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Hierarchical Autoinduction in *Ralstonia solanacearum*: Control of Acyl-Homoserine Lactone Production by a Novel Autoregulatory System Responsive to 3-Hydroxypalmitic Acid Methyl Ester

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Bacteria employ autoinduction systems to sense the onset of appropriate cell density for expression of developmental genes. In many gram-negative bacteria, autoinduction involves the production of and response to diffusible acylated-homoserine lactones (acyl-HSLs) and is mediated by members of the LuxR and LuxI families. *Ralstonia* (*Pseudomonas*) *solanacearum*, a phytopathogenic bacterium that appears to autoregulate its virulence genes, produces compounds that promote expression of several heterologous acyl-HSL-responsive reporter gene constructs. High-pressure liquid chromatography of highly concentrated ethyl acetate extracts revealed that culture supernatants of strain AW1 contained two compounds with retention times similar to *N*-hexanoyl- and *N*-octanoyl-HSL. To investigate the role of these acyl-HSLs in *R. solanacearum* virulence gene expression, transposon mutants that were deficient for inducing an acyl-HSL-responsive reporter in *Agrobacterium tumefaciens* were generated. Three loci involved in normal acyl-HSL production were identified, one of which was shown to contain the divergently transcribed *solR* and *solI* genes, the *luxR* and *luxI* homologs, respectively. A 4.1-kb fragment containing *solR* and *solI* enabled all of the mutants (regardless of the locus inactivated) and a naturally acyl-HSL-defective strain of *R. solanacearum* to produce acyl-HSLs. Inactivation of *solI* abolished production of all detectable acyl-HSLs but affected neither the expression of virulence genes in culture nor the ability to wilt tomato plants. AW1 has a functional autoinduction system, because (i) expression of *solI* required SolR and acyl-HSL and (ii) expression of a gene linked to *solR* and *solI*, designated *aidA*, was acyl-HSL dependent. Because AidA has no homologs in the protein databases, its discovery provided no clues as to the role of acyl-HSLs in *R. solanacearum* gene regulation. However, expression of *solR* and *solI* required the global LysR-type virulence regulator PhcA, and both *solR* and *solI* exhibited a cell density-associated pattern of expression similar to other PhcA-regulated genes. The acyl-HSL-dependent autoinduction system in *R. solanacearum* is part of a more complex autoregulatory hierarchy, since the transcriptional activity of PhcA is itself controlled by a novel autoregulatory system that responds to 3-hydroxypalmitic acid methyl ester.

Ralstonia (*Pseudomonas*) *solanacearum* causes vascular wilt in more than 200 plant species in the tropics, subtropics, and warm temperate regions of the world (27). Its agronomically important hosts include tomato, potato, tobacco, peanut, and banana. This pathogen's ability to cause wilt has been attributed mainly to its production of a high-molecular-mass acidic extracellular polysaccharide (EPS I), which can occlude vascular tissues and prevent water flow (reviewed by Schell [50]). Extracellular proteins also have a major role in pathogenesis (31), but individual cell wall-degrading enzymes (e.g., polygalacturonase [PGL] and endoglucanase [EGL]) appear to be nonessential, because inactivation of single genes only decreased the rate of wilting (13).

The expression of virulence determinants in *R. solanacearum* is controlled by a complex regulatory network in which PhcA, a LysR-type transcriptional regulator, plays a central role (50). Inactivation of *phcA* decreases production of EPS I and multiple extracellular proteins, thus reducing virulence in planta (4). We recently reported that *eps* and *egl* biosynthetic genes are differentially expressed during exponential multiplication; their transcription is low at cell densities of $<10^7$ CFU/ml but increases up to 50-fold during the next four generations (8, 20). This pattern of gene expression is reminiscent of the autoinduction phenomenon first observed in bioluminescent marine species of *Vibrio* (37) and subsequently found in many terrestrial bacteria (17, 21, 47, 57).

Autoinduction is observed when bacteria within a population coordinately express selected genes in response to the extracellular concentration of *N*-acyl-homoserine lactones (acyl-HSLs) that they synthesize. Since acyl-HSLs are thought to diffuse freely across bacterial membranes (21, 32), intracellular concentrations that trigger autoinducer-dependent gene expression occur only when a population reaches a sufficiently high cell density within a confined space. This type of synchronized activity within a bacterial population also has been described as quorum sensing (21, 30, 54). Autoinducers that differ in the length and structure of the acyl chain have been reported in more than a dozen bacteria (21, 52). Among phytopathogens, acyl-HSLs are involved in regulating the production of exoenzymes and carbapenem antibiotics in *Erwinia carotovora* (44, 59), capsular biosynthesis in *Erwinia stewartii* (3), and conjugal transfer of the tumor-inducing plasmid in *Agrobacterium tumefaciens* (22, 28, 60). In *E. carotovora* and *E. stewartii*, the inability to produce acyl-HSLs results in loss of virulence in planta (3, 44).

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristics	Source or reference
R. solanacearum		
AW1	Nx^r derivative of wild-type AW (race 1)	14
MoD3	Wild-type banana pathogen (Moko disease); acyl-HSL^-	6
AW1-130	*eps-130*::Tn*3*HoHo1 Lac^+ Nx^r Cb^r	12
AW1-28	*egl-28*::Tn*5-B20* Lac^+ Nx^r Km^r	10
AW1-80	*phcA80*::Tn*5* Nx^r Km^r	4
AW1-AI1 to -AI5	Random Tn*5* mutants; acyl-HSL-deficient Nx^r Km^r	This study
AW1-AI8	*solI8*::SP acyl-HSL^- Nx^r Sp^r	This study
AW1-AI395	*aidA395*::Tn*3*HoHo1 Lac^+ Nx^r Cb^r	This study
AW1-AI8,395	*solI8*::SP *aidA395*::Tn*3*HoHo1 acyl-HSL-deficient Lac^+ Nx^r Sp^r Cb^r	This study
AW1-AI3,395	*solR3*::Tn*5 aidA395*::Tn*3*HoHo1 acyl-HSL-deficient Lac^+ Nx^r Km^r Cb^r	This study
AW1-80,395	*phcA80*::Tn*5 aidA395*::Tn*3*HoHo1 acyl-HSL-deficient Lac^+ Nx^r Km^r Cb^r	This study
Plasmids		
pAI3	AW1 genomic DNA in pLAFR3; Tc^r	This study
pAI3-lac395	pAI3::Tn*3*HoHo1 *aidA395*::*lacZ* Lac^+ Tc^r Cb^r	This study
p395B	pAI3-lac395 Δ*Bam*HI fragment (*solR* and *solI* deleted); Lac^+ Tc^r Cb^s	This study
pAI31	10.3-kb pAI3 *Eco*RI fragment in pLAFR3; Tc^r	This study
pAI3B2	7.6-kb pAI3 *Bam*HI fragment in pLAFR3; Tc^r	This study
pAI3-Tn	18.5-kb *Eco*RI fragment containing Tn*5* and flanking DNA from AW1-AI3 cloned in pUC9; Ap^r Km^r	This study
pK311a and pK311b	4.1-kb pAI31 *Sal*I fragment in pKS (opposite orientations); Ap^r	This study
pK311	pK311a in pLAFR3; Ap^r Tc^r	This study
pK311ΔS	pK311a Δ*Sac*I fragment (deletes *solR* 3′ end and *aidA*); Ap^r	This study
pKsolI-SP	pK311ΔS::SP[a] (SP in *Sma*I site within *solI*) Ap^r Sp^r	This study
pLsolI	2.0-kb pK311b *Bam*HI fragment containing *solI* in pLAFR3; Tc^r	This study
pLsolI-lac	pLsolI::Tn*3*HoHo1 *solI*::*lacZ* Lac^+ Tc^r Cb^r	This study
pSVsolR	1.5-kb pK311a *Sma*I-*Stu*I fragment in pSK; Ap^r	This study
pLsolR	1.5-kb pSVsolR *Eco*RI-*Hin*dIII fragment containing *solR* in pLAFR3; Tc^r	This study
pLsolR-lac	pLsolR::Tn*3*HoHo1 *solR*::*lacZ* Lac^+ Tc^r Cb^r	This study

[a] SP, an Sp^r cartridge from mini-Tn*5* (11).

In *Vibrio fischeri*, which has the best-understood autoinduction system (54, 58), synthesis of the primary acyl-HSL (*N*-3-oxohexanoyl-HSL) requires *luxI*, which is the first gene in an operon that encodes the bioluminescence enzyme system. LuxI was recently demonstrated to synthesize an acyl-HSL from 3-oxohexanoyl-acyl carrier protein and *S*-adenosylmethionine (48). Once the autoinducer exceeds a threshold concentration within a cell, it activates the transcriptional activity of LuxR, which dramatically increases expression of the *luxI* operon and results in bioluminescence. Homologs of LuxI and LuxR are present in almost all of the bacteria that exhibit quorum sensing (21). Although clearly related, the overall similarity among the proteins within each of these gene families is so low (18 to 35%) that heterologous probes or PCR primers have not been used successfully in the search for new genes (47, 57).

We recently reported that 3-hydroxypalmitic acid methyl ester (3-OH PAME) is a novel autoregulator that controls virulence in *R. solanacearum* (9, 20). Mutants that do not synthesize 3-OH PAME do not produce the PhcA-regulated virulence factors EPS I and EGL and do not wilt tomato plants. Addition of excess 3-OH PAME at the beginning of incubation restored the expression of PhcA-regulated genes and reduced the density at which expression began by about fivefold. However, the failure of 3-OH PAME to activate PhcA-regulated gene expression immediately suggested that one or more additional factors regulate density-associated gene expression. We subsequently tested *R. solanacearum* for acyl-HSL production and found that it makes extracellular compounds that activate expression of acyl-HSL-responsive gene constructs in *V. fischeri* and *A. tumefaciens* (23). We describe here the identification of three loci in *R. solanacearum* required for normal production of acyl-HSLs and that one locus encodes LuxR and LuxI homologs that constitute an autoinduction system. Although eliminating acyl-HSL production did not affect the virulence of *R. solanacearum*, its autoinduction system was found to be controlled by PhcA and is thus part of a hierarchical signal transduction pathway regulated by 3-OH PAME.

MATERIALS AND METHODS

Strains and plasmids. Selected bacterial strains and plasmids used in this study are listed in Table 1. Unless noted otherwise, *Escherichia coli* DH5α (Gibco/BRL) was used as the host for all plasmids. To construct pLsolI, the 2.0-kb *Bam*HI fragment from pK311b that contains *solI* was subcloned in pLAFR3, a broad-host-range vector (56) (see Fig. 2B). To subclone *solR*, the 1.5-kb *Sma*I-*Stu*I fragment from pK311a was first inserted into the *Eco*RV site of pSK (Stratagene) to generate pSVsolR. The 1.5-kb insert was then recovered from pSVsolR by *Eco*RI-*Hin*dIII digestion and ligated into similarly digested pLAFR3 to generate pLsolR. In both pLsolI and pLsolR, *solI* and *solR* are transcribed from their native promoters toward the *lacZ* promoter in pLAFR3.

To create strain AW1-AI8, we first deleted the *Sac*I fragment from pK311a to remove *solR* (3′ end), *aidA*, and the *Sma*I site in the vector polylinker to generate pK311ΔS. A spectinomycin (SP) resistance gene cartridge, prepared by *Sma*I digestion of mini-Tn*5* (11), was then inserted into the unique *Sma*I site within *solI* in pK311ΔS to create pKsolI-SP. Finally, the *solI8*::SP allele was moved into the genome of AW1 by site-specific recombination (7) and one of the resulting Sp^r Cb^s mutants was designated AW1-AI8. The site of insertion was verified by Southern analysis, and the mutant was confirmed to be acyl-HSL minus.

Culture conditions and assay methods. *R. solanacearum* and *A. tumefaciens* strains were routinely grown at 30°C in BG broth (1% Bacto Peptone, 0.1% Casamino Acids, 0.1% yeast extract, and 0.5% glucose), 1/10 BG (as in BG, except that Bacto Peptone was at 0.1%), or BGT agar (BG plus 1.6% agar and 0.005% tetrazolium chloride). Cells were grown in EG broth (5) for EPS I, EGL, and PGL assays. *E. coli* and *Pseudomonas aeruginosa* strains were grown at 37°C in Luria-Bertani broth or Luria-Bertani agar. Minimum medium (MM) (10) with 0.8% agar was used for making soft-agar suspensions of either the *A. tumefaciens* or *R. solanacearum* acyl-HSL-responsive reporter strains. Antibiotics used were ampicillin (AP) (100 μg/ml); carbenicillin (CB) (10 μg/ml); kanamycin (KM) (50

μg/ml); nalidixic acid (NX) (20 μg/ml); SP (50 μg/ml); or tetracycline (TC) (15 μg/ml).

EPS I production and EGL and PGL activities were quantified from broth cultures as previously described (5, 10). To assess in planta virulence, 26 to 30-day-old tomato transplants (*Lycopersicon esculentum* cv. Marion) were inoculated by depositing a 2-μl drop containing 2×10^5 CFU on the stub of a cut petiole (46). For routine determination of β-galactosidase activity, cells were grown in BG to an optical density at 600 nm of ~1.0, permeabilized with chloroform and sodium dodecyl sulfate, and assayed with the substrate *o*-nitrophenyl-β-D-galactopyranoside (10, 38). Expression of *solI* and *solR lacZ* fusions during exponential multiplication in batch cultures was determined as described by Clough et al. (8). Briefly, cultures were started at an initial density of $\sim 10^3$ CFU/ml in 1/10 BG and incubated at 30°C and 250 rpm. Cells were then collected for CFU determination and β-galactosidase assays (using 4-methylumbelliferyl-β-D-galactopyranoside) every 60 to 90 min from 14 to 26 h of incubation.

Acyl-HSL characterization. For qualitative determination of acyl-HSL production we used *A. tumefaciens* NT1 (pSVB33, pJM749), which has *traR* and the regulated *tra*::*lacZ749* fusion (43). Fresh cultures of this acyl-HSL-responsive reporter strain were first grown overnight in 10 ml of 1/10 BG with antibiotics to an optical density at 600 nm of ~1.0, and the entire culture was mixed with 100 ml of 50°C soft MM agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (48 μg/ml). This suspension was immediately dispensed into 96-well microtiter plates (190 μl/well) or petri plates (25 ml/plate), allowed to solidify at room temperature for 30 min, and incubated for 6 h at 30°C. Strains to be tested for acyl-HSL production were patched onto the agar surface, and the plates were incubated for an additional 16 to 24 h at 30°C. Because this *A. tumefaciens* reporter strain responds to the presence of most known acyl-HSLs by increasing expression of the *tra*::*lacZ749* fusion up to 400-fold (43, 52), the agar around patches of acyl-HSL-producing strains turns blue.

To determine the number of acyl-HSLs made by *R. solanacearum*, bacteria were grown in 1.2 liters of liquid MM supplemented with 0.05% Casamino Acids and 0.05% yeast extract for 40 h (30°C, 250 rpm) and the cells were removed by centrifugation (15,000 × *g*, 20 min) and then filtration (0.2-μm pore size). Total acyl-HSLs were isolated by the procedure described for *V. fischeri* and *P. aeruginosa* (40) with minor changes. Culture filtrates were extracted twice with 600 ml of acidified ethyl acetate (0.1 ml of glacial acetic acid/liter), and the extracts were pooled, dried with sodium sulfate, and evaporated to dryness on a rotary evaporator. This primary residue was dissolved in 4 ml of ethyl acetate, transferred to 1.0- by 7.5-cm glass tubes, evaporated to dryness with a vacuum centrifuge, and dissolved in 120 μl of ethyl acetate to make a 10,000-fold concentrated extract. The concentrated extract (20 to 75 μl) was evaporated to dryness, dissolved in 250 μl of 20% (vol/vol) methanol in water, and injected into a high-pressure liquid chromatograph (HPLC) equipped with a Beckman Ultrasphere C_{18} column. The column was developed with a linear gradient of 20 to 100% (vol/vol) methanol and water, and fractions were collected every 2 min for 140 min. Solutions containing synthetic acyl-HSL standards were analyzed on the same HPLC column. Each fraction was tested for acyl-HSLs by using each of the following acyl-HSL-responsive reporter systems: *E. coli* VJS533 (pHV200I$^-$), which carries *V. fischeri luxR luxI'CDABEG* (40); *E. coli* MG4 (pKDT17), which has *P. aeruginosa lasR lasB*::*lacZ* (40); and *E. coli* DH5α(pECP61.5), which carries *P. aeruginosa rhlR rhlA*::*lacZ* (41a). Fractions were also tested by spotting onto lawns of the *A. tumefaciens* reporter and the native *R. solanacearum* acyl-HSL-responsive strain AW1-AI8,395 (*solI8*::SP *aidA395*::*lacZ*).

Cloning and identification of loci required for normal acyl-HSL production. To create acyl-HSL-deficient mutants, *R. solanacearum* AW1 was mutagenized with Tn*5* carried on the suicide vector pSUP2021 (53) as previously described (14), except that matings were for 3 h to minimize the generation of siblings. Mutants first were selected on BGT-NX-KM agar and then were tested for acyl-HSL production by the microtiter plate bioassay with the *A. tumefaciens* reporter strain (see above). Acyl-HSL-deficient mutants were picked from the surface of the bioassay plates and purified by streaking on BGT-NX-KM agar.

To isolate cosmids that restored acyl-HSL production to the Tn*5* mutants, a genomic library of *R. solanacearum* AW1 in pLAFR3 was mobilized en masse from *E. coli* DH5α into the mutants by triparental mating (7). Conjugation was for 5 h at 30°C, after which the bacteria were washed from the mating plates with sterile distilled water and the cell suspension was spread on MM-KM-TC plates. Individual transconjugant colonies were patched onto BGT-KM-TC agar and then screened for acyl-HSL production on the *A. tumefaciens* reporter strain.

To localize the region of cosmid pAI3 (see Fig. 2) required to restore acyl-HSL production, it was mutagenized with Tn*3*HoHo1 (55) as described in Carney and Denny (7). Cosmids with the transposon in the desired region were identified by mating individual *E. coli* C2110(pAI3::Tn*3*HoHo1) transconjugants with an *R. solanacearum* acyl-HSL-deficient strain (either the Tn*5* mutant AW1-AI3 or the banana pathogen MoD3) and selecting for rare progeny that did not produce acyl-HSL. Matings were done by spreading $\sim 10^9$ CFU of a recipient strain onto plates of MM-TC-CB, dispensing 1 μl of $\sim 10^9$ CFU/ml of *E. coli* HB101 (pRK2013) (18) at each mating site, and then patching a different C2210 transconjugant on each helper spot. After 3 days at 30°C, the *R. solanacearum* transconjugants were selected by patching onto MM-KM-TC-CB plates and then screened for the absence of acyl-HSL production by the microtiter plate bioassay with the *A. tumefaciens* reporter strain. The same procedure was used to mutagenize pLsolI and pLsolR (described below), except that for pLsolI the mutagenized plasmids were mated into AW1-AI8 (*solI8*::SP) and transconjugants were selected on MM-SP-TC-CB plates.

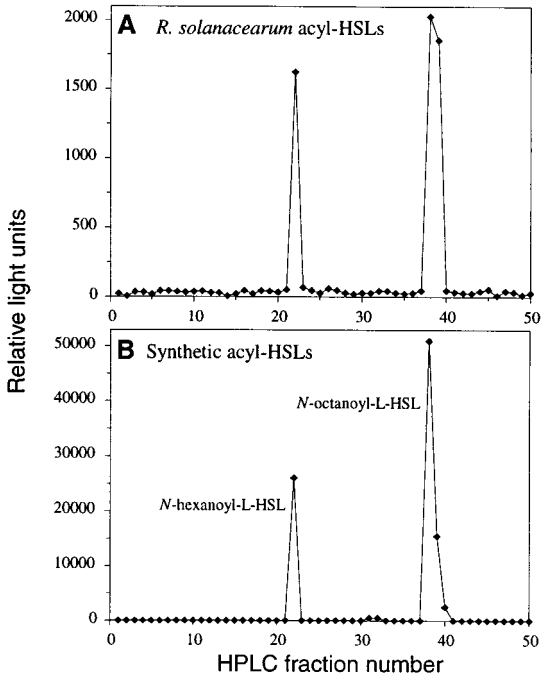

FIG. 1. HPLC analysis of acyl-HSLs. The preparations contained acyl-HSLs extracted from culture supernatants of wild-type *R. solanacearum* (A) or synthetic standards (B). Concentrated solutions were chromatographed on a C_{18} column, and 1-ml fractions were collected every 2 min for a total of 140 min. Each fraction was then assayed for activity by using the bioluminescent acyl-HSL-responsive *E. coli* strain VJS533(pHV200I$^-$) (26a). Fractions 51 to 70 were omitted from the figures because they contained no detectable acyl-HSLs.

Recombinant DNA techniques and DNA sequence analysis. Standard protocols were used for cloning, electroporation, competent cell preparations, restriction mapping, Southern blotting, and plasmid and genomic DNA isolation (7, 35). Allelic replacement of *R. solanacearum* chromosomal genes with mutant alleles carried on either plasmid or genomic DNAs was performed as described previously (8–10). Enzymes and buffers were purchased from New England Biolabs or Boehringer Mannheim.

Deletion derivatives of pK311a and pK311b were sequenced on an Applied Biosystems, Inc., automated DNA sequencer at the University of Georgia Molecular Genetics Instrumentation Facility using pKS and pSK universal primers and custom-designed internal primers. Database search and sequence analyses were performed by using the Wisconsin Package (version 8; Genetics Computer Group) and MacDNAsis Pro (Hitachi Software Engineering Co.). Protein homology searches used the BLAST program (1).

Nucleotide sequence accession number. The complete sequence of the 4.1-kb *Sal*I fragment has been assigned GenBank accession no. AF021840.

RESULTS

***R. solanacearum* produces two acyl-HSLs.** A standard protocol was used to extract acyl-HSLs from cell culture supernatants of an EPS-deficient mutant of *R. solanacearum* AW1, and the highly concentrated ethyl acetate extracts were fractionated on a C_{18} HPLC column. When each fraction was assayed with four previously characterized acyl-HSL-responsive reporter genes, only two peaks of acyl-HSL activity were found: the *V. fischeri* reporter (Fig. 1A) responded strongly to both compounds, the two *P. aeruginosa* reporters responded very weakly to the same two compounds, and the *A. tumefaciens* reporter detected only the more nonpolar peak (fractions 38 and 39) (results not shown). The two compounds detected by the *V. fischeri* reporter had retention times that matched authentic *N*-hexanoyl- and *N*-octanoyl-HSL run on the same col-

umn (Fig. 1B). These same two peaks were found when the extracts were retested with an *R. solanacearum* strain with a native acyl-HSL-responsive reporter construct (AW1-AI8,395; see below).

Tn*5* tagging of three loci in *R. solanacearum* required for normal acyl-HSL production. Attempts to identify a clone with the *R. solanacearum luxI* homolog by screening a cosmid library in *E. coli* directly for production of acyl-HSLs recognized by the *A. tumefaciens* reporter strain and by transforming *E. coli* MG4(pKDT17) (25) with *R. solanacearum* genomic clones were unsuccessful. Therefore, we used transposon mutagenesis to create *R. solanacearum* acyl-HSL-deficient mutants, with the expectation that one of the tagged loci would contain *luxR* and *luxI* homologs. Out of the 3,000 random Tn*5* mutants screened with the *A. tumefaciens* reporter strain, five acyl-HSL-deficient mutants (AW1-AI1 to AW1-AI5) were recovered.

The Tn*5* insertions were responsible for the reduced acyl-HSL production, because when wild-type *R. solanacearum* was transformed with genomic DNAs from each mutant, inheritance of kanamycin resistance was completely linked with the mutant phenotype. Southern analyses of genomic DNAs using a Tn*5* hybridization probe revealed that each mutant contained a single Tn*5* insertion, which was in a 9.5-kb *Eco*RI fragment in AW1-AI1, -AI4 and -AI5; in a 7.5-kb fragment in AW1-AI2; and in a 12.5-kb fragment in AW1-AI3 (19). These results suggested that at least three loci had been mutated, and this hypothesis was supported by the colony morphologies of the mutants (19). When grown at 30°C on MM plates containing 0.005% tetrazolium chloride, wild-type AW1 and mutant AW1-AI3 were completely white and mucoid. In contrast, colonies of AW1-AI2 were dark red and nonmucoid, and colonies of AW1-AI1, -AI4, and -AI5 were red with white margins and partially mucoid. However, the phenotype of AW1-AI2 was temperature sensitive, since growth at 25°C resulted in colonies that were white and mucoid like the wild type.

Mobilization of a wild-type *R. solanacearum* genomic library into the five acyl-HSL-deficient mutants confirmed that three loci had been mutated. One group of three cosmids restored acyl-HSL production to AW1-AI1, -AI4, and -AI5 but not to the other two mutants; AW1-AI1 was chosen to represent this locus. A second group of five cosmids restored acyl-HSL production only to AW1-AI2, and a third group of two overlapping cosmids (represented by pAI3) (Fig. 2A) restored acyl-HSL production to all five mutants. No cosmids that restored acyl-HSL production exclusively to AW1-AI3 were recovered. Significantly, only pAI3 also enabled the naturally acyl-HSL-negative strain MoD3 to produce acyl-HSLs recognized by the *A. tumefaciens* reporter strain. These results suggested to us that pAI3 carries the *luxI* and/or the *luxR* homologs, so this cosmid was further characterized.

Identification of the *lux* gene homologs, *solR* and *solI*, in *R. solanacearum*. The locus in pAI3 for acyl-HSL production was located by Tn*3*HoHo1 mutagenesis (Fig. 2A). Eight insertions eliminated the ability of pAI3 to convert MoD3 to an acyl-HSL producer, and restriction mapping indicated that they all clustered near the internal *Bam*HI site (designated B2 in Fig. 2). A similar effect was observed when the DNA to the left of the B2 *Bam*HI site in pAI3 was deleted to create pAI3B2 (Fig. 2B). In contrast, four adjacent Tn*3*HoHo1 insertions to the right of the B2 *Bam*HI site in pAI3 (insertions 333, 334, 392, and 393) did not interfere with the ability of this cosmid to restore acyl-HSL production in AW1-AI3. These results suggested that the locus in pAI3 involved in acyl-HSL production spans the B2 *Bam*HI site and contains at least two genes.

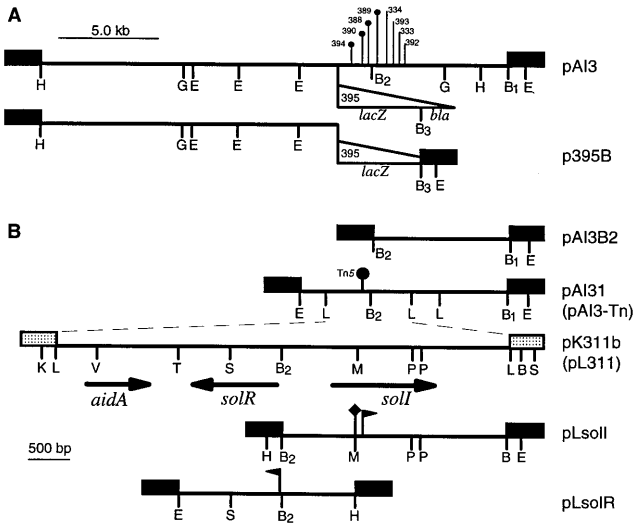

FIG. 2. Cloning the *solR* and *solI* genes from *R. solanacearum*. (A) Restriction map of pAI3, which restored acyl-HSL production by AW1-AI3, and transposon insertions. The eight numbered vertical lines indicate Tn*3*HoHo1 insertions that eliminated the ability of pAI3 to make MoD3 produce acyl-HSLs. In AW1-AI3, acyl-HSL production was restored by pAI3 with insertions denoted only by lines, whereas pAI3 with insertions denoted by lollipops did not restore acyl-HSL production. The enlarged flag labeled 395 indicates the acyl-HSL-dependent *lacZ* fusion in *aidA*. The *Bam*HI site labeled B3 in this Tn*3*HoHo1 insertion was used to delete two *Bam*HI fragments containing *bla*, *solR*, and *solI* to generate p395B. (B) Subclones of the *solR* and *solI* region. The 7.6-kb *Bam*HI fragment from pAI3 cloned in pAI3B2 did not restore acyl-HSL production by AW1-AI3, whereas the 10.3-kb *Eco*RI fragment in pAI31 did. The comparable 10.3-kb *Eco*RI fragment was cloned from the genome of AW1-AI3 to create pAI3-Tn, and the location of the Tn*5* insertion (filled circle) was mapped. The 4.1-kb *Sal*I fragment was subcloned from pAI31 into pKS to create pKS311a and pKS311b and into pLAFR3 to create pL311. Arrows below pK311b show the positions of the ORFs for *aidA*, *solR*, and *solI*. The flags in pLsoII and pLsoIR show the positions and orientations of Tn*3*HoHo1 *lacZ* fusions used to study the expression of *solI* and *solR*. The diamond in pLsoII represents the site of insertion of an Sp^r gene cartridge in *solI*; this mutant allele was introduced into the wild-type genome to generate AW1-AI8. Filled and shaded rectangles represent pLAFR3 and pKS vector sequences, respectively. Abbreviations for restriction sites: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; L, *Sal*I; M, *Sma*I; P, *Pst*I; S, *Sac*I; T, *Stu*I; V, *Eco*RV.

The mutation in AW1-AI3 is in the same locus, because restriction mapping of the *Eco*RI fragment containing Tn*5* (cloned on pAI3-Tn) revealed that the transposon is inserted about 0.3 kb to the left of the B2 *Bam*HI site (Fig. 2B). In addition, when the Tn*3*HoHo1 insertions 333, 334, and 390 (Fig. 2A) were introduced into the AW1 genome by allelic replacement, the resulting Cb^r mutants were acyl-HSL deficient when tested on the *A. tumefaciens* reporter strain. Because pAI3::*lacZ333* and pAI3::*lacZ334* still complemented AW1-AI3 (i.e., the insertions are not in the gene mutated in AW1-AI3), the loss of acyl-HSL production when these insertions were transferred into the genome supported the likelihood that this locus has at least two genes involved in acyl-HSL production.

A 4.1-kb fragment that contains the locus identified by the transposon insertions was subcloned from pAI3 to create pKS311a/b and pL311 (Fig. 2B). When pL311 was moved into AW1-AI1, -AI2, and -AI3 it restored acyl-HSL production; it also converted MoD3 to an acyl-HSL producer. DNA sequencing of pK311a and pK311b revealed two open reading frames (ORFs) with homology to *luxR* and *luxI* genes. One ORF, designated *solR*, spans 711 bases, starting 6 bases to the left of the B2 *Bam*HI site, and is predicted to encode a 25.9-kDa protein that is homologous to members of the LuxR family

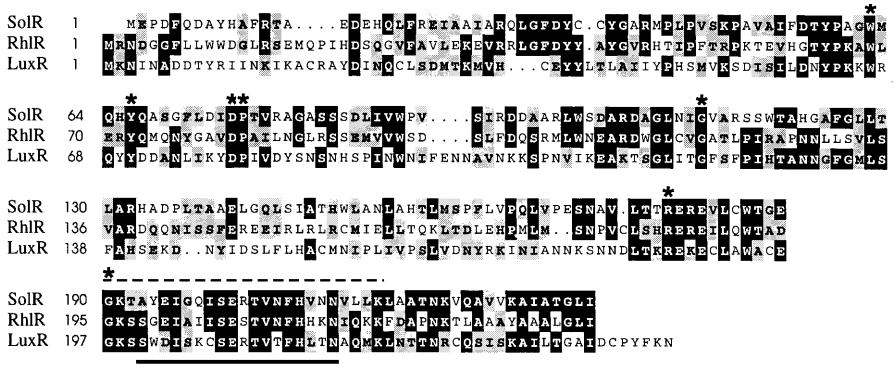

FIG. 3. Alignment of the predicted amino acid sequence of SolR with two representative members of the LuxR family. Gray and black shading indicate similar and identical amino acids, respectively. The solid line indicates the putative helix-turn-helix motif in LuxR (22); the dashed line indicates the LuxR family helix-turn-helix motif in SolR that was identified by PROSITE (2). Amino acids that are invariant in the LuxR homologs studied to date (21) have an asterisk above them. Alignments were performed by using the Genetics Computer Group PILEUP program. The additional sequences shown are RhlR from *P. aeruginosa* (L08962) and LuxR from *V. fischeri* (Y00509).

(Fig. 3) (21). Over the entire protein, SolR has the highest similarities with RhlR (57.5%), PhzR (56.8%), LuxR (56.8%), and SdiA (55.8%) and the lowest similarities with TraR (43.6%) and YenR (43.9%). SolR has all seven invariant amino acids typical of the LuxR family and higher homology in the region containing the putative helix-turn-helix DNA-binding domain near its 3′ end (Fig. 3). After having located *solR*, it was apparent that the Tn5 insertion in AW1-AI3 is in this gene. Unlike the previously constructed plasmids, *solR* was cloned separately from *solI* on a 1.5-kb fragment in pLsolR (Fig. 2B).

The second ORF with similarity to *lux* genes, designated *solI*, spans 615 bases; it is divergently oriented to *solR* and is separated from it by 396 nucleotides. Within 100 bases upstream of *solI* are putative −10 and −35 sites very similar to *E. coli* consensus sequences (Fig. 4A). In addition, partly overlapping the −35 site is a 20-bp imperfect palindrome with high similarity to the *lux* box (operator) found in the promoter region of several genes regulated by LuxR homologs (24, 25, 34, 41a, 45). The palindromic nucleotides C and G (separated by 14 bp) that are essential for operator function in *P. aeruginosa lasB* (45) were conserved in the putative *lux* box of *solI* (Fig. 4A). SolI is predicted to be a 22.4-kDa protein that is homologous to members of the LuxI family of acyl-HSL synthases (Fig. 4B) (21); SolI is most similar to RhlI (55.5%), LasI (55.5%), LuxI (54.4%), and PhzI (51.6%). Similarity was lower with homologs from the plant pathogens *A. tumefaciens* (48.7%), *E. carotovora* (46.2%), and *E. stewartii* (43.1%). Parsek et al. (39a) recently showed that 15 LuxI homologs have 10 invariant amino acids and that for RhlI at least 7 of these residues are critical for synthase activity. Significantly, SolI has all of the invariant amino acids typical of this gene family (Fig. 4B). A 2-kb fragment containing only *solI* was cloned in pLsolI (Fig. 2B).

Inactivation of *solI* eliminated acyl-HSL production but did not affect virulence. Acyl-HSL production by AW1-AI3 was strongly reduced due to inactivation of *solR* (see above), but a well-defined *solI* mutant had not been characterized. To create a strain specifically mutated in *solI*, we inserted an Sp^r gene cartridge into the *Sma*I site of pLsolI and then site specifically recombined this allele into the genome of AW1 to create AW1-AI8. A 10,000-fold-concentrated ethyl acetate extract of culture supernatants of AW1-AI8 was fractionated on a C_{18} HPLC column, and no acyl-HSLs were detected when the

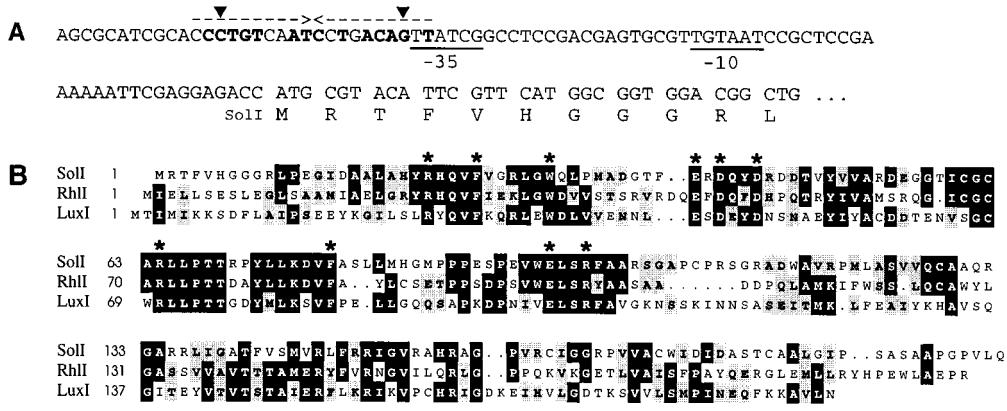

FIG. 4. Sequence analysis of *solI*. (A) Nucleotide sequence of the promoter region and 5′ end of *solI*. The putative −10 and −35 promoter sequences are underlined. An imperfect 20-bp palindrome that is similar to the *lux* box in *V. fischeri* is overlined with dashed arrows; bases also found in the *lux* box are shown in boldface type (25). Triangles indicate two palindromic bases that are also present in the *P. aeruginosa lasB* operator, where they were found to be essential (45). (B) Alignment of the predicted amino acid sequence of SolI with two representative members of the LuxI family. Gray and black shading indicate similar and identical amino acids, respectively. The 10 amino acids that are invariant in all 15 LuxI homologs studied to date (39a) have an asterisk above them. The alignment was performed by using the Genetics Computer Group PILEUP program and was then modified slightly to coincide with that of Parsek et al. (39a). The additional sequences shown are RhlI from *P. aeruginosa* (U40458) and LuxI from *V. fischeri* (Y00509).

TABLE 2. Effects of *solI* and *solR* mutations in *R. solanacearum* on production of extracellular EPS, EGL, and PGL

Strain	Mutation	Effect of mutation on production of[a]:		
		EPS I	EGL	PGL
AW1	None	410 ± 48	500 ± 24	492 ± 45
AW1-AI3	*solR*	512 ± 31	541 ± 36	869 ± 62
AW1-AI8	*solI*	548 ± 17	608 ± 40	822 ± 53

[a] EPS I production is given in micrograms of extracellular galactosamine polysaccharide per milligram of cell protein; EGL and PGL production is given in nanomoles of reducing sugars released from carboxymethylcellulose or polygalacturonate, respectively, per minute per milligram of cell protein. Values are means ± standard deviations of three replicates.

fractions were analyzed by using the *A. tumefaciens*, *V. fischeri*, and *R. solanacearum* acyl-HSL-responsive reporter systems. Therefore, it appears that SolI is essential for production of the acyl-HSLs detected in the supernatant of the wild-type parent.

Production of EPS I, EGL, and PGL virulence determinants by *R. solanacearum* was unaffected by mutations in either *solI* or *solR* (Table 2). To detect more subtle changes in gene regulation, we examined expression of *egl* and *eps* at different cell densities during exponential multiplication but found that inactivation of *solI* did not affect the temporal expression of either gene (19). In addition, both AW1-AI3 and AW1-AI8 wilted tomato plants as rapidly as the wild type. Therefore, under the conditions tested, acyl-HSLs were not required for production of virulence factors in culture or for virulence in planta by *R. solanacearum* AW1.

An acyl-HSL-dependent autoinduction system in *R. solanacearum* is downstream of PhcA in a regulatory hierarchy. Since acyl-HSLs were not essential for virulence, we questioned whether SolI and SolR are really members of a functional autoinduction system in *R. solanacearum*. To address this concern, we first examined expression of *solI* and *solR* by creating *lacZ* fusions in each gene (by insertion of Tn*3*HoHo1 [Fig. 2B]) and moved the resulting reporter plasmids into various *R. solanacearum* strains. Expression of *solR*::*lacZ* was not affected by mutations in either *solI* or *solR*, but expression of *solI*::*lacZ* was reduced more than 200-fold in either mutant background (Table 3). Addition of a crude extract containing *R. solanacearum* acyl-HSLs increased *solI*::*lacZ* expression in a *solI* mutant but not in a *solR* mutant background. Therefore, expression of *solI* is controlled by a positive feedback loop that requires SolR (presumably as a transcriptional activator) and the acyl-HSL made by SolI. This pattern of gene regulation is similar to the autoinduction systems in *V. fischeri*, *A. tumefaciens*, and *P. aeruginosa* (22, 28, 37, 51, 54, 58).

TABLE 3. Expression of *solR* and *solI* in *R. solanacearum* wild-type and mutant backgrounds

Strain (treatment)	Mutation	Reporter plasmid[a]	
		pLsolR-lac	pLsolI-lac
AW1	None	113.0 ± 6.2	457.7 ± 15.8
AW1-AI3	*solR*	127.3 ± 4.7	2.1 ± 0.1
AW1-AI3 (HSL[b])	*solR*	ND[c]	2.2 ± 0.1
AW1-AI8	*solI*	136.6 ± 6.5	2.2 ± 0.1
AW1-AI8 (HSL)	*solI*	ND	568.8 ± 58.2
AW1-80	*phcA*	15.1 ± 2.2	4.4 ± 0.7

[a] Values are β-galactosidase activities in Miller units; data are the average of at least three replicates ± standard deviations.
[b] Five microliters of a 10,000-fold concentrated acyl-HSL preparation from *R. solanacearum* was added to each 10-ml culture at the beginning of incubation.
[c] ND, not done.

TABLE 4. Expression of the autoinducer-dependent gene, *aidA*, in *R. solanacearum* wild-type and mutant backgrounds[a]

Strain (treatment)	Mutation	Location of *aidA*::*lacZ* fusion[b]	
		Plasmid	Genome
AW1	None	768.2 ± 155.1	293.6 ± 36.6
AW1-AI3	*solR*	3.1 ± 0.1	1.2 ± 0.1
AW1-AI8	*solI*	3.1 ± 0.2	1.2 ± 0.1
AW1-AI8 (acyl-HSL[c])	*solI*	533.0 ± 28.8	194.7 ± 14.5
MoD3	Unknown	4.1 ± 0.3	ND[e]
MoD3[d]	Unknown	1,240.3 ± 60.7	ND
AW1-80	*phcA*	ND	1.5 ± 0.2

[a] Values are β-galactosidase activities (in Miller units) ± standard deviations.
[b] With one exception, the *aidA395*::*lacZ* fusion was in the plasmid p395B (which lacks *solR* and *solI*) or had been inserted in the genome of each strain by allelic replacement. Strains used for examining expression of genomic fusions were AW1-AI395 (*aidA395*::*lacZ*), AW1-AI3,395 (*solR*::Tn*5* *aidA395*::*lacZ*), AW1-AI8,395 (*solI8*::SP *aidA395*::*lacZ*), and AW1-80,395 (*phcA80*::Tn*5* *aidA395*::*lacZ*).
[c] Five microliters of a 10,000-fold concentrated acyl-HSL preparation from *R. solanacearum* was added to the 10-ml cultures.
[d] The *aidA395*::*lacZ* reporter was in pAI3-lac395 (*solR*$^+$ and *solI*$^+$) instead of p395B.
[e] ND, not done.

The involvement of *solR* and *solI* in autoinduction was confirmed when we discovered a physically linked locus that is acyl-HSL regulated. While we were screening pAI3::Tn*3*HoHo1 mutants, we found that one fusion (*lacZ395*) was strongly expressed in AW1-AI3 and MoD3 (Table 4), but restriction mapping showed that it was to the left of *solR* (Fig. 2). To determine whether the locus containing *lacZ395* required *solR* and *solI* for expression, two *Bam*HI fragments containing *solI*, *solR*, and *bla* (from Tn*3*HoHo1) were deleted from pAI3-lac395 to create p395B (Fig. 2A), which no longer could code for the production of acyl-HSLs. When p395B was moved into various mutants, the remaining *lacZ395* fusion was strongly expressed in the wild-type AW1, but expression was reduced >200-fold in AW1-AI3 (*solR*), AW1-AI8 (*solI*), and MoD3 (Table 4). Significantly, expression of *lacZ395* in AW1-AI8 was restored to wild-type levels by the addition of a crude acyl-HSL extract (Table 4) or by plating in the presence of an acyl-HSL-producing strain (not shown). The same pattern of gene expression was observed when the *lacZ395* fusion was recombined into the genome of selected strains (Table 4). Consequently, the locus containing the *lacZ395* fusions was designated as *aidA* (for autoinducer dependent) pending the identification of its function. DNA sequencing of the region to the left of *solR* suggested that *aidA* is a 501-bp ORF transcribed convergently to *solR* (Fig. 2B). Unfortunately, database searches with the putative AidA protein sequence did not identify any significant sequence similarities or functional motifs.

Inactivation of *phcA* decreased expression of *solR*, *solI*, and *aidA* 8-, 100 (Table 3)-, and about 200 (Table 4)-fold, respectively. In addition, when cultures were started with $<10^3$ CFU/ml, expression of *solI* and *solR* in a wild-type background was low until a cell density of about 3.0×10^7 CFU/ml was reached and then increased 10- to 15-fold during the next few generations (Fig. 5). Cell density-associated expression of *solI* and *solR* was activated at about the same cell density as other PhcA-regulated genes, but the large decrease in expression of these genes when the cell density of the culture exceeded 2×10^8 CFU/ml is unusual (8). Thus, a bona fide SolR and SolI autoinduction system exists in *R. solanacearum*, and it is a previously unknown component of the PhcA regulon.

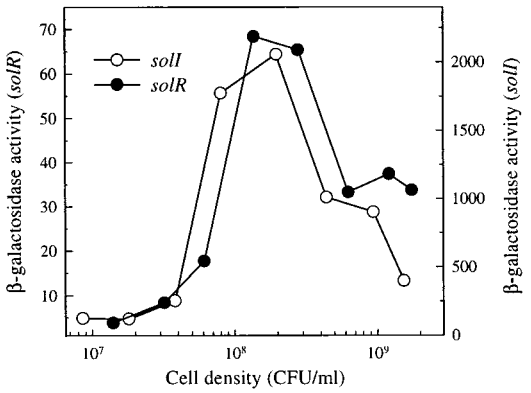

FIG. 5. Expression of *solI* and *solR* in batch cultures of *R. solanacearum*. The strains used were AW1(pLsolI-lac) and AW1(pLsolR-lac). Cultures were started with about 10^4 CFU/ml, and aliquots were removed throughout the late exponential phase to determine viable counts and β-galactosidase activities (8). β-Galactosidase activities were determined with 4-methyl-umbelliferyl-β-D-galactopyranoside as the substrate and are expressed as picomoles of 4-methylumbelliferone released per minute per 10^8 cells.

DISCUSSION

We recently found that the novel 3-OH PAME autoregulator system in *R. solanacearum* is essential for cell density-associated expression of PhcA-regulated genes but is not the only signal required to activate gene expression (8, 9, 20). We therefore investigated whether an acyl-HSL-dependent autoinduction system might also be involved in regulating virulence genes. To test this hypothesis rigorously required completely eliminating acyl-HSL production by *R. solanacearum*. However, this can be difficult, because many bacteria make multiple acyl-HSLs (16, 21, 52), and some have more than one acyl-HSL synthase gene that may or may not be a *luxI* homolog (24, 34). Consequently, we were more concerned with detecting all of the acyl-HSLs made by wild-type *R. solanacearum* and whether they were eliminated by inactivation of *solI* than we were with determining their structure.

After HPLC, we detected only two acyl-HSLs in the extracts of wild-type *R. solanacearum*, using five different acyl-HSL-responsive reporter systems that collectively respond to acyl-HSLs with side chains ranging in length from C_4 to C_{14} and that are either unsubstituted or carry 3-hydroxy or 3-oxo groups (26, 33, 34, 40, 41, 52). Based on their relative mobilities in HPLC and which reporters responded to them, the compounds made by strain AW1 were similar to hexanoyl-HSL and octanoyl-HSL. Shaw et al. (52) also detected two acyl-HSLs in extracts from *R. solanacearum* strain K60 by C_{18} thin-layer chromatography and the *A. tumefaciens* reporter strain and suggested that these compounds are 3-hydroxyoctanoyl-HSL and octanoyl-HSL. In a preliminary test, we found that AW1 produced two acyl-HSLs that migrated similarly to those from K60 when analyzed by the C_{18} thin-layer chromatography bioassay and that neither of these compounds was detectable in an extract from our *solI* mutant even when the preparation was concentrated 10-fold more than described by Shaw et al. (52). Although more work is required to determine the number and identity of all the acyl-HSLs produced by wild-type *R. solanacearum*, our results clearly indicate that inactivation of the putative autoinducer synthase encoded by *solI* eliminates production of the major acyl-HSLs by strain AW1.

Among the acyl-HSL-deficient transposon mutants, we determined that three loci (in AW1-AI1, -AI2, and -AI3) that contribute to normal acyl-HSL production had been inactivated. The locus mutated in AW1-AI3 appeared to affect only acyl-HSL production, whereas insertions in the other two loci also affected colony morphology. Because the locus in AW1-AI3 was found to contain the desired *luxR* and *luxI* homologs, it seems likely that the other two loci encode regulators of acyl-HSL production and that pAI3 suppresses the acyl-HSL-deficient phenotype of AW1-AI1 and AW1-AI2 due to overproduction of SolI and SolR. Indeed, we found that the mutated locus in AW1-AI1 appears to code for an alternative sigma factor (19). Preliminary characterization of the mutated locus in AW1-AI2 suggested that it encodes a membrane-associated protein, but analysis of a partial DNA sequence did not find any significant sequence similarities in the databases.

In *R. solanacearum*, *solI* and *solR* are separated by 396 bases and are transcribed in opposite directions. This contrasts with the organization of the *luxR* and *luxI* gene homologs in most other bacteria studied to date, which are convergently transcribed or are transcribed in the same direction (21, 47). The only other example of divergent organization is that for *luxR* and *luxI* in *V. fischeri*. The intergenic region in *V. fischeri* has several regulatory elements that control bioluminescence (54, 58), the most important being the 20-bp palindromic *lux* box, located about −40 bases from the *luxI* transcription start site (25), which is implicated as the binding site for LuxR (54). The presence of a putative *lux* box centered about −45 bases from the possible transcription start of *solI* is consistent with our results showing that SolR and acyl-HSLs are essential for activating transcription at the *solI* promoter. Similar positive feedback loops have been observed for *luxI*, *traI*, and *lasI* (15, 22, 28, 51), but in *E. stewartii* expression of *esaI* does not require *esaR*, the physically linked *luxR* homolog (3). In other bacteria, *luxR* homologs are positively and/or negatively autoregulated (3, 22, 41a, 54, 58). It appears that *solR* is not autoregulated, but further analysis of its expression in a variety of mutant backgrounds will be necessary to confirm this.

Mutations in *solR* and *solI* did not affect the extracellular levels of known virulence determinants, the cell density-associated expression of *egl* and *eps*, or the ability of *R. solanacearum* to wilt tomato plants. Indeed, other than the loss of acyl-HSLs, *solR* and *solI* mutants had no discernible phenotype. Nevertheless, *R. solanacearum* has a functional acyl-HSL-dependent autoinduction system because, besides regulating transcription of *solI*, SolR and acyl-HSLs activated expression of *aidA* >200-fold. The presence of a regulated locus (other than *solI*) close to *solR* is similar to the gene organization found in several pseudomonads (39, 42). Unfortunately, because *aidA* lacks homology to other genes, finding it provided no clues as to the role of the acyl-HSL-dependent autoinduction system in *R. solanacearum.*

Given our preliminary finding that *R. solanacearum phcA* mutants make reduced amounts of acyl-HSLs (23), it was not surprising to find that *solR* and *solI* are transcriptionally regulated by PhcA. Consequently, like several other PhcA-regulated genes (8), *solR* and *solI* are differentially expressed during exponential multiplication, with expression remaining low until a cell density of about 3×10^7 CFU/ml is reached and then rapidly increasing. Thus, the acyl-HSL-dependent autoinducer system in *R. solanacearum*, like those in most other well-studied bacteria (21), is subject to additional regulatory inputs. We have not identified all of the necessary factors or conditions that trigger increased expression of PhcA-regulated genes during late-exponential-phase multiplication. Having now eliminated acyl-HSL-dependent autoinduction in this process, one other possibility is that, like most LysR-type transcriptional regulators (49), PhcA needs a low-molecular-weight coinducer to activate transcription at target promoters. Indeed, in some

A. tumefaciens strains the acyl-HSL-dependent autoinduction system is regulated by OccR, a typical LysR-type transcriptional regulator for which the coinducer is the family of opines produced by transformed plant cells (22). However, PhcA is an atypical LysR-type regulator (4), and its coinducer (if there is one) has to be endogenously produced since density-associated expression occurs in unamended cultures.

The 3-OH PAME autoregulatory system controls PhcA-regulated genes via the PhcS and PhcR two-component regulators that somehow repress expression and/or function of *phcA* (9, 20). Thus, the acyl-HSL-dependent autoinduction system in *R. solanacearum* is part of the PhcA regulon, which is itself regulated in response to 3-OH PAME. The only other bacterium shown to have two complete autoinduction systems in a hierarchical cascade is *P. aeruginosa*, where one acyl-HSL system (LasR and LasI) directly regulates a second acyl-HSL system (RhlR and RhlI) that responds to two different acyl-HSLs (33, 41a). A variation on this theme may exist in *E. carotovora*, because some evidence suggests that two LuxR homologs (CarR and possibly ExpR [= RexR]) that regulate overlapping sets of genes respond to a single acyl-HSL produced by ExpI (= CarI) (29, 36, 47). Hierarchical autoinduction cascades may be found in additional bacteria as these systems are examined in more detail. Although the purpose for the complex, hierarchical autoregulatory system in *R. solanacearum* is unclear, that this phytopathogen has evolved such an elaborate system of checks and balances for autoregulation suggests that it plays an important role in the biology of this bacterium. In particular, since acyl-HSL-dependent autoinduction is activated coincidentally with that of virulence genes, its role is more likely to be during pathogenesis in planta.

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