HSL-dependent fashion (29, 42). In *Agrobacterium tumefaciens*, acyl-HSL-mediated autoinduction is required for the conjugal transfer of the resident Ti plasmid (23, 24, 41, 57). Since Ti plasmid-encoded functions govern the pathogenic potential of *A. tumefaciens*, the acyl-HSL-mediated dissemination of the Ti plasmid within the *Agrobacterium* population is a factor in promoting the interaction between the pathogen and its host plants. *Pseudomonas aureofaciens*, a rhizosphere bacterium, produces phenazine antibiotics which play a role in microbial competition and rhizosphere survival (40). Biosynthesis of these antibiotics occurs in a cell density-dependent manner and is regulated by a LuxR homolog, called PhzR (40), and an unidentified acyl-HSL (55).

The plant-pathogenic bacterium *E. stewartii* produces an extracellular heteropolysaccharide (EPS) capsule (16, 32) which plays several roles in the development of Stewart's wilt on sweet corn (10). The EPS provides a barrier that protects the pathogen against plant host defense factors (9, 10), partially contributes to the induction of water-soaking symptoms early in the development of Stewart's wilt (13), and obstructs the free flow of water in the host vascular system, causing necrosis and wilting during the systemic phase of the infection (10).

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Erwinia stewartii		
SS104	Wild-type pathogenic isolate	16
DC283	Nal^r derivative of SS104	16
ESVB51	*esaI*::Tn*5*seq1 homogenotized isolate of DC283	This study
Agrobacterium tumefaciens NT1(pJM749,pSVB33)	Indicator strain for detecting acyl-HSLs	41
Escherichia coli		
DH5α	F^- ϕ80d*lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 gyrA96* Δ(*lacZYA-argF*)	44
S17-1	RP4 Mob^+	47
JM109	F′ *lacI*^{q} Δ(*lacZ*)*M15* Δ(*lac-proAB*) *thi gyrA96 endA1 hsdR17* (r_K^- m_K^-) *relA supE44 traD36*	56
2174(pPH1JI)	*met pro* Gm^r Sp^r	8
MC1061	F^- *araD139* Δ(*araABC-leu*)*7679 galU galK* Δ(*lac*)*X74 rpsL thi*	35
Plasmids		
pUC18	Ap^r, ColE1 origin; cloning vehicle	56
pBluescript SK+	Ap^r, ColE1 origin; cloning vehicle	Stratagene
pDSK519	Km^r, IncQ	30
pRK415	Tc^r, IncP1α	30
pLAFR3	Tc^r, IncP1α	49
pES14-1	*Sau*3A-*Bam*HI cosmid clone, acyl-HSL^+	16
pLKC481	Source of *lacZY*-Km^r cassette	53
pES14-1XN51	*esaI*::*lacZY*-Km^r derivative of pES14-1	This study
pCM1	Source of *cat* cartridge	12
pSVB1	*Pst*I fragment from pES14-1 in pBluescript SK+; acyl-HSL^+	This study
pSVB2	*Pst*I fragment from pES14-1 in pLAFR3; acyl-HSL^+	This study
pSVB5-18	2.6-kb *Bam*HI-*Pst*I fragment in pUC18; acyl-HSL^+	This study
pSVB12-1	pSVB5-18; *esaI*::*cat* (t.o.)	This study
pSVB12-2	pSVB5-18; *esaI*::*cat* (r.o.)	This study
pSVB15-1	pSVB5-18; *esaR*::*lacZY* (t.o.)	This study
pSVB15-2	pSVB5-18; *esaR*::*lacZY* (r.o.)	This study
pSVB16	*Pst*I-*Eco*RI fragment from pSVB12-2 in pDSK519	This study
pSVB19	12-kb *Eco*RI fragment from pES14-1 in pRK415	This study

[a] Nal^r, nalidixic acid resistant; Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Tc^r, tetracycline resistant; Gm^r, gentamicin resistant; Sp^r, spectinomycin resistant; t.o., transcriptional orientation; r.o., reverse orientation.

During a general screen for acyl-HSL production by plant-pathogenic bacteria, we noticed that *E. stewartii* produces significant amounts of a factor or factors with autoinducer activity. Since other pathogens express extracellular virulence factors in an acyl-HSL-dependent fashion, we were interested in determining whether the production of EPS and virulence in *E. stewartii* are regulated by an acyl-HSL-mediated autoinduction system. We report here the isolation of an acyl-HSL from *E. stewartii* culture supernatants and its identification as 3-oxohexanoyl HSL. We located and determined the DNA sequence of a gene, called *esaI*, encoding the 3-oxohexanoyl HSL biosynthetic function. The amino acid sequence of EsaI is similar to that of other known proteins that are required for acyl-HSL biosynthesis. In addition, we determined the DNA sequence of a linked gene, called *esaR*, encoding a potential cognate transcriptional regulator that is related to the LuxR family of regulatory proteins. We further show that the disruption of *esaI* blocks the biosynthesis of 3-oxohexanoyl HSL and any other detectable acyl-HSL signal molecules. This deficiency suppresses EPS production and abolishes the capacity of *E. stewartii* to induce Stewart's wilt disease in the plant host.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and reagents. Strains of *E. stewartii*, *Escherichia coli*, and *A. tumefaciens* used in this study are listed in Table 1. Cultures of *E. stewartii* and *A. tumefaciens* were grown in L broth (44) or on nutrient agar plates (Difco Laboratories, Detroit, Mich.) at 28°C. *E. coli* strains were grown on the same medium at 37°C. AB medium (41) supplemented with 0.2% mannitol or 0.2% glucose was used as the minimal liquid and solid medium to grow *A. tumefaciens* and *E. stewartii* strains. *E. coli* cells were grown on M9 liquid minimal medium with glucose (44). Where applicable, antibiotics were added at the following concentrations: for *A. tumefaciens*, carbenicillin at 100 μg/ml and kanamycin at 50 μg/ml; for *E. stewartii*, nalidixic acid at 30 μg/ml, tetracycline at 15 μg/ml, kanamycin at 20 μg/ml, and gentamicin at 30 μg/ml; and for *E. coli*, tetracycline at 10 μg/ml, ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, and chloramphenicol at 30 μg/ml.

Biological assay to detect 3-oxohexanoyl HSL production. The assay used was described previously in detail (28, 41). The indicator strain, *A. tumefaciens* NT1(pJM749,pSVB33), contains a *lacZ* fusion in a *tra* gene of pTiC58 carried on pJM749 (7). Plasmid pSVB33 encodes TraR (41), a transcriptional activator that activates *tra* in the presence of any one of several acyl-HSLs, including 3-oxooctanoyl HSL (41, 57). AB-mannitol minimal agar plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase; 40 μg/ml) were seeded with the indicator strain. Crude or partially purified culture supernatants containing active acyl-HSLs, or colonies of bacteria capable of producing these signal molecules, generate a blue zone at the site of application when the plates are incubated at either 28 or 37°C.

Isolation, purification, and characterization of 3-oxohexanoyl HSL. A culture of wild-type *E. stewartii* DC283 was grown for 48 h in 3 liters of AB-glucose medium, and the cells were removed by centrifugation. The supernatant was extracted twice with equal volumes of ethyl acetate, and the organic phase was collected and taken to dryness by rotary vacuum evaporation at 43°C. The resulting residue was redissolved in 5 ml of ethyl acetate and applied to a silica gel column (2.5 by 40 cm) (Aldrich Silica Gel Merck grade 10180, 70/230 mesh). The column was eluted with ethyl acetate, and fractions with biological activity were pooled and evaporated to dryness. This residue was redissolved in methanol-H_2O (30:70, vol/vol), and the activity was further purified by reverse-phase column chromatography (1 by 30 cm), using bonded phase-octadecyl (C_{18}) silica gel, 40-μm average particle size, obtained from Baker Chemical Co., Phillipsburg, N.J. Isocratic elution with methanol-H_2O (30:70, vol/vol) yielded biologically active fractions that were pooled and evaporated to dryness. This preparation was submitted to the Mass Spectrometry Facility at the University of Illinois for mass spectral (MS) analysis using electron spray fast atom bombardment (FAB) and tandem MS to characterize the compound.

Recombinant DNA methods and DNA sequence analysis. Plasmid DNA was isolated by the alkaline lysis method (44) and, when desired, was further purified by using Qiagen columns (Qiagen, Inc., Chatsworth, Calif.). Restriction digestions and DNA ligations were performed according to the manufacturers' specifications. Enzymes were purchased from GIBCO BRL (Gaithersburg, Md.) or Boehringer Mannheim (Indianapolis, Ind.). DNA fragments were sequenced by the dideoxy method, using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Co., Cleveland, Ohio). DNA oligonucleotide primers were obtained from the Genetic Engineering Facility at the University of Illinois. DNA sequences were analyzed with the DNA Strider program (33), Blast protocols (2) for database searches, and the GCG software package from Genetics Computer Group Inc. (Madison, Wis.) to generate amino acid sequence alignments and to estimate relatedness of proteins.

Transposon mutagenesis and genomic replacement. The 3-oxohexanoyl HSL-active cosmid clone pES14-1 was mutagenized with transposon Tn*5*seq1 (35). Strain MC1061(pES14-1) was infected with λ::Tn*5*seq1, and isolates containing transposition events were selected on nutrient agar containing neomycin (15 μg/ml) and tetracycline (10 μg/ml). Colonies growing on this medium were pooled and grown in liquid culture with neomycin and tetracycline. Total plasmid DNA was isolated from the collected cells and used to transform *E. coli* DH5α. Transformants that were resistant to kanamycin and tetracycline contained Tn*5*seq1 insertions in pES14-1 as confirmed by restriction endonuclease analysis. Sites of transposon insertions were mapped by *Bam*HI and *Pst*I restriction analysis. Plasmids were mobilized into *E. stewartii* strains, and genomic replacement was forced by introducing into the resulting merodiploid strains the plasmid pPH1JI, which is incompatible with the cosmid vector. Proper marker exchanges were identified by antibiotic resistance phenotype and confirmed by restriction endonuclease digestions of total genomic DNA and Southern hybridization using a Tn*5*seq1-specific DNA fragment as a probe.

Reporter fusion construction. The *esaI* gene contained on pSVB5-18 was disrupted by inserting into the unique *Sal*I restriction site a chloramphenicol acetyltransferase gene (*cat*) cassette, excised as a *Sal*I fragment from plasmid pCM1 (12). Plasmids were isolated with the cassette in both orientations and were designated pSVB12-1 for the transcriptional orientation and pSVB12-2 for the reverse orientation. A fusion between *esaR* and *lacZ* was constructed by excising the *lacZY* cassette from pLKC481 (53) with *Xma*I to generate compatible ends for cloning into the unique *Mro*I site located in *esaR*. In the transcriptional orientation, this insertion generated a translational fusion, and the resulting plasmid was designated pSVB15-1, while the plasmid with the cassette inserted in the reverse orientation was designated pSVB15-2. To separate the *esaR* gene from the contiguous and overlapping *esaI* gene, we took advantage of a unique *Eco*RI site provided by the *cat* cassette located in *esaI* of pSVB12-2. This allowed us to clone *esaR* as a *Pst*I-*Eco*RI fragment into pDSK519 to generate pSVB16.

β-Galactosidase assays. Cells were grown in M9 minimal medium, diluted to an optical density at 600 nm (OD_{600}) of 0.05, and allowed to grow to an OD_{600} of 0.6. At this point, 1 ml of culture was removed and assayed for β-galactosidase activity by the procedure of Stachel et al. (48).

CAT assay. Cultures prepared in M9 minimal medium as described for the β-galactosidase assays were supplemented with partially purified 3-oxohexanoyl HSL 1 h after dilution. Incubation was continued, and samples were removed when the cultures reached OD_{600} values of 0.2 and 0.6. Cells were lysed by sonication, and total protein concentrations were determined by using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, Calif.). Levels of CAT in cell-free lysates were measured by using the CAT enzyme-linked immunosorbent assay kit marketed by 5 Prime→3 Prime, Inc., as recommended by the manufacturer.

Detection of EPS biosynthesis. Strains of *E. stewartii* were grown on CPG agar (1% glucose, 1% tryptone, 0.1% Casamino Acids, 1.5% agar) (10) at 28°C. Colonies producing EPS have a slimy, fluidal, mucoid appearance, while those deficient in EPS have a distinct nonmucoidal, creamy colony morphology.

Pathogenicity assays. Seedlings of the highly susceptible sweet corn hybrid "Platinum Lady" (50) were inoculated with cell suspensions grown in AB-glucose minimal medium containing the appropriate antibiotics. Leaf tissue was inoculated with a device consisting of fine needles held in a bunch. The inoculation device was dipped into the bacterial suspension, and leaves were lightly pricked to deposit the inoculum. When required, strain ESVB51 was preinduced by growing a 1-ml culture in the presence of 100 μl of semipure, sterile 3-oxohexanoyl HSL. The acyl-HSL was also applied to inoculation sites at daily intervals. As a negative control, sham-inoculated leaves were treated with daily applications of 3-oxohexanoyl HSL. Plants were maintained either in a greenhouse under ambient conditions or in a controlled environment growth chamber at 29°C, high humidity, and 16-h light/8-h dark cycle.

Nucleotide sequence accession numbers. DNA sequences were deposited in the GenBank database under accession numbers L32183 for *esaI* and L32184 for *esaR*.

RESULTS

Detection and characterization of 3-oxohexanoyl HSL activity in culture supernatants of *E. stewartii* DC283. Culture supernatants of wild-type *E. stewartii* DC283 produced large, diffuse zones when spotted onto acyl-HSL indicator plates. This observation suggested that *E. stewartii* produces an acyl-HSL related to the *A. tumefaciens* signal, 3-oxooctanoyl HSL. We purified this active substance from spent culture supernatants of *E. stewartii* DC283 by ethyl acetate extraction, silica gel column chromatography, and C_{18} reverse-phase chromatography. Electron spray FAB MS of the purified sample yielded a strong quasimolecular (M + H) ion of 214 *m*/*z*. FAB tandem MS of the *m*/*z* 214 peak generated daughter ions characteristic of 3-oxohexanoyl HSL, including the M + H ions *m*/*z* 102 for the protonated homoserine lactone species and *m*/*z* 113 for the 3-oxohexanoyl moiety (Fig. 1). Thus, the *E. stewartii* factor is identical to the 3-oxohexanoyl HSL autoinducers produced by *V. fischeri* (19) and *E. carotovora* (29).

Localization and subcloning of the gene encoding 3-oxohexanoyl HSL biosynthesis. We screened a *Sau*3A-partial genomic cosmid library of *E. stewartii* SS104 in pLAFR3 (16) maintained in *E. coli* JM109 to identify clones that confer biosynthesis of the *E. stewartii* acyl-HSL to *E. coli*. Pools of 20 *E. coli* JM109 colonies, each harboring an individual cosmid clone, were patched onto indicator plates. Two pools with acyl-HSL activity were identified. Subsequent single-colony purifications yielded two separate isolates that contained cosmids which when analyzed had nearly identical DNA restriction patterns. We focused on one of these isolates and characterized the cosmid clone, designated pES14-1, by restriction analysis (data not shown). A 12-kb *Eco*RI fragment of pES14-1, when subcloned into pRK415, yielded a clone, designated pSVB19 (Fig. 2A), that conferred on *E. coli* production of a factor with acyl-HSL activity. Thin-layer chromatographic analysis of ethyl acetate extracts from culture supernatants of this strain is consistent with the cloned DNA fragment encoding the production of 3-oxohexanoyl HSL (46). This fragment contains three internal *Pst*I fragments. A subclone containing the 6.0-kb *Pst*I fragment, designated pSVB1, also conferred production of the 3-oxohexanoyl HSL. The smallest fully functional subclone, pSVB5-18, contains the 2.6-kb *Bam*HI-*Pst*I fragment in pBluescript SK^+ (Fig. 2).

DNA sequence analysis. Dideoxy DNA sequence analysis of a 1,807-bp segment of pSVB5-18 showed two open reading frames (ORFs) (Fig. 2). ORF1 begins with an ATG at bp 167 and terminates at bp 800 with two stop codons, TGA and TAA. This ORF can encode a 210-amino-acid protein of 23,829 Da. ORF2, which is translatable in the opposite direction, begins with an ATG at bp 1520 and terminates with two stop codons (TAG and TGA) at bp 768. It can encode a protein of 251 amino acids with a mass of 28,085 Da. The two ORFs are oriented convergently, and they show an unusual overlapping region of 31 bp at their 3′ ends. The initiation codon of ORF1 is preceded by a typical ribosomal binding site, AGAGG, located seven nucleotides upstream. Potential promoter sequences for this ORF are located upstream of the *Hin*dIII site (bp 105) and include a −10 region (AATAAT) beginning with bp 77 and a −35 region (TTGACA) at bp 46. The translation initiation codon for ORF2 is preceded by a ribosomal binding site sequence (TGGAG), a −10 hexamer (TACTAT) at bp 1569, and a −35 region (TTGTAA) at bp 1601. In addition, a 20-nucleotide imperfect inverted repeat (ACCTGCACTAT AGTACAGGC) spans the −10 region. This repeat shows 15-of-20 nucleotide identity with the canonical *lux* box sequence (15, 24).

A search of the GenBank database with the amino acid sequence of ORF1 revealed several protein homologs with known acyl-HSL biosynthetic functions. Pairwise alignments of these indicate that EagI (52) is 60%, CarI (52) and YenI (GenBank accession number X76082) are 45%, ExpI (42) is

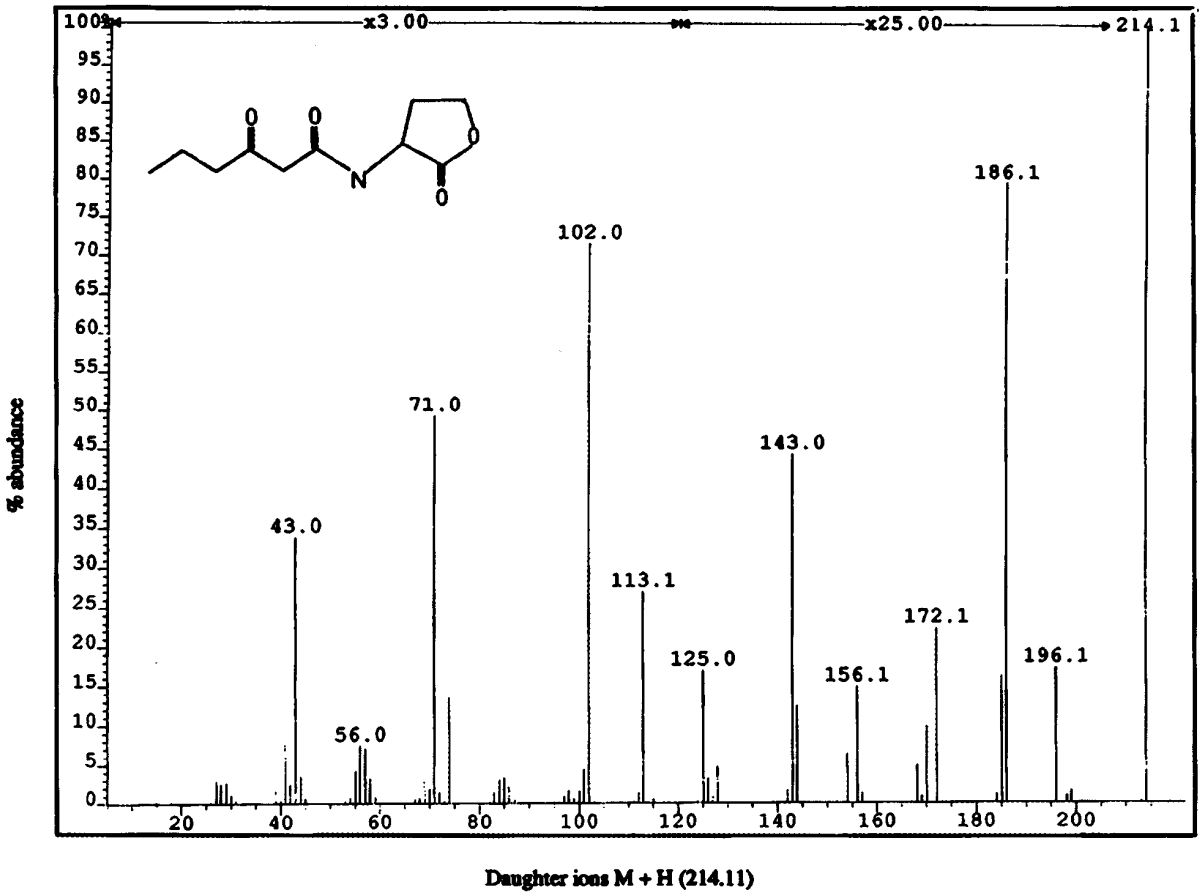

FIG. 1. MS analysis of a biologically active component purified from culture supernatants of *E. stewartii* DC283. FAB tandem MS of the *m/z* = 214 molecular component produced molecular ions of *m/z* 102 and *m/z* 113 characteristic of 3-oxohexanoyl HSL (19).

43%, TraI (23, 28) and LuxI (4, 22) are 25%, LasI (39) is 24%, and PhzI (GenBank accession number L33724) is 19% identical to the protein encoded by ORF1 (Fig. 3A). Since pSVB5-18 confers production of 3-oxohexanoyl HSL and ORF1 could encode a protein related to other acyl-HSL biosynthetic determinants, we designate ORF1 as the *E. stewartii* gene *esaI*. Sequence comparison indicated that the protein encoded by ORF2 is related to the LuxR family of regulatory proteins, which include LasR (25) and PhzR (40) with 27%, LuxR (4, 15, 22) with 24%, RhiR (14) with 23%, and TraR (23, 41) with 21% identity (Fig. 3B). We designate ORF2 as the *E. stewartii* gene *esaR*, encoding a potential 3-oxohexanoyl HSL-responsive transcriptional regulator.

EsaR regulates *esaR*, but *esaI* does not require EsaR for expression. The term autoinduction describes a system in which acyl-HSLs produced by the bacteria accumulate in the surrounding environment. When these signal molecules reach a critical concentration, the bacteria respond by inducing the expression of the appropriate gene systems (18, 20, 36, 37). We approached the question of whether expression of *esaI* requires EsaR and the acyl-HSL signal by constructing a translational fusion between *lacZ* and *esaR* and a transcriptional fusion between a *cat* cassette and *esaI*. These gene fusions were created in plasmid pSVB5-18, which contains a wild-type insert encoding both *esaR* and *esaI* (Fig. 4). The resulting *esaR* fusion constructs were designated pSVB15-1, with the *lacZ* fusion in the transcriptional orientation, and pSVB15-2, with the *lacZ* fusion in the reverse orientation (Fig. 4). The *esaR*::*lacZ* fusion on pSVB15-1 was expressed at high levels in the absence of a functional *esaR* gene, but this expression decreased 17-fold when wild-type *esaR* was provided in *trans* on pSVB16 (Table 2). This result suggests that *esaR* is self-regulated by repression. The disruption of *esaR* had no effect on 3-oxohexanoyl HSL biosynthesis encoded by the *esaI* gene on the same plasmid. *E. coli* strains carrying these plasmids produced blue zones on indicator plates indistinguishable in size and intensity from those produced by the strain containing the parent plasmid pSVB5-18 (data not shown).

E. coli strains carrying the *esaI*::*cat* gene fusion either on pSVB12-1 (transcriptional orientation) or pSVB12-2 (reverse orientation) failed to produce detectable levels of 3-oxohexanoyl HSL. Strains harboring pSVB12-1 produced roughly 8 μg of CAT per mg of protein regardless of cell density (OD_{600} of 0.2 versus 0.6), preincubation with exogenous 3-oxohexanoyl HSL, or presence of multiple copies of *esaR* expressed from pSVB16 in *trans* (Table 3). These results suggest that expression of *esaI* is not regulated by EsaR.

Transposon mutagenesis and genomic replacement to generate an *esaI* mutant of *E. stewartii* DC283. We disrupted the genomic *esaI* locus of *E. stewartii* to determine if 3-oxohexanoyl HSL controls the expression of essential virulence functions. The cosmid clone, pES14-1, was mutagenized with Tn*5*seq1 (35). Several kanamycin-resistant insertion mutants did not produce detectable levels of 3-oxohexanoyl HSL. The insertions in these mutant plasmids mapped to the region encoding *esaI*. One such plasmid, pES14-1XN51 (Fig. 2A), was used to

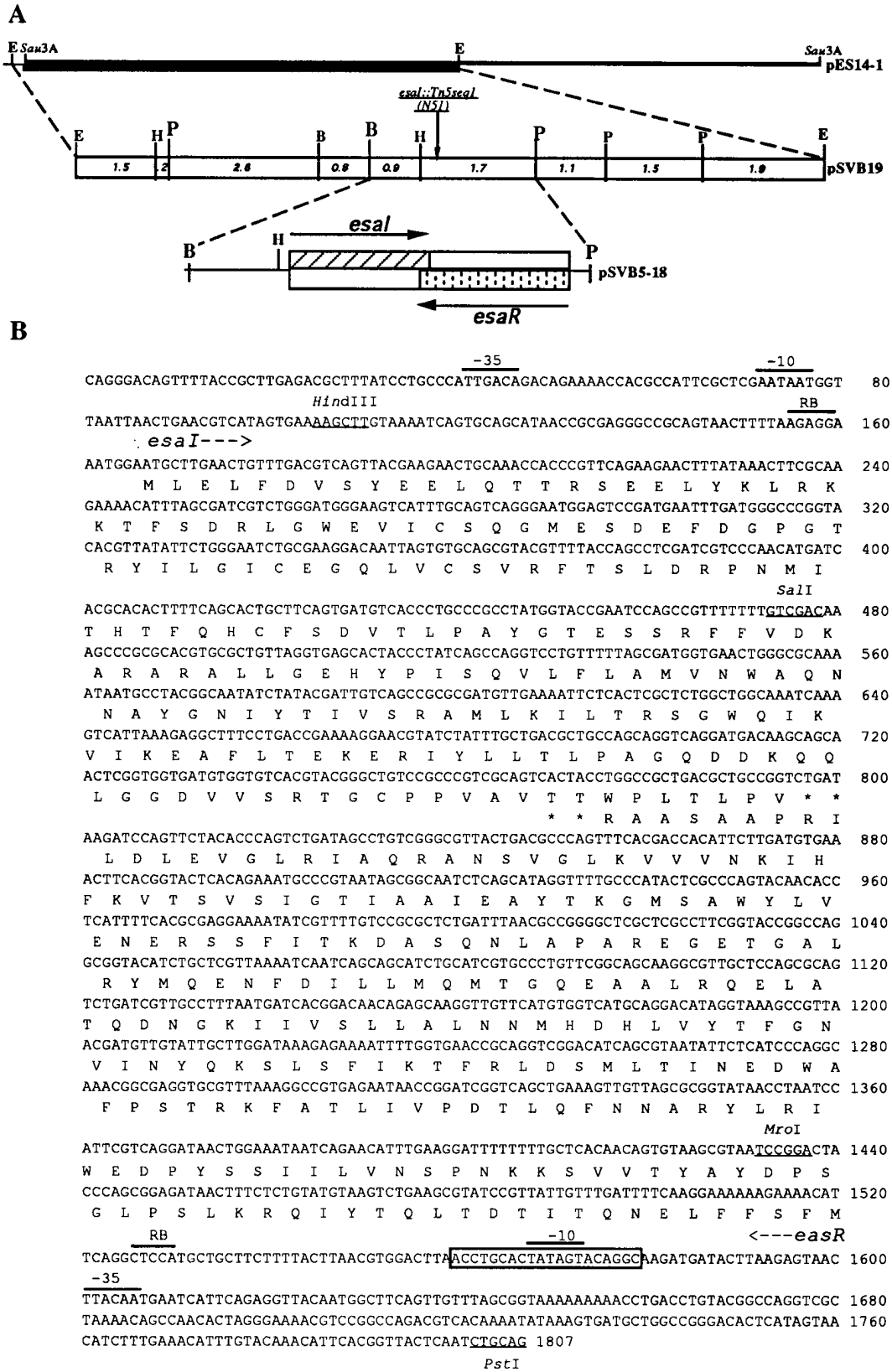

FIG. 2. (A) Physical map of the 12-kb *Eco*RI fragment contained within cosmid clone pES14-1. The locations and convergent gene organization of *esaI* and *esaR* are indicated. The map position of the Tn*5*seq1 insertion N51 in *esaI* is indicated by a vertical arrow. (B) DNA and predicted amino acid sequences of *esaI* and *esaR* contained on the 2.6-kb *Bam*HI-*Pst*I fragment of pSVB5-18. The transcriptional polarities of *esaI* and *esaR* are indicated by the arrows (note that the coding sequence for *esaR* is the complement of that shown). Predicted ribosomal binding sites (RB) and putative promoter elements are overlined, and pertinent restriction sites are underlined. An inverted repeat showing strong sequence similarity to the *lux* box consensus sequence and that overlaps the −10 region of *esaR* is boxed. Abbreviations: H, *Hin*dIII; P, *Pst*I; B, *Bam*HI.

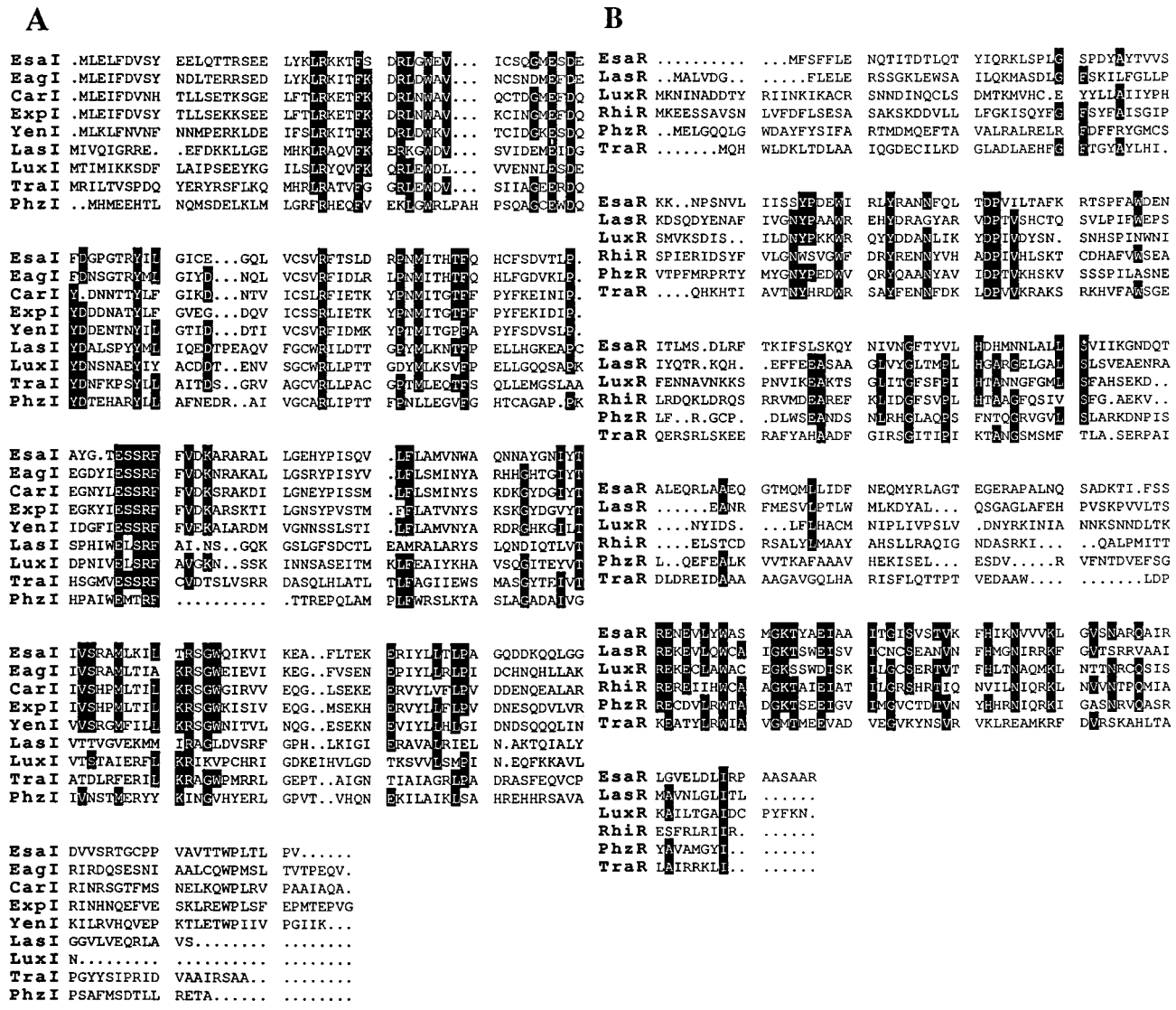

FIG. 3. Relatedness of proteins translatable from the ORFs encoded by pSVB5-18. (A) Alignment of EsaI with LuxI and other homologs. (B) Alignment of EsaR with LuxR and other known homologs. Identical amino acids are indicated by white letters on black background.

mutate the wild-type allele in *E. stewartii* DC283 as described in Materials and Methods. Southern analysis of genomic DNA and also total plasmid DNA isolated from candidate mutant derivative strains of DC283 verified the proper genomic replacement and also confirmed that *esaI* is a chromosomal locus and is not plasmid-borne (data not shown). One confirmed homogenote, designated ESVB51, did not produce detectable levels of 3-oxohexanoyl HSL when assayed on the *Agrobacterium* indicator plates. Production of 3-oxohexanoyl HSL could be restored by expressing in *trans* a wild-type copy of *esaI* encoded on pSVB2 (data not shown).

EPS biosynthesis requires 3-oxohexanoyl HSL. Synthesis of EPS is the primary determinant of pathogenicity in *E. stewartii* (9, 10). We tested the *esaI* mutant strain ESVB51 on medium (10) that stimulates capsular polysaccharide synthesis. The wild-type strain DC283 and the *trans*-complemented mutant strains ESVB51(pSVB2) and ESVB51(pES14-1) all display a mucoid phenotype resulting from the production of capsular polysaccharide. In contrast, the *esaI* mutant strain ESVB51 exhibits a distinct nonmucoid, creamy colony morphology at the distal end of the streak but becomes progressively more mucoid the closer it grows to the 3-oxohexanoyl HSL-producing wild-type strain on the plate (Fig. 5). When assayed on separate plates, the *esaI* mutant is completely nonmucoid (data not shown). Moreover, when the CPG medium was supplemented with exogenous 3-oxohexanoyl HSL, the *esaI* mutant produced colonies that were phenotypically EPS^+ (data not shown).

The 3-oxohexanoyl HSL-deficient strain ESVB51 is nonpathogenic. We conducted pathogenicity tests on the sweet corn variety "Platinum Lady," which is highly susceptible to *E. stewartii* (50). *E. stewartii* mutant strain ESVB51 when inoculated to this corn variety does not produce lesions on the plant host, while the wild-type strain DC283 and the mutant strain complemented with pSVB2 produce typical symptoms of Stewart's wilt (Fig. 6). Strain ESVB51 harboring the pLAFR3 vector alone did not produce symptoms (data not shown). Extensive water-soaking and chlorosis did result when ESVB51 was preincubated with 3-oxohexanoyl HSL prior to inoculation and exogenous 3-oxohexanoyl HSL was added at daily intervals

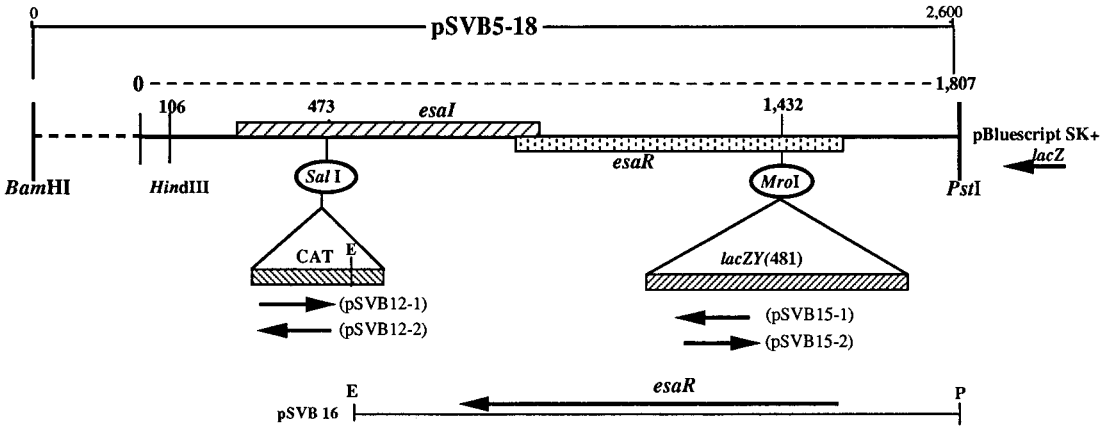

FIG. 4. Organization of the *E. stewartii esaI-esaR* loci of pSVB5-18 and the locations of reporter fusions. The dashed line indicates the 1,807-bp region that was sequenced. Insertion orientations are indicated by arrows. Plasmids shown in parentheses contain reporter fusions with transcriptional orientations indicated by the arrows. The map also shows the location of the *Eco*RI site used to construct pSVB16.

over the duration of the infection (Fig. 6). However, the pronounced wilting and necrosis characteristic of infections with the wild-type parent did not occur. Treatment of sham-inoculated leaf tissue with exogenous 3-oxohexanoyl HSL did not elicit any detectable plant response (data not shown).

DISCUSSION

Autoinduction mediated by acyl-HSL signal molecules appears to be a common form of gene regulation among plant-associated gram-negative bacteria (24, 51). We show in this report that the pathogen *E. stewartii* regulates the expression of traits involved in pathogenesis by an acyl-HSL-mediated signal pathway. We have identified linked chromosomal genes from this bacterium designated *esaI* and *esaR*. EsaR is a member of the LuxR family of regulatory proteins that mediate gene expression by autoinduction. EsaI is a member of a family of proteins that confer the synthesis of acyl-HSLs varying only in length and hydrophobicity of their constituent acyl side chains (3, 19, 28, 31, 39, 57). EsaI is responsible for the production of 3-oxohexanoyl HSL, an autoinducer identical in structure to those produced by other members of the family *Enterobacteriaceae*, including *V. fischeri*, *E. carotovora*, and *Enterobacter agglomerans* (19, 24, 51, 52).

In spite of the sequence similarities, the autoinduction system in *E. stewartii* seems to differ from those in other organisms. First, the organization of the two regulatory genes is considerably different. In *E. stewartii*, *esaR* and *esaI* are closely linked and convergently transcribed, with the two genes sharing sequence overlap at their 3′ ends (Fig. 2B). This contrasts organisms in which the genes for the transcriptional regulator and the production of the acyl-HSL signal either are linked and divergently transcribed (22, 38) or are completely unlinked (23, 28). Second, *esaI* appears to be expressed constitutively and apparently is not regulated by EsaR and 3-oxohexanoyl HSL (Table 3). In contrast, the expression of *luxI* in *V. fischeri*, *traI* in *A. tumefaciens*, and *lasI* in *P. aeruginosa* depends on their cognate gene activators LuxR (21), TraR (23, 28), and LasR (45), respectively, and the appropriate autoinducer signal.

Our genetic analyses indicate that EsaR can autorepress its own expression. However, our experiments were conducted with high-copy-number vectors, so these results must be viewed with some caution. Nonetheless, LuxR was observed to autorepress expression of its own gene under similar high-copy-number conditions (17). Interestingly, the promoter region of *esaR* contains a region of dyad symmetry that closely resembles the canonical *lux* box (Fig. 2). The role of this element in regulating expression of *esaR* is not known. However, this *lux* box-like

TABLE 2. *esaR* expression is autorepressed by EsaR

E. coli reporter strain	Reporter orientation[a]	β-Galactosidase activity (U/OD_{600})[b]	
		−pSVB16[c]	+pSVB16
DH5α(pSVB5-18)	NR	23	NT[d]
DH5α(pSVB15-1)	t.o.	3,803	230
DH5α(pSVB15-2)	r.o.	183	NT

[a] NR, no reporter (parent clone); t.o., transcriptional orientation; r.o., reverse orientation.
[b] Cells were assayed at an OD_{600} of 0.6.
[c] pSVB16 is a clone of *esaR* in pDSK519.
[d] NT, not tested.

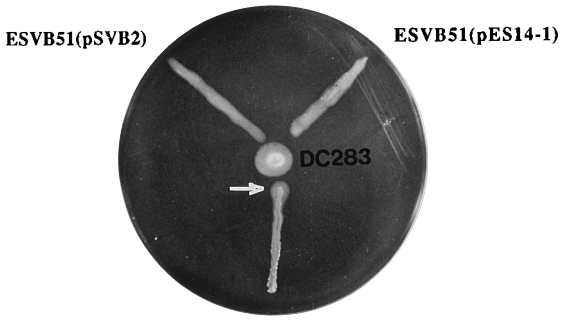

FIG. 5. Production of EPS by wild-type and mutant strains of *E. stewartii*. Strains were streaked onto CPS agar and incubated at 28°C as described in Materials and Methods. The wild-type *E. stewartii* strain DC283 was patched in the plate center. The mutant strain ESVB51 and its derivatives expressing a wild-type copy of *esaI* carried on pSVB2 and pES14-1 were applied as streaks beginning close to the DC283 center colony and extending toward the edge of the plate. Mucoid, slimy colony morphology displayed by DC283 and the complemented strains ESVB51(pSVB2) and ESVB51(pES14-1) is indicative of EPS production. Nonmucoid, creamy colony morphology indicates the lack of EPS production and characterizes the colony morphology of the ESVB51 mutant strain. Note that ESVB51 shows a progressively more mucoid colony morphology (arrow) in the region close to strain DC283 which produces and releases 3-oxohexanoyl HSL into the medium.

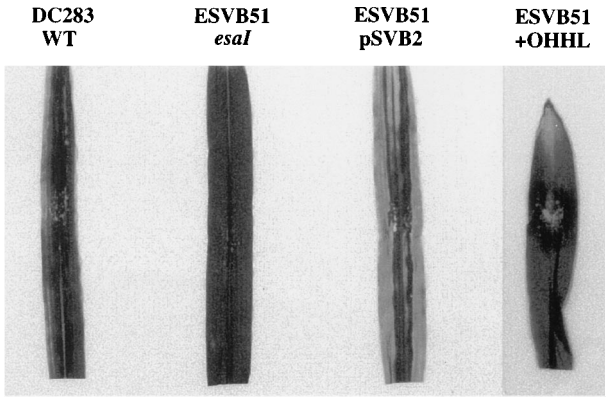

FIG. 6. Pathogenesis of wild-type and mutant *E. stewartii* strains on sweet corn. The leaves of the sweet corn hybrid "Platinum Lady" were inoculated with *E. stewartii* strains as described in Materials and Methods. The wild-type strain (DC283) produces symptoms that are characterized by water-soaked lesions and longitudinal necrotic lesions which appear as light yellow streaks. Leaves inoculated with the *esaI* mutant strain ESVB51 are symptomless, while leaves inoculated with this strain complemented with pSVB2 develop strong symptoms of water-soaking and necrosis. Leaves inoculated with ESVB51 and treated with 3-oxohexanoyl HSL (OHHL) show strong water-soaking symptoms, as indicated by the dark zone surrounding the area of inoculation, but fail to develop the longitudinal necrotic lesion. The photograph was taken 5 days after inoculation.

palindrome overlaps the putative −10 region. This contrasts with the placement of *lux* box-type elements upstream of or in association with the −35 regions in activator-controlled autoinducible promoters. It is tempting to speculate that EsaR represses its own expression by interacting with the *lux* box-like element at the −10 region.

In the related, well-characterized acyl-HSL-dependent systems, the regulated phenotypes generally promote the interaction between the microbe and its specific host. Consistent with this, our results indicate that production of 3-oxohexanoyl HSL by *E. stewartii* is a critical component in fostering its association with susceptible host plants. Mutants unable to produce 3-oxohexanoyl HSL are nonpathogenic. Moreover, preinduction of the mutant strain followed by addition of exogenous 3-oxohexanoyl HSL to the site of inoculation resulted in water-soaking symptoms and chlorosis but not the severe systemic wilting characteristic of infections by wild-type *E. stewartii*. Full virulence requires that the bacteria spread systemically throughout the vascular system. Perhaps exogenous application of 3-oxohexanoyl HSL is insufficient to deliver effective concentrations of signal to the bacteria in the vascular system.

TABLE 3. *esaI* is expressed constitutively

E. coli reporter strain[a]	3-Oxohexanoyl HSL[b]	CAT concn (μg/mg of cellular protein) at OD_{600} of[c]:	
		0.2	0.6
DH5α(pSVB12-1)	−	8.4	8.0
	+	NT[d]	8.4
DH5α(pSVB12-1,pSVB16)	−	7.8	9.5
DH5α(pSVB12-2)	−	1.9	2.3
DH5α(pSVB12-2,pSVB16)	−	<0.1	<0.1

[a] pSVB12-1 and pSVB12-2 confer 3-oxohexanoyl HSL biosynthesis. pSVB16 is a clone of *esaR* in pDSK519.

[b] 3-Oxohexanoyl HSL was supplied exogenously; cells were grown to an OD_{600} of 0.6.

[c] Cells were grown in M9-glucose minimal medium.

[d] NT, not tested.

Pathogenesis in *E. stewartii* correlates with the ability to produce and secrete large amounts of EPS. Our results indicate that production of the EPS virulence factor requires the 3-oxohexanoyl HSL signal. Virulence in the closely related plant pathogen *E. caratovora* also requires induction by 3-oxohexanoyl HSL (42). However, in this organism, the acyl-HSL-signalling system regulates the production and secretion of several extracellular cell wall-degrading enzymes. Moreover, a mutant defective in producing the acyl-HSL signal is avirulent, and pathogenicity can be restored by treating the site of inoculation with exogenous 3-oxohexanoyl HSL (42). Although these two organisms rely on different mechanisms to cause disease, the processes share the unifying principle of being regulated by acyl-HSL signalling systems. Thus, a pattern emerges in which acyl-HSL signals appear to regulate specialized phenotypes that dictate bacterial behavior required for occupying particular niches rather than traits required for general growth and development.

Our work is the first demonstration that biosynthesis of capsular polysaccharides in a gram-negative bacterium is controlled by an acyl-HSL signal. The expression of the *cps* locus in *E. stewartii* depends on an elaborate regulatory circuit consisting of at least five components, including RcsA to RcsD, with RcsC and RcsB resembling a conventional sensor kinase and response regulator two-component signal transduction system (32, 54). The relationship between autoinduction regulation and the Rcs regulatory circuit is not clear. It is possible that acyl-HSL-dependent control of EPS production or translocation exerts a level of regulation that may directly interface with and even dominate the Rcs regulatory cascade in *E. stewartii*. Alternatively, the HSL-mediated control of EPS biosynthesis may operate by a distinct, separate pathway that overrides the Rcs regulatory cascade. Bacterial capsules play a major role in infectious diseases of animals and humans mainly by providing a protective cover to evade the host defense system (6). It will be interesting if biosynthesis of capsular polysaccharides in these gram-negative pathogens is regulated in an acyl-HSL-dependent manner.

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