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A negative regulator mediates quorum-sensing control of exopolysaccharide production in *Pantoea stewartii* subsp. *stewartii*

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ABSTRACT   Classical quorum-sensing (autoinduction) regulation, as exemplified by the *lux* system of *Vibrio fischeri*, requires *N*-acyl homoserine lactone (AHL) signals to stimulate cognate transcriptional activators for the cell density-dependent expression of specific target gene systems. For *Pantoea stewartii* subsp. *stewartii*, a bacterial pathogen of sweet corn and maize, the extracellular polysaccharide (EPS) stewartan is a major virulence factor, and its production is controlled by quorum sensing in a population density-dependent manner. Two genes, *esaI* and *esaR*, encode essential regulatory proteins for quorum sensing. EsaI is the AHL signal synthase, and EsaR is the cognate gene regulator. *esaI*, Δ*esaR*, and Δ*esaI-esaR* mutations were constructed to establish the regulatory role of EsaR. We report here that strains containing an *esaR* mutation produce high levels of EPS independently of cell density and in the absence of the AHL signal. Our data indicate that quorum-sensing regulation in *P. s.* subsp. *stewartii*, in contrast to most other described systems, uses EsaR to repress EPS synthesis at low cell density, and that derepression requires micromolar amounts of AHL. In addition, derepressed *esaR* strains, which synthesize EPS constitutively at low cell densities, were significantly less virulent than the wild-type parent. This finding suggests that quorum sensing in *P. s.* subsp. *stewartii* may be a mechanism to delay the expression of EPS during the early stages of infection so that it does not interfere with other mechanisms of pathogenesis.

Many Gram-negative bacteria control the expression of specific gene systems in a population-dependent manner by a regulatory mechanism known as autoinduction or quorum sensing (1). At the core of this process are self-produced signals, commonly called autoinducers, which when available at or above intrinsic threshold concentrations, enable cognate transcriptional effectors to activate otherwise silent genes (for recent reviews, see refs. 1–4). The autoinducer signals from diverse bacteria are generally *N*-acyl homoserine lactones (AHLs), which differ in the length and substitution of their respective acyl side chains (5–7).

AHL-mediated quorum sensing first was described for the luminous symbiotic marine bacterium *Vibrio fischeri*. In this system, expression of the *lux* operon, which encodes enzymes involved in light production, requires at least two proteins, LuxI and LuxR. LuxI is the enzyme responsible for the synthesis of *N*-3-oxohexanoyl-L-homoserine lactone (HSL), the primary AHL produced by *V. fischeri* (8, 9). LuxR is a transcriptional activator that requires the AHL coinducer to initiate the expression of the *lux*-encoded functions (3). Anal-

ogous AHL-dependent activation mechanisms govern Ti plasmid conjugal transfer in *Agrobacterium tumefaciens* (10), the expression of virulence factors in *Pseudomonas aeruginosa* (11), the induction of antibiotic biosynthesis in *P. aureofaciens* (12) and *Erwinia carotovora* (13), exoenzyme synthesis in *E. carotovora* (14), and the expression of functions important in root nodulation and growth inhibition in *Rhizobium leguminosarum* (15). Additional bacterial quorum-sensing mechanisms are described in other recent reviews (1–4, 16, 17).

Quorum sensing controls the production of the capsular polysaccharide stewartan by *Pantoea stewartii* subsp. *stewartii* (18) [this bacterium formerly was named *Erwinia stewartii* (19)]. *P. s.* subsp. *stewartii* is the causative agent of Stewart's wilt of sweet corn and leaf blight of maize. Wilt symptoms and lesions on mature leaves are the result of blockage of xylem elements by bacterial extracellular polysaccharide (EPS) (20, 21). The stewartan biosynthetic pathway is encoded by the *cps* gene cluster (22), and mutations at this locus generally lead to loss of pathogenicity (23). The regulation of *cps* gene expression is complex and involves a number of transcriptional factors. Some of these resemble components of classical two-component signal transduction systems homologous to and cross-functional with the Rcs transcriptional activators required for colanic acid synthesis in *Escherichia coli* (24–26). However, for stewartan synthesis, the Rcs regulatory cascade appears to be secondary to a master control mechanism mediated by EsaR. EsaR is a homologue of LuxR that requires *N*-3-oxohexanoyl-L-HSL to function (19). The enzyme responsible for AHL production is encoded by the *esaI* gene, and a mutation in this gene has pleiotropic effects, eliminating AHL production, mucoidy, and pathogenicity (19).

Previous observations suggested that EsaR, in contrast to all known LuxR-type transcriptional activators, may function as a negative regulator in absence of the AHL signal (19). We report here genetic evidence that confirms this prediction. Specifically, we show that a wild-type *P. s.* subsp. *stewartii* strain synthesizes EPS in a cell density-dependent manner, and that mutations in *esaR*, either alone or in combination with an *esaI* mutation, lead to growth-independent synthesis of EPS; these mutant strains produce capsules and slime constitutively, even at low cell density. In contrast, a mutation in the *esaI* gene alone that eliminates AHL signal production abolishes EPS synthesis (19). The fact that an *esaR* mutation leads to full expression of the phenotype, independent of AHL, constitutes classical genetic proof that EsaR operates as a negative regulator of EPS synthesis. We conclude that quorum sensing, mediated by a LuxR-like protein, can operate by a mechanism

of repression rather than by gene activation. The precise molecular basis for EsaR-mediated negative regulation of EPS synthesis is currently under investigation.

Because our data suggested that EPS production *in planta* might require a critical population density, we evaluated the pathogenicity of *esa* mutants in plant inoculation assays. We report here that the strains with a mutated *esaR* gene, which produce EPS constitutively, induced significantly less wilting in sweet corn than the parent strain. We therefore propose that quorum sensing in *P. s.* subsp. *stewartii* may play a role in delaying the production of EPS so that it does not interfere with, or limit, early disease development.

MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions. *P. s.* subsp. *stewartii* strains used were DC283 (wild type) (23); ESN51 (*esaI*::*Tn5seqN51*) (same as ESVB51, ref. 19); ESΔR (*esaRΔHpa*I–*Pst*I); and ESΔIR (*esaI/esaRΔKpn*I). *A. tumefaciens* strain NT1(pDCI41E33) served as the indicator strain for AHL detection (27). *E. coli* strains were DH5α (28), S17–1 (29), and 2174 (pPH1JI) (30). Broad host range vector pRK415 (31) was used for cloning the *esaI/esaR* locus, and a derivative plasmid, designated pRK415K, was modified to remove the *Kpn*I cloning site in preparation for the *Kpn*I-specific *esaI/esaR* deletion mutagenesis. The suicide plasmid pKNG101 (32) served as vehicle to introduce deletion mutations into the *P. s.* subsp. *stewartii* chromosome by allelic replacement. Luria–Bertani broth, CPG broth (1% Difco peptone, 1% glucose, 0.1% Difco casamino acids), nutrient agar, and culture conditions were described previously (19, 22, 23).

TLC Assay to Detect AHLs. The TLC assay for AHLs was performed as described by Shaw *et al.* (33). This assay uses the indicator strain *A. tumefaciens* NT1(pDCI41E33) for visualization of the various autoinducers. One milliliter of culture supernatant was extracted with an equal volume of ethyl acetate, and a 1-μl aliquot of this extract was applied to a C18 reverse-phase TLC plate (Whatman KC18F Silica Gel 60 Å, catalog no. 4803–800). The synthetic AHL standards were a gift from P.D. Shaw (University of Illinois at Urbana-Champaign).

Deletion Mutagenesis and Allelic Replacement. A mutation in the *esaR* locus was created by deletion of the *Hpa*I–*Pst*I fragment within the *esaR* coding region (nucleotides 1977–2582 as shown in Fig. 1*A*). The mutated DNA was cloned into

the suicide vector, pKNG101 to create plasmid pSVB40 (Fig. 1*C*). This plasmid then was mobilized into *P. s.* subsp. *stewartii* strain DC283 and stable Km^R transconjugants, resulting from integration of the plasmid, were selected. Growth on 5% sucrose subsequently selected for excision of pKNG101. Southern blot hybridization was used to screen for and verify allelic replacements.

A double-mutation within *esaI* and *esaR* was created by deleting the 581-nt *Kpn*I fragment that spans the 3′ coding regions of both genes (Fig. 1*A*). The mutated DNA fragment was cloned into pKNG101 to create plasmid pSVB33 (Fig. 1*D*). This mutation then was introduced into *P. s.* subsp. *stewartii* strain DC283 as above. Allelic replacement of the *esaI/esaR* deletion was confirmed by Southern blot analysis.

Quantitative Measurement of EPS Production. Cultures of *P. s.* subsp. *stewartii* strains were grown in Luria–Bertani broth overnight. The cells then were washed twice in equal volumes of 0.9% NaCl and diluted 10-fold in 0.9% NaCl. About 10^8 cells were used to inoculate 2-liter flasks containing 400 ml of CPG broth (23). EPS was recovered from 800 ml of a culture of grown to an OD_{560} of 0.1, 400 ml grown to an OD_{560} of 0.2 and 0.3, and 200 ml grown to an OD_{560} of 0.4 and 0.6. Cells were collected by centrifugation at $8,000 \times g$ for 30 min. The unbound EPS present in the culture supernatant was precipitated from 40 ml of spent medium with three volumes of absolute ethanol. To recover the capsular EPS fraction bound to the bacterial cells, the cell pellets were resuspended in 50 ml of high-salt buffer (10 mM KPO_4, pH 7.0/15 mM NaCl/1 mM $MgS0_4$) and blended in an Omni Mixer at setting 5 for 30 min at 0°C. Cells were removed by centrifugation at $12,000 \times g$ for 30 min. Dislodged EPS was precipitated from the supernatant with three volumes of ethanol. The EPS precipitates were collected by centrifugation at $12,000 \times g$ for 30 min and then resuspended in 10 ml of sterile H_2O. The amount of total carbohydrates contained in each sample was determined by the phenol/sulfuric acid method (34) followed by spectrophotometric analysis at wavelength 488 nm using a standard curve prepared from known quantities (10–100 μg) of D-glucose. The cfu/ml in each sample was determined by plating serial dilutions of cell suspensions on nutrient agar plates. The data represented in Fig. 2*A* are from three separate experiments.

Virulence Assays on Sweet Corn Seedlings. Sweet corn seedlings (*Zea mays* cv. Seneca Horizon) were grown in a mixture of peat, field soil, and fine vermiculite (1:1:1) in a controlled environment chamber at 29°C, 90% relative humidity, 16-h light and 8-h dark cycle, 355 μE·m^{-2}·sec^{-1} light intensity. They were inoculated at 8 days after planting by using the eyelet end of a sewing needle, which delivered 1 μl of inoculum containing 1×10^6 cells. Pseudostems were wounded twice at right angles *ca.* 1 cm above the soil line. Eighteen to 20 plants were inoculated with each strain. Symptom severity was rated on the following scale: 0 = no symptoms, 1 = a few restricted lesions; 2 = scattered water-soaking symptoms; 3 = numerous lesions and slight wilting; 4 = moderately severe wilt; 5 = death.

RESULTS

Growth Phase-Dependent Analysis of EPS and AHL Synthesis. We previously established that the *esaI/esaR* locus encodes elements essential for autoinduction regulation of EPS synthesis in *P. s.* subsp. *stewartii* (19). A general feature of such regulated phenotypes is that their expression is cell density dependent. However, there were no previous indications of such a growth dependence for EPS synthesis in *P. s.* subsp. *stewartii*. We therefore measured the amount of EPS produced by strain DC283 at different stages of growth. Cultures were inoculated by using less than 10^6 cells/ml and grown for 15–24 h to reach densities between 1×10^7 and 5×10^8 cells/ml. The results are summarized in Fig. 2*A*, which
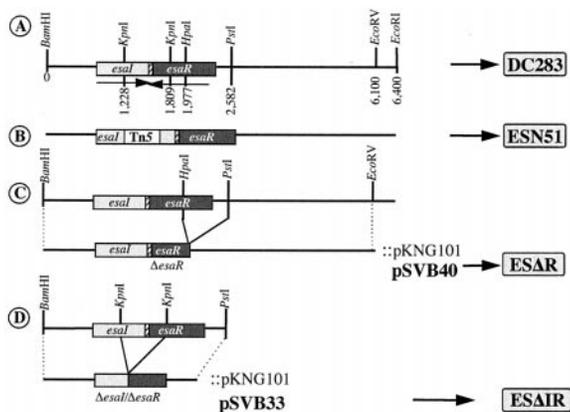

Fig. 1.   Restriction map of the *esaI/esaR* locus (*A*). The direction of transcription of *esaI* and *esaR* is indicated by the arrows. As indicated, a Tn5 insertion in the coding sequence of *esaI* generates mutant strain ESN51 (*B*); a *Hpa*I–*Pst*I deletion in the *esaR* gene is the basis for generating mutant strain ESΔR (*C*); a *Kpn*I deletion in *esaI/esaR* becomes the basis for generating the mutant strain ESΔIR (*D*). The stippled region indicates where the coding segments of the two genes overlap.

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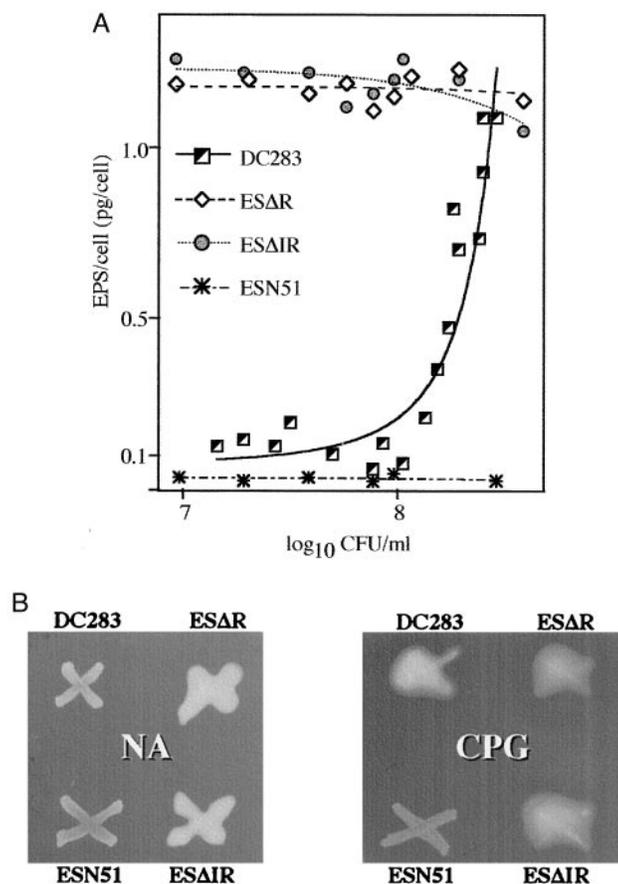*Proc. Natl. Acad. Sci. USA 95 (1998)*   7689

FIG. 2. (*A*) Growth-dependent synthesis of EPS. The strains were grown in CPG medium to different cell densities. Cultures of wild-type strain DC283 (—■—), strain ESN51 (---*---), strain ESΔR (---◇---), and ESΔIR (···◉···) were analyzed for the presence of bound and free EPS. The data points indicated for each strain are the combined data obtained from three separate experiments. The amount of EPS (*y* axis) is expressed as pg/cell. The number of colony-forming units (CFU), expressed as Log$_{10}$ cfu/ml, was determined by dilution plating on nutrient agar. (*B*) Qualitative analysis of EPS production on nutrient agar medium (*Left*) and CPG medium (*Right*). The wild-type strain DC283 exhibits a mucoid phenotype only on CPG medium, whereas the Δ*esaR* and Δ*esaIR* mutants, ESΔR and ESΔIR, are mucoid on both media. The mutant strain ESN51 is unable to synthesize EPS under either condition.

shows that strain DC283 typically yielded 0.1 pg EPS/cell during the early stages of growth; only after reaching *ca.* 2–3 × 10^8 cells/ml did EPS production increase to 1.1 pg /cell, indicating that induction of EPS synthesis occurred during late log phase.

We also determined the pattern of AHL production during growth and the concentration of signal required to promote EPS synthesis. Ethyl acetate extracts of supernatants of bacterial cultures grown to various cell densities were separated and analyzed by TLC as detailed above. The major AHL species produced by *P. s.* subsp. *stewartii* was *N*-3-oxohexanoyl-L-HSL (Fig. 3*A*). It was detectable even at low cell densities (OD$_{560}$ = 0.05) and accumulated linearly with growth. Only negligible amounts of the *N*-3-oxooctanoyl-L-HSL, another common autoinducer, could be detected in high-density cultures (OD$_{560}$ ≥ 0.3). We estimated the amount of AHL by using the method of Shaw *et al.* (33). The *N*-3-oxohexanoyl-L-HSL produced by strain DC283 accumulated in a linear fashion during growth in CPG medium (Fig. 3*B*). The minimum concentration of AHL required for EPS synthesis was *ca.* 2 μM, which occurred when cultures reached an OD$_{560}$ of 0.3 (2–3 × 10^8 cells/ml) (Fig. 3*A*). When strain DC283 was grown

in CPG medium that was supplemented with 2 μM synthetic *N*-3-oxohexanoyl-L-HSL, EPS production occurred at 5 × 10^7 cells/ml (OD$_{560}$ = 0.1), whereas unsupplemented cultures grown to this same density remained repressed for EPS production (data not shown).

Mutagenesis of the *esaR* Locus and Genetic Evaluation of Mutant Strains. We previously reported that EPS synthesis in the *esaI*::Tn5*seq* mutant (Fig. 1*B*), ESN51, is impaired because of the deficiency in AHL synthesis. This finding indicated that EPS production in *P. s.* subsp. *stewartii* is AHL dependent (19). To determine the role of the linked *esaR* gene in this process, we created an *esaR* mutation by deleting an 875-nt *Hpa*I–*Pst*I fragment, which removed the promoter of this gene along with an extensive portion of the coding region (pSVB40, Fig. 1*C*). This mutation was transferred into the chromosome of wild-type *P. s.* subsp. *stewartii* strain DC283 by allelic replacement. The resulting *esaR* mutant, designated ESΔR, was evaluated for its ability to synthesize AHL and EPS. The TLC plate in Fig. 4 contained 20-fold concentrated samples of ethyl acetate extracts from culture supernatants of strains DC283, ESΔR, and ESN51. This assay shows that DC283 and ESΔR produced virtually identical types and amounts of AHLs. These two strains differed, however, in the manner by which they regulate EPS synthesis. Strain ESΔR exhibited a supermucoid phenotype not only on CPG medium but also on nutrient agar, which does not normally stimulate slime production in DC283 (Fig. 2 *A* and *B*). In addition, ESΔR synthesized fully induced levels of EPS during the early stages of growth when DC283 remains repressed (Fig. 2*A*).

Construction and Characterization of an *esaI*/*esaR* Double-Mutant. Because the Δ*esaR* mutant produced normal levels of AHL, it was possible that EsaR is not involved in regulating EPS synthesis. To further investigate the role of EsaR as a cognate regulator for quorum-sensing control of EPS synthesis, we created a double-mutation in the *esaI*/*esaR* locus by deleting an internal 521-nt *Kpn*I fragment encompassing the 3′ portions of both genes (Fig. 1*D*). Because this deletion removes the putative DNA-binding domain of EsaR, the truncated protein is unlikely to function as a gene regulator. The mutation, carried on plasmid pSVB33, was introduced into the chromosome of strain DC283 by allelic replacement. The resulting mutant, designated ESΔIR, was tested for AHL and EPS production. As shown in Fig. 4, strain ESΔIR did not make detectable amounts of AHL, because of the *esaI* mutation. More significantly, strain ESΔIR exhibited the same supermucoid phenotype as strain ESΔR (Fig. 2 *A* and *B*), indicating that an *esaR* mutation bypasses the need for AHL. This finding was in contrast to strain ESN51 (*esaI*⁻/*esaR*⁺), which also is deficient in AHL synthesis (Fig. 4), but remained repressed for EPS production even when grown on CPG medium (Fig. 2 *A* and *B*). Table 1 summarizes the phenotypes associated with the wild-type and *esa* strains evaluated in this study.

Comparative Virulence of Wild-Type and *esa* Strains of *P. s.* subsp. *stewartii*. Because the virulence of *P. s.* subsp. *stewartii* has been correlated with EPS production (35) and *hrp* gene function (36), and *esaI* mutants were less virulent than *cps* mutants, we were interested in determining what effect early overproduction of stewartan would have on virulence. Sweet corn seedlings were inoculated with 10^6 cells of DC283 (*esa*⁺), ESN51 (*esaI*), ESΔR (Δ*esaR*), and ESΔIR (Δ*esaIR*) by wounding the stem. Symptoms were rated at intervals up to 13 days after infection (Fig. 5). The wild-type strain was fully virulent, producing water-soaked lesions after 4 days and completely wilting the plants by 10 days. In contrast, the *esaI* mutant was completely avirulent and unable to cause either lesions or wilting. The two *esaR* mutants were intermediate in virulence; the Δ*esaR* mutant was able to produce some lesions, but not systemic wilting, whereas the Δ*esaIR* mutant could cause only a few scattered lesions. Relative areas under the disease
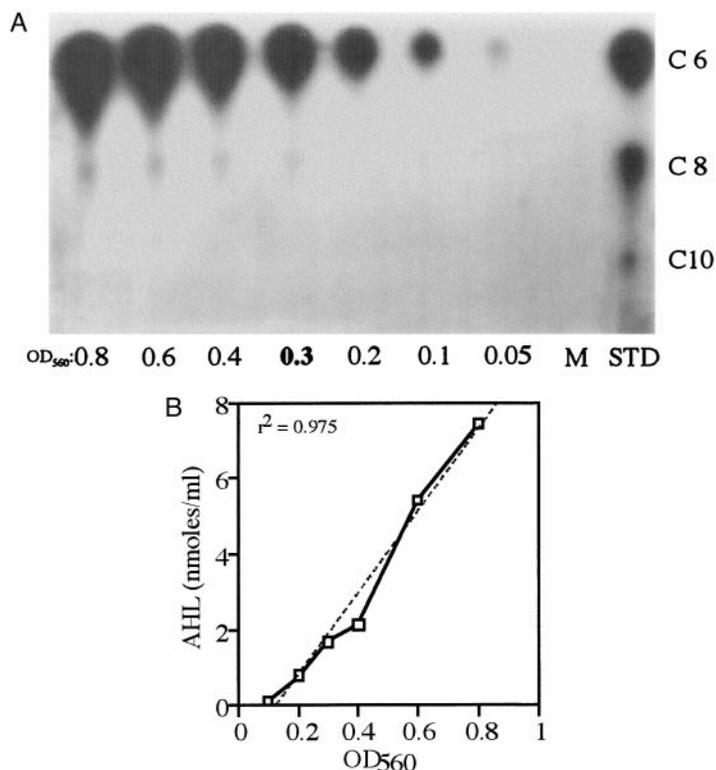
FIG. 3.   TLC analysis of AHLs produced during growth. (*A*) Samples taken from cultures at various optical densities (OD_{560}) were chromatographed on C18 reverse-phase thin layer plates in the presence of methanol/water (60:40 vol/vol) and visualized with an *A. tumefaciens* reporter strain bioassay. Lanes from right to left include: STD, synthetic *N*-3-oxoacyl HSL standards indicated by their respective acyl side chains C6, C8, and C10; M, an extract of fresh culture medium; lanes indicated as 0.05–0.8 include extracts obtained from cultures grown to these specific optical densities (OD_{560}). (*B*) Graph of the amount of AHL present in cultures grown to different optical densities (OD_{560}). The estimated concentration of AHL detected is expressed in nmol/ml culture grown to the corresponding OD_{560}.

progress curves shown in Fig. 5 were 35.6, 1.3, 11.5, and 26.5 for DC283, ESN51, ESΔR, and ESΔIR, respectively. Bacteria reisolated from infected plants retained their original Esa phenotypes. The differences in virulence between *esa*ΔR mutants and the wild type strain were not apparent at higher inoculum levels (>10^7 cells/plant, data not shown). Likewise, in water-soaking assays, where bacterial suspensions in 0.2% Tween 40 were dropped into whorls of 8-day-old seedlings (22), the mutants were indistinguishable from each other and

the parent strain in their ability to incite water-soaked lesions (data not shown).

DISCUSSION

Selective, cell density-dependent gene expression is an inherent feature of quorum-sensing regulated phenotypes. We reported that EPS production in *P. s.* subsp. *stewartii* is regulated by an autoinducer produced by EsaI (19); yet, there were no previous indications that EPS synthesis was, in fact, growth dependent. The results of this study demonstrate that wild-type *P. s.* subsp. *stewartii* produces appreciable EPS only after its population reaches 2×10^8 cells/ml. At this stage, the intrinsic AHL concentration is *ca.* 2 μM and consists primarily of *N*-3-oxohexanoyl-L-HSL. Although subnanomolar concentrations of *N*-3-oxooctanoyl-L-HSL were also present, we
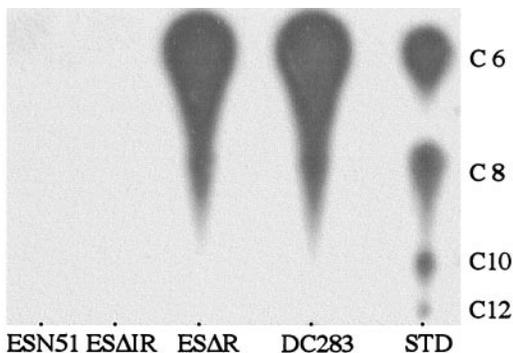

FIG. 4.   TLC analysis of AHL samples extracted from cultures of strain DC283 (wild type), and mutant strains ESΔR, ESΔIR, and ESN51. The lane designated STD contains synthetic preparations of *N*-3-oxoacyl-HSLs identified by their respective acyl side chains. The standards included 0.52 pmol of 3-oxo-C6-HSL, 0.03 pmol of 3-oxo-C8-HSL, 3 pmol of 3-oxo-C10-HSL, and 16 pmol of 3-oxo-C12-HSL. AHLs were detected only in cultures of strains DC283 and ESΔR. All samples applied were extracted from cultures grown to an OD_{560} of 0.6 and concentrated about 20-fold.

Table 1.   The effect of mutations in the *esaI/esaR* locus on AHL production, EPS synthesis, and pathogenicity

Strain	*esaI/esaR**	AHL synthesis	EPS[†] production	Pathogenicity[‡]
DC283	+ +	+	+	4.0
ESN51	− +	−	−	0.1
ESΔR	+ −	+	+++	1.1
ESΔIR	− −	−	+++	2.6

*+ indicates that the strain contains a wild-type allele of *esaI/esaR*; − indicates a mutated allele.

[†]The strain produces wild-type, cell density-dependent levels of EPS (+), no EPS; (−); and constitutive, supermucoid levels of EPS (+++) (see Figs. 2 and 5).

[‡]Symptoms on sweet corn seedlings were rated at 10 days on a five-point scale as described in the text.
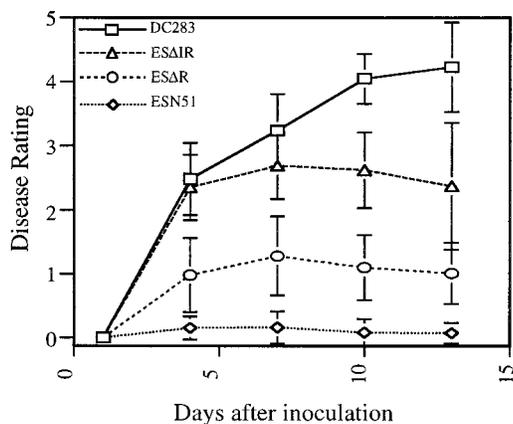
FIG. 5.   Disease progress curves. Sweet corn seedlings (cv. Seneca Horizon) were inoculated with strain DC283 (—□—), ESN51 (···◇···), ESΔR (---○---), and ESΔIR (---△---) as described in *Materials and Methods*. Symptoms were rated at 1, 4, 7, 10, and 13 days after inoculation by using the following disease severity scale: 0 = no symptoms; 1 = a few restricted lesions; 2 = moderate water-soaking symptoms; 3 = numerous lesions and slight wilting; 4 = moderately severe wilt; 5 = plant death. Eighteen to 20 plants were inoculated with each strain. The *Y*-error bars indicate the SD of the individual data points.

believe that this autoinducer has little effect on EPS synthesis, because addition of 2 μM synthetic *N*-3-oxohexanoyl-L-HSL alone fully induced EPS production in DC283 at low cell density. This is not to discount the possibility that minimal levels of *N*-3-oxooctanoyl-L-HSL, or even yet unidentified AHLs, may play a role in some aspect of quorum-sensing regulation. We also found that the accumulation of AHL in cultures was linearly correlated with bacterial growth, which strongly implies that AHL biosynthesis is constitutive and not autoinduced. This conclusion is further supported by the observation that an *esaR* mutation has no affect on the synthesis of *N*-3-oxohexanoyl-L-HSL (Fig. 4). This finding contrasts with other quorum-sensing systems, including the Lux paradigm system, in which full expression of the AHL synthase gene requires induction by the AHL coinducer and the cognate regulator.

The most significant finding of this study is that EsaR behaves genetically as a repressor, unlike all other LuxR-like regulators, which function as transcriptional activators. The Δ*esaR* and Δ*esaIR* mutants synthesize EPS constitutively at all cell densities examined. If EsaR were to function as a transcriptional activator required for expression of the *cps* operons, these same mutations should have a loss-of-function (EPS⁻) phenotype. Additional evidence that EsaR acts a negative regulator comes from the phenotype of strain ESN51. This mutant is deficient in AHL synthesis as a result of an insertion in *esaI*, but it carries a wild-type allele of *esaR*. The presence of a functional EsaR protein in the absence of AHL leads to stringent repression of EPS synthesis in this strain, regardless of cell density; and significantly, this strain remains uninduced in EPS synthesis even on the CPG medium, which strongly induces the synthesis of EPS by the wild-type strain (Fig. 2*B*). This repression is readily relieved by addition of AHL to the culture (19).

The finding that EsaR is a negative regulator is particularly intriguing given the structural similarity between EsaR and LuxR-type activators and the differential biophysical and molecular criteria for transcriptional repressors and activators. For example, if EsaR functions as a repressor, directly controlling the *cps* operons, then it must assume a DNA-binding conformation in the absence of the AHL coinducer and a conformation unsuited for DNA binding in the presence of AHL. Just the opposite appears true for the typical LuxR-type

activators (37, 38). Comparison of the amino acid sequence of EsaR with several of LuxR-class proteins does not reveal any salient structural differences that could account for these gross mechanistic differences. In fact, the amino acid sequence within the putative N-terminal AHL-binding domain and the predicted C-terminal helix–turn–helix structure are remarkably conserved among all these proteins (1, 2, 19). It could be argued that EsaR may function both as a transcriptional repressor in absence of AHL and as an activator in its presence. We feel, however, that this is an unlikely scenario because *esaR* deletion mutations lead to a fully induced, supermucoid phenotype at all cell densities. Thus, an activating role of EsaR in this context could be minimal at best.

At this point the only known *lux* box-like palindrome sequence in *P. s.* subsp. *stewartii* is located in the promoter region of *esaR*, where it overlaps and includes the −10 promoter consensus sequence. No apparent *lux* box-like elements have been identified in the DNA sequence of the *cps* gene cluster, which encodes all of the known structural genes for the biosynthesis of EPS. This may mean that EsaR does not directly act on the *cps* genes, but may instead operate at the other end of a regulatory cascade, such as the Rcs regulatory circuit previously described in *E. coli* (24) and *P. s.* subsp. *stewartii* (25). It is conceivable that EsaR governs the expression of one of the *rcs* genes, or else functions posttranslationally by limiting or interfering with the ability of RcsA-RcsB dimers to activate *cps* transcription. Similarly, a number of additional regulatory components influence the overall expression of the *cps*-encoded functions; any one of these may be potential targets for control by EsaR. Experiments are in progress to define the precise molecular role of EsaR as a regulator of EPS synthesis.

Stewartan is an important virulence factor for *P. s.* subsp. *stewartii* during the later stages of pathogenesis. It is thought to hold water and nutrients in the intercellular spaces after water soaking has been elicited in the leaves, and it provides hydrostatic pressure to disrupt plugged xylem vessels and separate parenchyma cells to facilitate the spread of bacteria within plant tissues. Wilting occurs when EPS plugs the xylem pit membranes. The virulence of *P. s.* subsp. *stewartii cps* mutants and field isolates has been correlated with EPS production and colony type (22, 35); lack of EPS usually results in loss of the ability to move systemically in the plant and causes severe wilting, although some nonmucoid strains still can incite limited water-soaked lesions. The almost complete avirulence of the *esaI* mutant in this study was comparable to that of an *rcsB* mutant (data not shown) and can be explained by its inability to produce EPS. Conversely, stimulating EPS production by increasing the copy number and expression of *rcsA* does not alter virulence, even though such strains overproduce EPS on Luria–Bertani agar (D.L.C., unpublished work). Therefore we were interested to determine whether *esaR* mutants, which also overproduce EPS, would behave similarly. It was surprising to find that they were greatly reduced in virulence and failed to move systemically throughout the plant when wound inoculated at fairly low inoculum levels (<10⁶ cells/plant). However, they still could cause water soaking in the whorl assay and wilting at high inoculum dosages (>10⁷ cells/plant). At this point, we cannot account for why the Δ*esaR* mutant was less virulent than the Δ*esaIR* mutant, but it may be because of a difference in infectivity, because the difference is not apparent at higher inoculum levels or in the whorl assay. These findings suggest that during the initial stages of pathogenesis EPS could be a hinderance to the pathogen, and quorum sensing could be an important means of delaying its production. Two steps in the infection process that may be affected by early EPS production are initial movement of bacteria through the xylem and elicitation of water soaking by the Hrp/Wts proteins. In the field, Stewart's wilt is spread primarily by the corn flea beetle, which intro-

duces the pathogen into the xylem and intercellular spaces of the leaves through wounds made when it feeds. The small number of bacteria that enter the xylem this way then must spread throughout the plant. At this stage of colonization, they may not be able to traverse pit membranes if they are fully capsulated. The next step in pathogenesis is probably the injection of Hrp/Wts pathogenicity proteins into host cells by a *hrp*-encoded type III secretion system (36) to cause cell death and release of nutrients. This transfer process requires cell-to-cell contact and could be very inefficient in the presence of a thick capsule or slime layer. It will be interesting to learn whether quorum sensing is a general mechanism for controlling the production of pathogenicity factors, or whether it is more a means to sense diffusion-limited surroundings that the bacteria encounter in a plugged xylem vessel or crevices between plant cells.

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