A negative regulator mediates quorum-sensing control of exopolysaccharide production in \textit{Pantoea stewartii} subsp. \textit{stewartii}

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**Communicated by Luis Sequeira, University of Wisconsin, Madison, WI, April 27, 1998 (received for review January 15, 1998)**

**ABSTRACT** Classical quorum-sensing (autoinduction) regulation, as exemplified by the lux system of \textit{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 \textit{Pantoea stewartii} subsp. \textit{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, \textit{esaI} and \textit{esaR}, encode essential regulatory proteins for quorum sensing. \textit{EsaI} is the AHL signal synthase, and \textit{EsaR} is the cognate gene regulator. \textit{esaI}, \textit{ΔesaR}, and \textit{ΔesaI-esaR} mutations were constructed to establish the regulatory role of \textit{EsaR}. We report here that strains containing an \textit{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 \textit{P. s. subsp. stewartii}, in contrast to most other described systems, uses \textit{EsaR} to repress EPS synthesis at low cell density, and that derepression requires micromolar amounts of AHL. In addition, derepressed \textit{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 \textit{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 autoinducers 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 \textit{Vibrio fischeri}. In this system, expression of the \textit{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 \textit{V. fischeri} (8, 9). LuxR is a transcriptional activator that requires the AHL coinducer to initiate the expression of the \textit{lux}-encoded functions (3).

**Abbreviations:** AHL, N-acylhomoserine lactone; EPS, extracellular polysaccharide; CPG, casamino acids/peptone/glucose; HSL, homoserine lactone.
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); ESA5 (esaRΔHpaI–PstI); and ESAΔIR (esaI/esaRΔKpnI). A. tumefaciens strain NT1(pDCI41E33) served as the indicator strain for with, or limit, early disease development.

Deletion Mutagenesis and Allelic Replacement. A mutation in the esaR locus was created by deletion of the HpaI–PstI fragment within the esaR coding region (nucleotides 1977–2582 as shown in Fig. 1A). The mutated DNA was cloned into the suicide vector, pKNG101 to create plasmid pSVB40 (Fig. 1C). This plasmid then was mobilized into P. s. subsp. stewartii strain DC283 and stable Km<sup>R</sup> 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 KpnI fragment that spans the 3′ coding regions of both genes (Fig. 1A). The mutated DNA fragment was cloned into pKNG101 to create plasmid pSVB33 (Fig. 1D). 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<sup>6</sup> 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<sub>560</sub> of 0.1, 400 ml grown to an OD<sub>560</sub> of 0.2 and 0.3, and 200 ml grown to an OD<sub>560</sub> of 0.4 and 0.6. Cells were collected by centrifugation at 8,000 × 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<sub>4</sub>, pH 7.0/15 mM NaCl/1 mM MgSO<sub>4</sub>) and blended in an Omni Mixer at setting 5 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 × g for 30 min and then suspended in 10 ml of sterile H<sub>2</sub>O. 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. 2A 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 μEm<sup>−2</sup>sec<sup>−1</sup> 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<sup>6</sup> 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<sup>6</sup> cells/ml and grown for 15–24 h to reach densities between 1 × 10<sup>8</sup> and 5 × 10<sup>8</sup> cells/ml. The results are summarized in Fig. 2A, which
shown that strain DC283 typically yielded 0.1 pg EPS/cell during the early stages of growth; only after reaching ca. 2–3 × 10⁷ 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 material in cultures grown to various cell densities was separated and analyzed by TLC as detailed above. The major AHL material in cultures grown to various cell densities were separated and analyzed by TLC as detailed above.

Mutagenesis of the esaR Locus and Genetic Evaluation of Mutant Strains. We previously reported that EPS synthesis in the esaI:Tn5seq mutant (Fig. 1B), 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 HpaI–PstI fragment, which removed the promoter of this gene along with an extensive portion of the coding region (pSVB40, Fig. 1C).

This mutation was transferred into the chromosome of wild-type P. s. subsp. stewartii strain DC283 by allelic replacement. The resulting esaR mutant, designated ESN51, 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, ESN51, and ESN51. This assay shows that DC283 and ESN51 produced virtually identical types and amounts of AHLs. These two strains differed, however, in the manner by which they regulate EPS synthesis. Strain ESN51 exhibited a supermucoid phenotype not only on CPG medium but also on nutrient agar, which does not normally stimulate slime production in DC283 (Fig. 2A and B). In addition, ESN51 synthesized fully induced levels of EPS during the early stages of growth when DC283 remains repressed (Fig. 2A).

Construction and Characterization of an esaI/esaR Double-Mutant. Because the ΔesaR 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 KpnI fragment encompassing the 3’ portions of both genes (Fig. 1D). 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 ESN51, was tested for AHL and EPS production. As shown in Fig. 4, strain ESN51 did not make detectable amounts of AHL, because of the esaI mutation. More significantly, strain ESN51 exhibited the same supermucoid phenotype as strain ESN51 (Fig. 2A and B), indicating that an esaI 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. 2A 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 10⁶ cells of DC283 (esaI), ESN51 (esaI), ESN51 (ΔesaR), and ESN51 (ΔesaR) 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 esaI mutants were intermediate in virulence; the ΔesaR mutant was able to produce some lesions, but not systemic wilting, whereas the ΔesaR mutant could cause only a few scattered lesions. Relative areas under the disease.
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 esaΔR 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

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>esaI/esaR</th>
<th>AHL synthesis</th>
<th>EPS production</th>
<th>Pathogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC283</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>4.0</td>
</tr>
<tr>
<td>ESN51</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>ESΔR</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>1.1</td>
</tr>
<tr>
<td>ESΔIR</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>2.6</td>
</tr>
</tbody>
</table>

*+ 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.
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-class regulators, which function as transcriptional activators. The ΔesaR and ΔesaIR mutants synthesize EPS constitutively at all cell densities examined. If EsaR were to function as a transcriptional activator required for expression of the cps operon, 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 mutation has no affect on the expression of one of the rcs genes, or else functions posttranslationally or 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 mucoid strains still can incite limited water-soaked lesions. The almost complete avirulence of the ΔesaR 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 (<10^6 cells/plant). However, they still could cause water soaking in the whorl assay and wilting at high inoculum dosages (>10^7 cells/plant). At this point, we cannot account for why the ΔesaR mutant was less virulent than the ΔesaIR 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.

We thank Dr. E. Conrad for his expert suggestions in isolating and quantifying bacterial polysaccharides; Dr. P. D. Shaw for the gift of synthetic AHLs; and Drs. J. M. Clark, Jr., A. Smyth, and C. Fuqua for quantifying bacterial polysaccharides; Dr. P. D. Shaw for the gift of Department of Agriculture.