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LasR Receptor for Detection of Long-Chain Quorum-Sensing Signals: Identification of *N*-Acyl-homoserine Lactones Encoded by the *avsI* Locus of *Agrobacterium vitis*

Michael A. Savka · Phuong T. Le · Thomas J. Burr

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Abstract Bacterial biosensor strains have greatly facilitated the rapid discovery, isolation, and study of quorum-sensing systems. In this study, we determined the relative sensitivity of a LasR-based *E. coli* bacterial bioluminescence biosensor JM109 (pSB1075) for 13 diverse long-chain *N*-acyl-homoserine lactones (AHLs) including oxygen-substituted and -unsubstituted AHLs containing 14, 16, and 18 carbons and with and without double bonds. Furthermore, we show by bioassay, HPLC, and GC/MS that four long-chain AHLs of the C16-HSL family are encoded by the *avsI* gene of *Agrobacterium vitis* strain F2/5, a non-tumorigenic strain that inhibits pathogenic strains of *A. vitis* from causing crown gall on grape. The four C16-HSLs include: C16-HSL, *N*-hexadecanoyl homoserine lactone; 3-oxo-C16-HSL, *N*-(3-oxo-hexadecanoyl)homoserine lactone; C16:1-HSL, *N*-(*cis*-9-octadecenoyl)homoserine lactone; and 3-oxo-C16:1-HSL, *N*-(3-oxo-*cis*-11-hexadecenoyl)homoserine lactone. Thus, the LasR-based bioluminescent biosensor tested in this study should serve as a useful tool for the detection of various long-chain AHLs with and without double bonds as well as those oxylated at the third carbon from uninvestigated species.

Electronic supplementary material The online version of this article (doi:10.1007/s00284-010-9679-1) contains supplementary material, which is available to authorized users.

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Introduction

Many bacteria employ a cell density sensing mechanism called quorum sensing (QS) [31, 37, 40]. Gram-negative bacteria have been shown to produce and respond to *N*-acyl-homoserine lactone (AHL) signal molecules that are associated with gene expression in QS regulation [31]. AHL-specific QS in Gram-negative bacteria implicate two proteins, an AHL synthase (LuxI homologs), and an AHL-responsive transcription factor (LuxR ortholog) that are involved with regulation of target genes. LuxR homologs contain AHL-specific binding domains. As the population density of a bacterial community increases, the AHLs accumulate to physiologically relevant threshold concentrations that allow interaction with cognate transcription factors [40]. In most cases, signal-bound LuxR-type proteins activate the target genes [34], while others function as transcriptional repressors in a ligand-free state [23]. The overall result is the coordinated activation or derepression of specific target genes in response to a bacterial quorum. QS systems are diverse and regulate a range of different virulence functions that affect plant and animal diseases [3, 19, 26].

Some LuxI orthologs catalyze the synthesis of a range of AHLs [24]. AHLs are characterized by a homoserine lactone moiety ligated to an acyl side chain. The specificity determinants of the AHL signals are the length of the acyl side chain ranging from 4 to 18 carbons, substitution at the C3 position and unsaturation within the acyl chain [6, 8]. AHLs are characterized as long- or short-chain AHLs depending on whether their acyl moiety consists of greater than and equal to, or less than eight carbons, respectively [28].

Most of the conserved amino acid residues in LuxR homologs are involved in AHL signal recognition and

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DNA binding. TraR binds DNA at inverted repeat regions in promoter regions of regulated genes [38, 42]. Recently, the crystal structure of the *Pseudomonas aeruginosa* LasR including the LasR-ligand-binding domain to 3-oxo-C12-HSL was revealed [1, 9]. The LasR and TraR proteins have ligand-binding pockets adapted to their respective cognate acyl-HSLs, 3-oxo-C12-HSL and 3-oxo-C8-HSL, respectively, and the LasR-binding pocket is larger ($\sim$670 Å^3) than the TraR pocket ($\sim$440 Å^3). The larger AHL-binding pocket of LasR may promote its ability to interact with long-chain non-cognate AHLs [1].

Bacterial species, including, *Agrobacterium vitis*, *Paracoccus denitrificans*, *Rhodobacter capsulatus*, *Rhizobium leguminosarum*, and *Sinorhizobium meliloti* have been shown to produce AHLs containing acyl side chains longer than 12 carbons [11, 17, 27]. The detection of long-chain AHLs is not facile since little is known about the dose–responses to these signals using the popular and flexible TraR and LasR biosensors [5, 15, 30, 32, 33, 44]. A biosensor designed for long-chain signal detection was constructed based on the *S. meliloti* SinI/R system [17]. The SinR-dependent biosensor is sensitive to 3-oxo-C14-HSL, 3-oxo-C16:1-HSL, C16-HSL, and C16:1-HSL; but not highly sensitive to C14-HSL and does not detect C18-HSL or signals with acyl side chains less than 14 carbons [17]. Due to the lack of broad-range long-chain AHLs biosensors, workers have used a radiotracer technique that depends on the incorporation of radioactive ^{14}C label from [^{14}C] methionine into AHLs to detect long-chain signals [21, 27].

Agrobacterium vitis is the agent of crown gall disease on grapevine, and results in poor vine growth and sometimes death [2]. *A. vitis* is unique among *Agrobacterium* spp. because in addition to inducing crown gall disease it also causes a tissue-specific necrosis of grape and a hypersensitive-like response (HR) on non-host plants like tobacco [13]. It was determined that grape necrosis and the HR are regulated by a complex QS system that includes multiple transcription factors belonging to the LuxR family [11, 43]. *A. vitis* has recently been shown to produce both long-chain (>8 carbons in acyl side chain) and short-chain AHLs [16]. The locus (*avsI*) encoding long-chain AHLs from F2/5 has recently been cloned and shows the greatest homology with the *sinI* locus of *Sinorhizobium meliloti* [11]. However, the identification of the long-chain AHLs encoded by *avsI* locus has not been investigated.

In this study, we first report on the relative sensitivity of a LasR-dependent *E. coli*-based bioluminescent biosensor JM109 (pSB1075) [41] for rapid detection of 12 non-cognate long-chain AHLs with and without double bonds as well as those oxygenated at the third carbon as compared to the LasR-cognate AHL signal, 3-oxo-C12-AHL [25]. Second, we show the utility of biosensor JM109 (pSB1075) to detect long-chain AHLs encoded by *A. vitis avsI* locus from culture supernatants. Third, by the use of HPLC and GC/MS analyses, we identified four members of the C16-HSL family of long-chain AHLs that are encoded by *avsI*.

Methods

Long-Chain *N*-Acyl-homoserine Lactones

AHLs with acyl side chain lengths of 12, 14, 16, and 18 with ketone and hydroxyl substitutions and with and without double bonds in the acyl side chain were synthesized as described previously [4]. In this article, we abbreviate an acyl-homoserine lactone with any chain length or degree of substitution as AHL. The acyl chain length is indicated by "Cn" for the number of carbons in the chain. For example, *N*-(tetrahydro-2-oxo-3-furanyl) dodecanamide is C12-HSL. The substitution type and position are designated 3-oxo or 3-hydroxy. For example, *N*-(3-oxododecanoyl) homoserine lactone is 3-oxo-C12-HSL. The names of the AHL signals and abbreviations of the signals used in this study include: *N*-dodecanoyl homoserine lactone, C12-HSL; *N*-tetradecanoyl homoserine lactone, C14-HSL; *N*-hexadecanoyl homoserine lactone, C16-HSL; *N*-octadecanoyl homoserine lactone, C18-HSL; *N*-(3-oxododecanoyl) homoserine lactone, 3-oxo-C12-AHL; *N*-(3-oxotetradecanoyl) homoserine lactone, 3-oxo-C14-HSL; *N*-(3-oxohexadecanoyl) homoserine lactone, 3-oxo-C16-HSL; *N*-(3-hydroxydodecanoyl) homoserine lactone, 3-OH-C12-HSL; *N*-(*cis*-9-tetradecenoyl) homoserine lactone, C12:1-HSL; *N*-(*cis*-9-hexadecenoyl) homoserine lactone, C14:1-HSL; *N*-(cis-9-octadecenoyl) homoserine lactone, C16:1-HSL; *N*-(3-oxo-*cis*-11-hexadecenoyl) homoserine lactone, 3-oxo-C16:1-HSL; and *N*-(3-oxo-*cis*-11-octadecenoyl) homoserine lactone, 3-oxo-C18:1-HSL.

Bacterial Strains and Media

Bacterial strains used in this study are listed in Table 1. All *Agrobacterium* strains were grown and maintained on potato dextrose agar. *A. vitis* strain F2/5 *avsI*$^-$ having an insertional disruption in *avsI* [11] was grown in the presence of kanamycin (50 μg ml^{-1}). The Ti plasmid-cured derivative of *A. tumefaciens* strain C58, NT1 containing plasmid pPZP201::*avsI* was grown in AB minimal medium in the presence of spectinomycin (400 μg ml^{-1}). LasR-dependent *E. coli*-based bioluminescent biosensor was used as developed by the Williams laboratory [41]. The JM109 (pSB1075) sensor utilizes the LasR receptor protein designed to activate the *lux* reporter operon in response to cognate signal 3-oxo-C12-HL. JM109 (pSB1075) was grown in LB broth (EMD Chemicals, Inc) supplemented with ampicillin (100 μg ml^{-1}).

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	References
E. coli		
JM109 (pSB1075)	*lasR* + *lasI::luxCDABE*	[41]
Agrobacterium vitis		
F2/5	Nontumorigenic, wild type	[29]
F2/5 *avsI*$^-$	F2/5 containing avsI::pVIK165, Km^R	[11]
S4	Tumorigenic, wild type	[35]
Agrobacterium tumefaciens		
NT1	C58 cured of Ti plasmid	[39]
NT1	(pPZP201::*avsI*) pPZP201 expressing *avsI*, Spc^R	[11]

Dose–Response Assays

Stock solutions of 13 long-chain AHLs were prepared in acidified ethyl acetate (aEtOAc) to obtain a 1-mM solution and a dilution series to 10 nM. The activation of bacterial sensors by various long-chain AHLs was assessed at molar concentrations from 100 pM to 50 μM. The AHLs were placed in round-bottomed tubes (Evergreen Scientific) and allowed to dry. A 1:10 dilution was made of the overnight cultures of the biosensor strains in LB broth giving an OD_{600} between 0.45 and 0.50. These diluted cell suspensions were added to tubes containing various long-chain AHLs. Tubes were covered loosely with Parafilm and incubated at 30°C for 5–6 h with shaking (150 rpm). The induced bioluminescence was measured by a luminometer TD-20/20 (Turner Designs) at a sensitivity setting of 50.1% with a 20-s integration period, and are reported as relative light units (RLU) per triplicate sample [7, 20, 28].

Extraction of AHLs from *Agrobacterium* Strains and Bioassays

Agrobacterium strains were grown on PDA plates for 4 days during which well-developed confluent slimy growth of F2/5 or NT1 developed. Bacterial cells were scraped off and mixed into 20 ml of PD broth medium to form homogenous cell suspensions, which were extracted with 20 ml of aEtOAc with agitation for 60 min at 150 rpm. The tubes were centrifuged for 10 min at 4000 rpm to form a well-defined interface between the aqueous broth and the aEtOAc. The recovered aEtOAc fractions were dried in a Savant Speed Vac (Savant Instruments, Inc.), and resuspended with aEtOAC to make 20-fold (20×) concentrated resuspension extracts [7, 20, 28]. From 1 to 32 μl of 20× aEtOAc extracts of *A. vitis* and *A. tumefaciens* strains (Table 1) were placed into the bottom of round-bottomed tubes. The extracts were dried overnight and the bioassays with JM109 (pSB1075) were performed the following day as described above.

Sample Preparation and HPLC and GC/MS Analysis

Five 200-ml cultures of *A. tumefaciens* NT1 (pPZP201::*avsI*) were grown on AB minimal medium containing spectinomycin (400 μg ml^{-1}) to an optical density (OD_{600}) of approximately 2.0. After clarification by centrifugation, the culture supernatants were filtered (0.2 μM) and passed through C-18 solid phase extractions (SPE) tubes (Strata C18-E, 2G, 12 ml, Phenomenex) with vacuum. After washing with one volume of sterile distilled water and drying by vacuum, the non-polar compounds were eluted with two volumes of 12 ml of acetonitrile (MeCN). The MeCN was evaporated off with N_2 and the residue resuspended in ethyl acetate. After clarification by centrifugation, the supernatant aEtOAc was evaporated to dryness, treated with 300 μl of MeCN, centrifuged again, treated with 100 μl of water, and centrifuged again. The resultant clear supernatant was used for HPLC [18].

HPLC was performed with a Waters 600E instrument, using a 10 × 250 mm C8 column (Phenomenex) with a MeCN water gradient of 20 to 100% over 40 min, at a pumping rate of 3 ml/min, collecting 2 ml fractions and monitoring absorbance at 220 nm. HPLC fractionation of NT1 (pPZP201::*avsI*) extract was eluted using 40% v/v acetonitrile/water for 2 min followed by a gradient of 100% acetonitrile. Forty-two 6-ml fractions were collected from the HPLC and evaporated using a SpeedVac and the residues were redissolved in 10 μl of ethyl acetate. Two microliters of the dissolved fractions were used in bioassays with the LasR-dependent JM109 (pSB1075) biosensor strain. Gas chromatography/mass spectrometry analysis was performed with a Hewlett Packard Series II model 5890 GC fitted with a 5971A Mass Selective Detector using a 12 m × 0.2 mm column of 5% PH ME Siloxane (Agilent). The HPLC fractions were first tested with selective ion monitoring (SIM) using mass 143. This mass fragment is formed by all AHLs through a McLafferty Type-II mechanism involving the amide carbonyl group [3, 22]. The fractions were then tested in scan mode to obtain the

fragmentation patterns of the peaks highlighted by SIM. The retention times and fragmentation patterns were compared to those of authentic AHLs to make an identification. Under our conditions, 3-oxo-AHLs decomposed in the injector of the GC to give methyl ketones with a carbon number one less than that of the acyl groups of the 3-oxo-HSLs. The retention times and fragmentation patterns of authentic methyl ketones were compared to those of peaks from the HPLC fractions, yielding a tentative identification.

Statistical Analyses

All experiments were repeated independently at least twice. Data were statistically analyzed using SAS version 9.1 (SAS Institute Inc. 2004. Cary, North Carolina, USA).

Results

Recognition of Long-Chain AHLs with Unsubstituted and 3-oxo-Substituted Acyl Side Chains

We tested four unsubstituted long-chain AHL, C12-HSL, C14-HSL, C16-HSL, and C18-HSL for detection by the LasR-dependent bioluminescent reporter. Sensitivity of LasR to signals C12-HSL, C14-HSL, and C16-HSL when compared to the cognate signal, 3-oxo-C12-HSL, are reduced about 10-fold for every two-carbon increase in the acyl-side chain length (Fig. 1a). This two-carbon increase was observed at the initial activation concentration and at concentrations to induce maximum light production (Fig. 1a). C18-HSL failed to activate the LasR-dependent biosensor even at the high concentrations of 10 and 50 μM (Fig. 1a and data not shown).

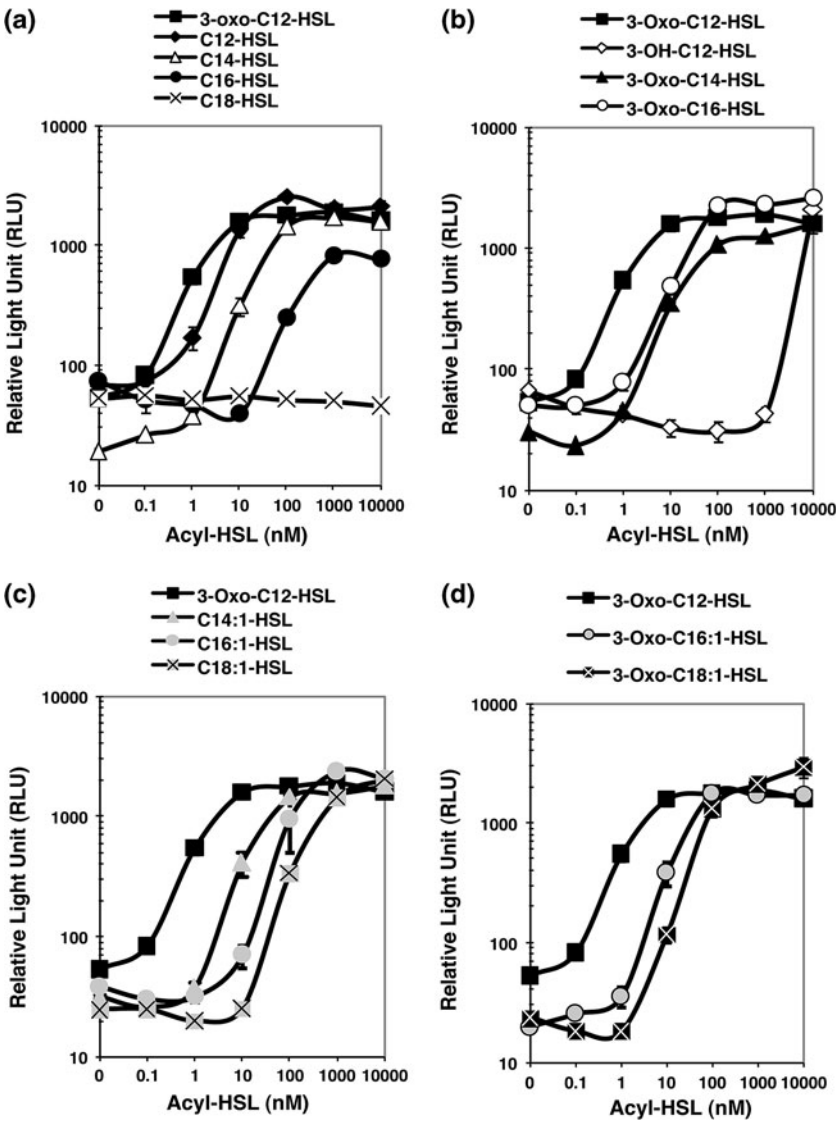

Fig. 1 Effect of acyl-chain length, substitutions, and double bond in long-chain AHLs on the response of bioluminescent biosensor based on the LasR receptor. Detection of synthetic C12-HSL (◆), C14-HSL (☆), C16-HSL (●), and C18-HSL (×), compared to cognate signal, 3-oxo-C12-HSL (■) (**a**); of 3-oxo-C14-HSL (▲), 3-oxo-C16-HSL (○), and 3-OH-C12-HSL (◇), compared to 3-oxo-C12-HSL (■) (**b**); of C14:1-HSL (○), C16:1-HSL (●), and C18:1-HSL (×), compared to 3-oxo-C12-HSL (■) (**c**); and of 3-oxo-C16:1-HSL (●), and 3-oxo-C18:1-HSL (×), compared to 3-oxo-C12-HSL (■) (**d**) in dose–response assays. Concentrations of long-chain AHLs except for C18-HSL significantly affected RLU ($P < 0.01$)

AHLs, 3-oxo-C14-HSL, and 3-oxo-C16-HSL, activated the LasR receptor at 10-nM concentrations (Fig. 1b). These activation concentrations are about 10-fold greater than the LasR cognate acyl-HSL, 3-oxo-C12-HSL (Table S1). Maximum bioluminescent levels induced by 3-oxo-C16-HSL were similar from acyl-HSL cognate signal, 3-oxo-C12-HSL (Fig. 1b). AHL 3-oxo-C14-HSL activated the *lux* operon in the LasR-dependent biosensor to relative light units (RLUs) over 30-fold when compared to background light production (Fig. 1b). 3-OH-C12-HSL was the only 3-OH acyl-HSL tested in this study, and this OH-substituted AHL failed to activate LasR-dependent bioluminescence except at very high concentrations, i.e., 10 and 50 μM (Fig. 1b and data not shown).

Recognition of Long-Chain AHLs with a Double Bond, and with 3-oxo Substitution and Double Bond in the Acyl Side Chain

Three AHLs were evaluated containing double bonds but lacking a substitution on the third carbon of the acyl side chain and included C14:1-HSL, C16:1-HSL, and C18:1-HSL. LasR sensitivity, as measured by the activation of bioluminescence in JM109 (pSB1075), to C14:1-HSL were approximately 10-fold less sensitive (1 nM) than 3-oxo-C12-HSL (100 pM) (Fig. 1c; Table S1). Signals C16:1-HSL and C18:1-HSL activated bioluminescence with about 100-fold less sensitive than the LasR cognate signal (Fig. 1c). The maximum activation peaked at similar values for all three long-chain AHLs with double bonds but required different concentrations ($\sim$100 nM) for C14:1-HSL and C16:1-HSL when compared to C18:1-HSL, which required the highest concentration (1 μM) of the three signals (Fig. 1c).

Two signals with 3-oxo substitution of the third carbon and containing a double bond in the acyl side chain were tested for activation of bioluminescence in the LasR-dependent biosensor. 3-oxo-C16:1-HSL and 3-oxo-C18:1-HSL activated bioluminescence in the LasR-dependent biosensor at 10 nM but to different RLUs values (385 and 118, respectively) thus exhibiting higher limits of detection for the 3-oxo-substituted signals containing double bonds with longer chain lengths as compared to the LasR-cognate AHL, 3-oxo-C12-HSL (Fig. 1d; Table S1).

LasR Detection of Long-Chain Signals Produced by *A. vitis avsI* Gene

We used JM109 (pSB1075) to test for the long-chain signal(s) produced by non-tumorigenic *A. vitis* strain F2/5 [36] and by a previously described *luxI* homolog cloned from F2/5 named *avsI* expressed in *A. tumefaciens* strain NT1 [11]. Acidified EtOAc (aEtOAc) extracts were prepared from 4- to 6-day old plate cultures and tested for activation of bioluminescence with the LasR-dependent biosensor JM109 (pSB1075). Ethyl acetate extract (20 μl of 20×) from *A. vitis* strains S4 and F2/5, an *avsI* site-directed mutant of F2/5 and *A. tumefaciens* NT1 with and without plasmid (pPZP201::*avsI*) were tested for bioluminescent activation in the LasR-dependent reporter. Strain F2/5 and NT1 containing the *avsI* locus induced significant RLU in JM109 (pSB1075), whereas the *avsI*$^-$ mutant and NT1 gave background values of RLUs comparable to the aEtOAc solvent only (C−) (Fig. 2a). Extracts from *A. vitis* strain S4 and 50 nM of pure LasR-cognate signal 3-oxo-

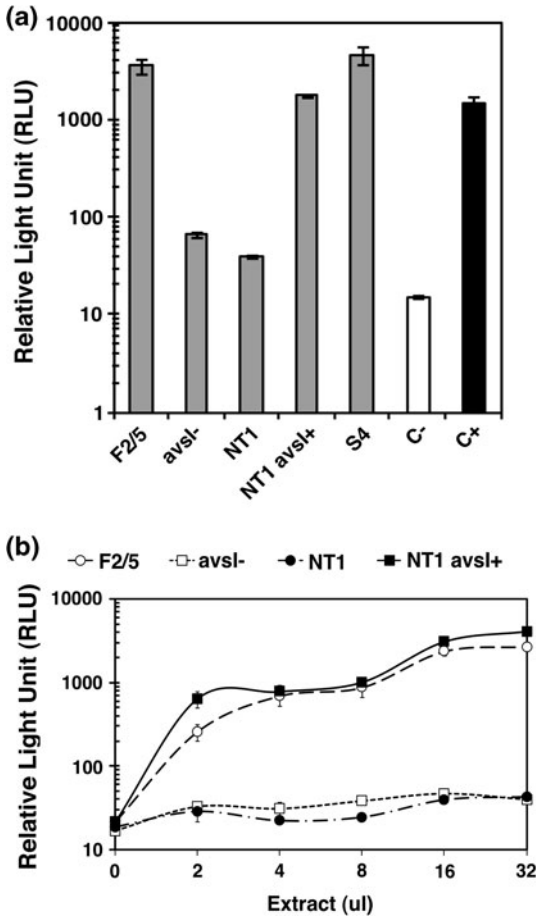

Fig. 2 Detection of long-chain AHLs with JM109 (pSB1075) produced from *A. vitis* F2/5 *avsI*. **a** Response of biosensor to *A. vitis* F2/5 wild type, its *avsI*$^-$ mutant and *avsI* cloned in *A. tumefaciens* NT1. Twenty microliters of crude aEtOAc extract from cultures of wild-type *A. vitis* strain F2/5 (F2/5); site-directed *avsI* mutant of F2/5 (*avsI*$^-$); non-acyl-HL producing *A. tumefaciens* strain NT1 (NT1); strain NT1 containing *avsI* locus on plasmid (NT1 *avsI*$^+$); tumorigenic wild-type *A. vitis* strain S4 (S4); aEtOAc solvent (C−); and 50 nM of pure 3-oxo-C12-HL in aEtOAc (C+). Relative light units (RLU) were significantly different among the strains ($P < 0.01$). **b** Response of increasing amount of extract on the bioluminescence of JM109 (pSB1075) from wild-type F2/5 (∘) and cloned *avsI* locus (•) in *A. tumefaciens* NT1. Additional abbreviations are in Fig. 4b. Relative light units (RLU) of all the strains were significantly affected by the amount of extract ($P < 0.01$)

C12-HSL (C+) activated bioluminescence to levels similar to extracts from strains F2/5 and NT1 containing pPZP201::*avsI* (Fig. 2a).

To estimate the amount of F2/5 and NT1 (pPZP201::*avsI*) extracts required to activate detectable RLU by JM109 (pSB1075), dose–response curves were obtained for extract prepared from F2/5 and F2/5 mutant, NT1 and NT1 (pPZP201::*avsI*). In this experiment, each microliter of extract is equivalent to 20 µl of culture supernatant; thus, detectable activation at 2 µl of 20× extract in a 200-µl volume bioassays is equal to a 1 to 5 ratio (Fig. 2b). Therefore, a 40-µl volume of extracted supernatant is sufficient to activate the LasR-dependent bioluminescence in biosensor JM109 (pSB1075) to detectable levels significantly above both the *avsI*$^{-}$ mutant and the *A. tumefaciens* strain NT1 (Fig. 2b).

avsI Locus of *A. vitis* F2/5 Encodes Four C16 AHLs

Previous studies were done to identify the structures of the AHL produced by the wild-type *A. vitis* strain F2/5. It was determined that the inclusion of non-chemically defined components such as yeast extract or tryptone to introduce certain required amino acids to the growth media led to problematic complications in further analysis of crude extract by high pressure liquid chromatography (HPLC) and mass spectrometry (MS) analysis [16]. Furthermore, it was demonstrated by us previously that NT1 (pPZP201::*avsI*) showed a single long-chain acyl-HSL by thin layer chromatography with a broad-range AHL detection system based on a TraR-receptor [11]. For this study, we grew NT1 (pPZP201::*avsI*) in AB minimal medium without yeast extract or tryptone to high cell density and used solid extraction procedure to prepare an NT1 (pPZP201::*avsI*) extract for HPLC analysis followed by GC and MS analyses. Distinct fractions (#31, 34, 35, and 38) activated bioluminescence to levels significantly above the background levels (Fig. 3).

To identify the chemical structures of the *avsI*-produced AHLs, one microliter of the dissolved fractions were systematically screened using gas chromatography (GC) and mass spectrometry (MS) analysis. We used the utility of the ion at *m/z* 143 as a marker fragment in the selective ion mode (SIM) for AHL identification and *m/z* 102 for that is typical of the homoserine lactone ring [14]. The retention times on the HPLC and GC/MS traces, when compared to the retention times of standard AHLs, confirmed the identity of the long-chain AHLs synthesized by the *avsI* gene of *A. vitis* strain F2/5. GC/MS integrated peak at retention time of 13.92 min from HPLC fraction number 38 obtained a fragmentation pattern consistent with long-chain AHL, C16-HSL (Fig. 4a). A peak with a retention time of 13.71 min from HPLC fraction number 35 obtained a fragmentation pattern consistent with C16:1-HSL (Fig. 4b).

HPLC fraction 34 yielded a peak at retention time of 7.31 min and a fragmentation pattern consistent with 2-pentadecanone, a unique fragment derived from the long-chain AHL, 3-oxo-C16-HSL (Table 2, and data not shown). Similarly, HPLC fraction 31, yielded a peak at a retention time of 7.26 min and a fragmentation pattern is consistent with cis-10-pentadecene-2-one, a unique fragment of long-chain AHL signal 3-oxo-C16:1-HSL (Table 2, and data not shown). Thus, we propose that AvsI synthase is capable of the synthesis of four C16-AHL signals, C16-HSL, C16:1-HSL, 3-oxo-C16-HSL, and 3-oxo-C16:1-HSL (Table 2). Further study will employ electrospray ionization tandem mass spectrometry (ESI MS/MS) [18] to further evaluate AHLs, 3-oxo-C16-HSL and 3-oxo-C16:1-HSL, from extracts of NT1 (pPZP201::*avsI*).

Discussion

In this article, we compared the responses of *E. coli* strain JM109 harboring the *lasR*+ and *lasI::luxCDABE* promoter-

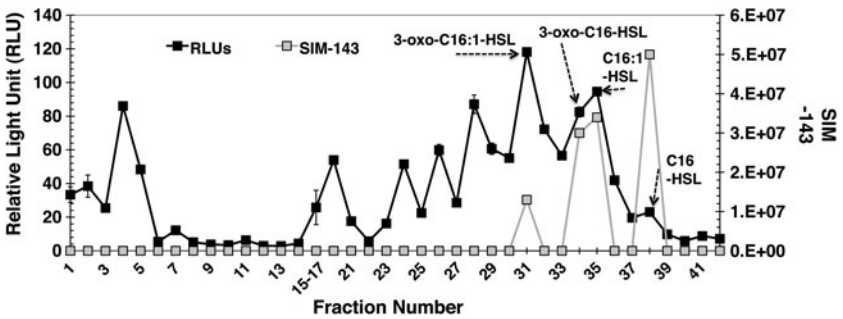

Fig. 3 Bioluminescence (▪) and selected ion monitoring (▫) integrations of high performance liquid chromatography (HPLC) analysis of an extract of *A. tumefaciens* NT1 containing *avsI*. Relative light units (RLU) and selected ion monitoring (SIM) were significantly different among the fraction numbers ($P < 0.01$)

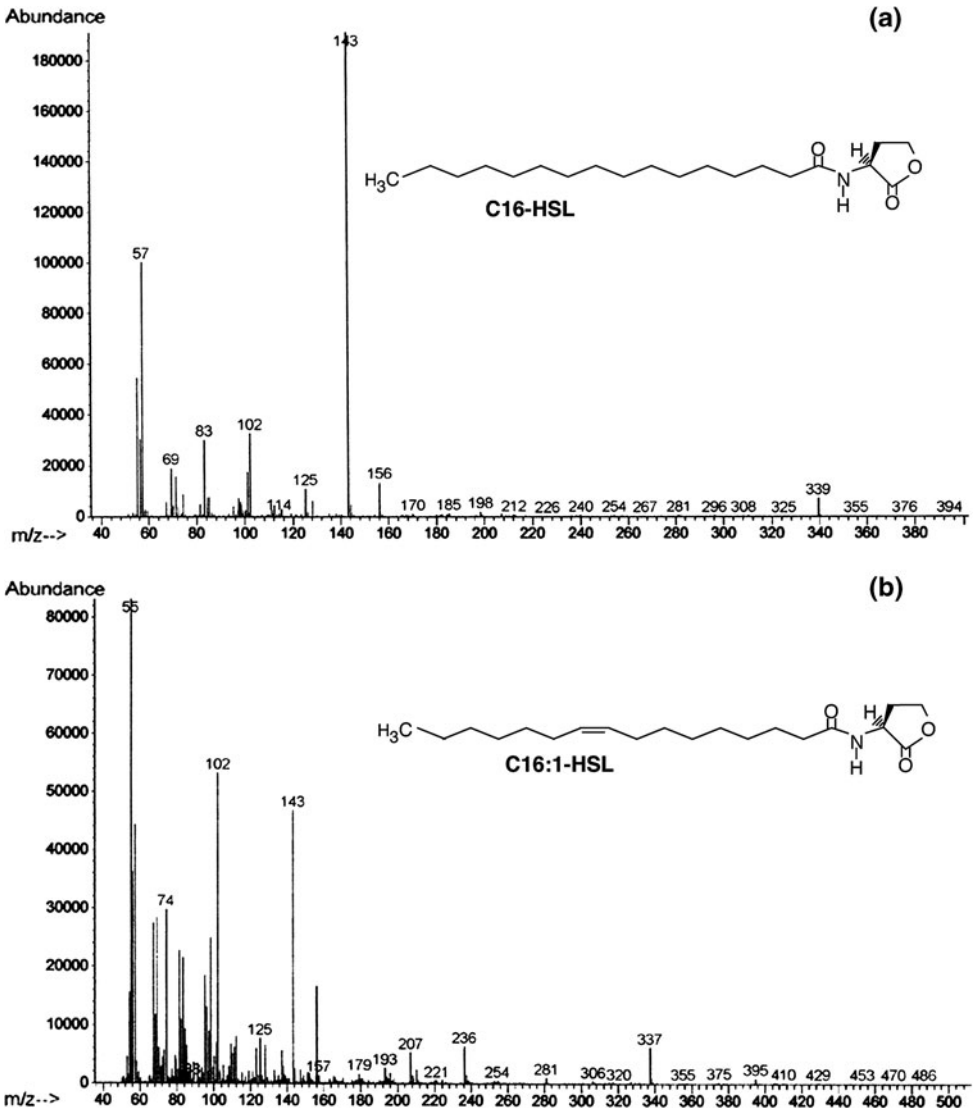

Fig. 4 Identification of *avsI*-produced AHLs by GC/MS analysis. The selective ion-monitoring (SIM) mode was at *m/z* 143. Fragmentation patterns of long-chain AHLs: C16-HSL (**a**) and C16:1-HSL (**b**). Analysis revealed molecular parent ion peaks [M + H] of *m/z* 339 for C16-HSL and *m/z* 337 for C16:1-HSL. A characteristic fragmentation product of *m/z* 143 derived from electron impact ionization with all AHLs via McLafferty Type-II mechanism involving the amide carbonyl group of AHL was clearly identified [22]

Table 2 High performance liquid chromatography (HPLC) fraction, integration value, gas chromatography/mass spectrometry fragmentation identification, and identified signals

HPLC fraction	Integration value	GC/MS identified product	Corresponding acyl-HSL[a]
31	1.3×10^7	*cis*-10-Pentadecene-2-one	3-oxo-C16:1-HSL
34	3.0×10^7	2-Pentadecanone	3-oxo-C16-HSL
35	3.4×10^7	C16:1-HSL	C16:1-HSL
38	5.0×10^7	C16-HSL	C16-HSL

[a] The long-chain AHLs produced from the *A. vitis* strain F2/5 *avsI* locus include: C16-HSL, *N*-hexadecanoyl homoserine lactone; 3-oxo-C16-HSL, *N*-(3-oxohexadecanoyl)homoserine lactone; C16:1-HSL, *N*-(*cis*-9-octadecenoyl)homoserine lactone; and 3-oxo-C16:1-HSL, *N*-(3-oxo-*cis*-11-hexadecenoyl)homoserine lactone

fusion (pSB1075) in dose–response assays with the cognate AHL, 3-oxo-C12-HSL to 12 diverse long chain AHLs. In general, as the acyl side chain increases by 2 carbons (using the cognate signal as a reference), the minimal limit of detection decreased by approximately 10-fold. This relationship is best observed with the unsubstituted AHLs (Fig. 1a). All 12 non-cognate long-chain AHLs tested, except for C18-HSL, presented a minimal detection limit between 1 and 100 nM (Table S1). AHL signal C18-HSL was not detected with JM109 (pSB1075) up to

concentrations of 50 μM, while signal 3-OH-C12-HSL activated light production only at concentrations at or greater than 10 μM (Fig. 1 and data not shown). The inability of C18-HSL to be detected by LasR may be due to low permeability into JM109 or the lack of sensitivity by LasR or its low solubility in water. Further analysis aided by structure–function studies will be useful in gathering information about LasR residues involved in differential AHL detection of non-cognate long-chain AHLs.

We used biosensor JM109 (pSB1075) strain to test *A. vitis* strain F2/5 and its cloned *avsI* locus expressed in the *A. tumefaciens* NT1 background for AHLs. *A. vitis* F2/5 is non-tumorigenic strain used to study HR on tobacco and necrosis on grape [12]. Extract from *A. vitis* type-strain, S4, induced light production to levels similar to strain F2/5 and to NT1 expressing *avsI* gene (Fig. 2a). As little as 2 μl of 20× extracts prepared from *A. vitis* strain F2/5 and its cloned *avsI* locus expressed in NT1-induced significant bioluminescence in JM109 (pSB1075) in comparison to the *avsI*$^{-}$ mutant and *A. tumefaciens* NT1 (Fig. 2b).

A. vitis strain F2/5 has been previously shown by us to produce six different AHL signals [16]. Three short-chain AHLs, C6-HSL, C8-HSL and 3-oxo-C8-HSL, as well as three long-chain, AHLs C14:1-HSL, C16:1-HSL and 3-oxo-C16:1-HSL, were identified from strain F2/5 [16]. In this study, we used HPLC and GC/MS to confirm the production of C16:1-HSL and 3-oxo-C16:1-HSL by strain F2/5 and to show that these two AHL signals are produced by the chromosomally encoded *avsI* gene. Furthermore, we show that the *avsI* locus expressed in *A. tumefaciens* NT1 produces two additional long-chain AHLs, C16-HSL, and 3-oxo-C16-HSL. Thus, the *avsI* locus of *A. vitis* strain F2/5 is proposed to direct the synthesis of four different AHLs all with 16 carbons in the acyl side chain; and, the *A. vitis* F2/5 strain, in all, is proposed to produce a total of eight structurally different AHLs [16] (Figs. 3, 4; Table 2).

The cellular environment in which AHLs are produced could affect the specificity of a given LuxI homolog synthase, because there are many different acyl–acyl carrier protein (acyl-ACP) pools [10, 14]. Although the differences in acyl-ACP pools are not known between *A. tumefaciens* C58 and *A. vitis* F2/5, we propose they do not differ enough to affect AHL profiles under comparison in this study. Furthermore, since the genomes of *A. tumefaciens* C58 and *A. vitis* S4 are quite similar and therefore substrates for AHL synthesis in both are likely to be similar, we do not expect differences in AHL production by the recombinant *avsI* locus in *A. tumefaciens* C58 background but further work in an *A. vitis* background is warranted.

We reported the production of AHLs, C16:1-HSL, and 3-O-C16:1-HSL [16], and now confirm the production of these two AHLs by *A. vitis* strain F2/5. We propose here that AvsI directs the production of two additional AHLs also with a C16 side-chain, C16-HSL, and 3-O-C16:1-HSL (Table 2). Three *luxR* homologs, *aviR*, *avhR*, and *avsR* have been described in F2/5 and all affect AHL signal profiles as determined by thin-layer chromatography detection. A mutant derivative of F2/5 lacking *aviR* showed fewer or lower concentrations of AHLs in comparison to wild type and resulted in complete loss of grape necrosis and tobacco HR [43]. The *avhR* mutant showed the same number of signals as wild type but the signal strength is noticeably stronger than the wild-type strain and *avhR* is involved in the expression of the tobacco HR-negative and grape necrosis responses [12]. The third *luxR* homolog identified in strain F2/5, *avsR* regulates expression of *avsI*, long-chain AHL signal production as well as induction of the tobacco HR and grape necrosis [11]. Since *avsI* is the only member of the *luxI* family found on the two circular chromosomes of *A. vitis*, we suspect that some of the four long-chain AHLs could bind regulators other than AvsR, such as AvhR and AviR, that we have reported [12, 43]. There are other R-type regulators in the *A. vitis* genome whose activity have not been determined that also may interact with one or more of the C16 AHLs signals identified to be produced by the *avsI* locus.

There are at least two additional AHL synthases in *A. vitis* F2/5 that direct the production of the short-chain AHLs, since clones containing either the tartrate catabolic plasmid, pTr, or the octopine catabolic plasmid, pOc, have shown by thin-layer chromatography detection to produce 3-oxo-C8-HSL [16, 20]. However, neither of these plasmids by themselves contains the determinants for HR or necrosis induction on tobacco or grape, respectively [16].

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