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Acyl-homoserine Lactones from *Erwinia psidii* R. IBSBF 435^T, a Guava Phytopathogen (*Psidium guajava* L.)

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The phytopathogen *Erwinia psidii* R. IBSBF 435^T causes rot in branches, flowers, and fruits of guava (*Psidium guajava* L.), being responsible for crop losses, and has no effective control. It was demonstrated that this strain produces two compounds [*S*-(−)-*N*-hexanoyl and *N*-heptanoyl-homoserine lactone], both belonging to the class of quorum-sensing signaling substances. A protocol using gas chromatography−flame ionization detection with chiral stationary phase is described for the absolute configuration determination of a natural acyl-homoserine lactone. Biological assays with specific reporter and synthesis of identified substances are also described. This is the first report on the *N*-heptanoyl-homoserine lactone occurrence in the *Erwinia* genus.

KEYWORDS: Acyl-homoserine lactones; *Erwinia psidii* R.; *Psidium guajava* L.; GC-MS; quorum-sensing

INTRODUCTION

The guava tree (*Psidium guajava* L.) is a perennial plant belonging to the Myrtaceae family, popularly known in Brazil as "goiabeira". It is cultivated in ca. 11 500 hectares, being an important commercial culture mainly for small farmers. Their fruits are appreciated in natura and as juices, candies, and jellies (*1*).

One of the major drawbacks to guava production is caused by the phytopathogen *Erwinia psidii* R. This disease is characterized by wilt of new branches (which become black or brown), lesions and cell death in leaves, and necrosis and mummification of flowers and fruits. The bacterium is introduced into the orchards by contaminated shoots and spreads during the pruning process (*2*).

It has become clear that bacteria do not survive as solitary microorganisms but exploit elaborate intercellular communication systems to adapt themselves to the fluctuations of environmental conditions by a process known as quorum-sensing. This process is based on the production of low-molecular weight diffusible substances, whose extracellular concentrations are related to the microorganism population density. Upon reaching the critical concentration, the signaling substances can be detected by the cells, inducing the population to initiate a concerted action (*3*). This control of phenotypic expression may be of extreme importance in pathogenic infestations, when the premature production of virulence factors can alert host defense systems before a high population density is reached (*4*).

In Gram-negative bacteria, the main class of signaling substances is acyl-homoserine lactones (acyl-HSL). The first study of phenotypic expression control by these substances was reported for the marine luminescent bacterium *Vibrio fischeri* B. In the free-living bacterium, light emission is not observed, however, in high cellular concentrations, *V. fischeri* displays bioluminescence with blue-green light. During growth, this microorganism releases an autoinducer, *N*-(3-oxo-hexanoyl)-HSL, which regulates the expression of genes responsible for bioluminescence once its critical concentration is reached. This control of phenotypic expression is essential for this microorganism in ecological relationships with the squid *Euprymna scolopes* B. Genes controlling synthesis and detection of the autoinducer are called *luxI* and *luxR*, respectively, and are well characterized in this bacterium (*5*, *6*).

Since its discovery, the quorum-sensing system has been demonstrated in a great number of Gram-negative bacteria, many of which employ acyl-HSL in intercellular communication. The structural diversity of these metabolites relies on the acyl side chain, while the homoserine lactone moiety is maintained. The most common structural variations are the chain length, oxo and hydroxy substituents at the 3 position, and unsaturations. This variety can be illustrated by *N*-butanoyl-HSL (biofilm synthesis control in *Pseudomonas aeruginosa* S.), *N*-(3-hydroxy-butanoyl)-HSL (bioluminescence control in *Vibrio harveyi* J. and S.), *N*-(3-oxo-octanoyl)-HSL (plasmid conjugal transfer in *Agrobacterium tumefaciens* S. and T.), and *N*-(3-hydroxy-7-*cis*-tetradecenoyl)-HSL (expression of rhizosphere genes in *Rhizobium leguminosarum* F.) (*7*).

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Many phytopathogens exploit acyl-homoserine lactone based communication systems. *Pectobacterium carotovorum* H. (*Erwinia carotovora* J.), which causes the soft rot disease, is one of the best-studied examples. It has been demonstrated that the carbapenem antibiotic and plant-cell-wall-degrading enzyme syntheses are under the control of the same autoinducer, the *N*-(3-oxo-hexanoyl)-HSL (*8*). Furthermore, the communication system can be disrupted by antagonists, such as halogenated furanones from the red algae *Delisea pulchra* M., thus decreasing antibiotic and exoenzyme production (*9*).

This example shows the importance of the quorum-sensing system during development of some phytopathogenic bacteria in hosts. Suppression of this mechanism is considered a new research field to control bacteriosis (*10*). Therefore, it is important to understand how these biological mechanisms occur, starting from the chemical nature of substances involved. The current communication deals with the acyl-homoserine lactones produced by *Erwinia psidii*, a particularly destructive phytopathogen of Brazilian guava crops (*P. guajava*). The study of possible quorum-sensing signaling substances from this bacterium was carried out employing the IBSBF 435^T type strain, isolated from infected guava trees.

MATERIALS AND METHODS

General Experimental Procedures. NMR spectroscopic data were acquired from a Varian Inova spectrometer, operating at 499.88 MHz for ^{1}H NMR and 125.71 MHz for ^{13}C NMR. $CDCl_3$ was used as the solvent and TMS as the internal reference (δ_H and δ_C 0.0 ppm). Chemical shifts δ were recorded in ppm, and coupling constants J were recorded in Hertz (Hz). Optical rotation was measured on a Perkin-Elmer 341 polarimeter at 17 °C, and the results were converted to 20 °C by the usual equations. Fourier transform infrared (FTIR) spectra were obtained with a Bomem MB Michelson spectrometer, using KBr (Merck, Darmstadt, Germany) as sample support. Silica gel for CC (0.035−0.070 mm) was from Merck. TLC analyses were made on silica gel 60 F_{254} plates (Merck) and were visualized under exposure to UV light (254 nm) or chemically with solution of *p*-anisaldehyde (5%), acetic acid (50 mL), and concentrated sulfuric acid (1 mL) and were heated to 90−100 °C for 5 min.

GC-MS Analysis. GC-MS analyses were carried out on an HP 6890/5973 instrument, equipped with a 30 m × 0.25 mm × 0.25 μm i.d. HP5 fused silica capillary column. Mass spectra were recorded over the 40−450 amu range at 3.54 scans/s, with an ionization energy of 70 eV. Helium was the carrier gas at a flow rate of 1 mL/min. The injector temperature was maintained at 250 °C. The initial oven temperature was 100 °C and was programmed to increase to 290 °C at 10 °C/min and then was held for 10 min. One-microliter samples were injected, without splitting.

Chiral GC-FID Analyses. Chiral gas chromatography analyses were carried out with an HP 6890 instrument with flame ionization detection (FID), equipped with a 25.0 m × 250.0 μm × 25.0 μm chiral capillary column Chrompack CP chirasil-dex coating 7502 (Chrompack International BV, 4330 EA Middelburg, The Netherlands). The initial oven temperature was 50 °C and was programmed to increase at 2 °C/min to 180 °C and then was held for 5 min. Hydrogen was the carrier gas at a flow rate of 1 mL/min. The injector and detector (FID) temperatures were kept at 220 °C and 240 °C, respectively. One-microliter samples were injected, with a 1:100 split ratio.

Bacterial Strains and Cultivation Media. The strain *Erwinia psidii* IBSBF 435^T (=ATCC 49406 type strain) was kindly provided by Dr. Júlio Rodrigues Neto, curator of the collection of cultures of Instituto Biológico de São Paulo, Campinas, Brazil. It was isolated from *Psidium guajava* L. 1982, Brazil and maintained in solid nutrient broth (NB) medium. Indicator strain *Agrobacterium tumefaciens* NTL4(pZLR4) was maintained in Luria-Bertani (LB) medium supplemented with gentamicin (50 μg/mL). *Pseudomonas aeruginosa* CCT 1987 was maintained in solid NB medium. NB medium (20 g/L) was from Oxoid (Hampshire, England). LB medium is composed of 1% peptone (Oxoid), 0.5% NaCl, and 0.5% yeast extract (Oxoid). Solid medium was prepared with 2% agar (Oxoid). X-Gal (5-bromine-4-chloro-3-indolyl-β-D-galactopyranoside) was purchased from Sigma (Aldrich Chemical Co., Milwaukee, WI).

Bioassay with Reporter *Agrobacterium tumefaciens* NTL4(pZLR4). Inoculums of strains *P.* aeruginosa CCT 1987, *A. tumefaciens* NTL4(pZLR4), and *E. psidii* IBSBF 435^T were prepared in test tubes with LB liquid medium (2 mL). These were maintained at 28 °C for 24 h. To four test tubes containing LB liquid medium (2 mL) the following solutions were added: tube 1 (blank control): 20 μL of inoculum of *E. psidii* and 20 μL of X-Gal (stock solution at 50 mg/mL in DMF); tube 2 (blank control): 20 μL of inoculum of *A. tumefaciens* NTL4(pZLR4) and 20 μL of X-Gal; tube 3 (positive control): 20 μL of inoculum of *A. tumefaciens* NTL4(pZLR4), 20 μL of X-Gal, and 20 μL of inoculum of *P.* aeruginosa CCT 1987; tube 4 (test): 20 μL of inoculum of *A. tumefaciens* NTL4(pZLR4), 20 μL of X-Gal, and 20 μL of inoculum of *E. psidii*. The four tubes (in duplicate) were incubated at 28 °C and the colorations were visually evaluated after 24 h.

Culture of *E. psidii* and Detection of Acyl-HSL. *E. psidii* IBSBF 435^T was grown in test tubes containing liquid NB medium (10 mL), was incubated at 30 °C for 24 h, and then was transferred to NB medium (1L) to be incubated at 30 °C under shaking at 100 rpm. After 24 h, the culture medium was centrifuged at 5000 rpm for 20 min under refrigeration (5 °C). The aqueous medium was extracted with ethyl acetate (3 × 500 mL). The combined organic phases (1.5 L) were washed with distilled water (1 × 500 mL), dried over anhydrous sodium sulfate, and evaporated under reduced pressure at 50 °C. The whole procedure was repeated eight times yielding a crude extract (0.728 g), which was separated by silica column chromatography (15 g) eluted with hexane, dichloromethane, and ethyl acetate, recovering 84 fractions of 50 mL, monitored by TLC. Similar fractions were combined and analyzed by GC-MS. Acyl-HSL were detected in fractions FRA32 (2.0 mg) and FRA33−35 (2.5 mg).

N-Hexanoyl-HSL. GC-MS (EI, 70 eV) *m*/*z*: 199 (M^+, 2), 156 (12), 143 (100), 125 (21), 115 (6), 102 (12), 101 (15), 99 (22), 57 (45), 43 (47).

N-Heptanoyl-HSL. GC-MS (EI, 70 eV, SIM) *m*/*z*: 213 (M^+, 1), 156 (10), 143 (100), 125 (20), 113 (15), 102 (14), 101 (15), 85 (16), 57 (52), 43 (40).

Acyl-HSL Synthesis. *General Procedure*. To a round-bottom flask (5 mL) containing distilled water (2.5 mL), triethylamine (1.05×10^{-4} mol), α-amino-γ-butyrolactone hydrobromide [racemic or *S*-(−), Aldrich Chemical Co., Milwaukee, WI] (1.05×10^{-4} mol), and hexanoic or heptanoic acids (1.57×10^{-4} mol) were added. To the stirred solution at room temperature, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (1.57×10^{-3} mol) was added. The reaction was further stirred at room temperature for 24 h. Extraction with ethyl acetate (3 × 10 mL) and the usual workup yielded pure acyl-HSL.

(±)-N-Hexanoyl-homoserine Lactone. 54% yield. GC-MS (EI, 70 eV) data were identical to the natural product. IR, ^{1}H, and ^{13}C NMR data were consistent with those previously reported (*11, 12*).

S-(−)-Hexanoyl-homoserine Lactone. 54% yield. IR, GC-MS, ^{13}C, ^{1}H NMR, DEPT-135, and DEPT-90 data were identical to those obtained for the racemic compound. $[\alpha]^{20}_D$ −22.86° (c. 0.35 MeOH).

(±)-N-Heptanoyl-homoserine Lactone. 64% yield. GC-MS (IE, 70 eV) data were identical to the natural product. IR (KBr) 3315 (m), 2955 (m), 1776 (s), 1646 (s), 1545 (m), 1173 (m), 1013 (m), 646 cm^{-1} (w). ^{1}H NMR (499.88 MHz, TMS, $CDCl_3$) δ_H (ppm) 0.88 (t, 3, J = 7.3 Hz, H-7′), 1.29 (m, 6, H-4′, H-5′, H-6′), 1.64 (quintet, 2, J = 7.6 Hz, H-3′), 2.14 (m, 1, H-4), 2.25 (t, 1, J = 8.9 Hz, H-2′), 2.84 (m, 1, H-4), 4.28 (ddd, 1, J = 5.8, 11.3, 9.5 Hz, H-5), 4.47 (t, 1, J = 8.9 Hz, H-5), 4.56 (ddd, H, J = 5.8, 11.6, 8.6 Hz, H-3), 6.15 (d, 1, J = 3.7 Hz, NH). ^{13}C NMR (125.71 MHz, TMS, $CDCl_3$) δ_C (ppm) 14.0 (C-7′); 22.4 (C-6′); 25.3 (C-3′); 28.8 (C-5′); 30.5 (C-4); 31.4 (C-4′); 36.1 (C-2′); 49.2 (C-3); 66.1 (C-5); 173.7 (C-1′); 175.5 (C-2).

Biological Assays with Extract, Fractions, and Synthetic Products. Biological activities of the synthetic products, ethyl acetate extract, and fractions from *E. psidii* culture medium with biosensor *A. tumefaciens* NTL4(pZLR4) were evaluated as described above. The tests were performed using solutions (20 μL) of each synthetic product

Table 1. Results of Chiral GC-FID Analyses and Absolute Configuration Determination of Natural *S*-(−)-*N*-Hexanoyl-HSL

	(±)-*N*-he xanoyl-HSL		*S*-(-)-*N*-hexanoyl-HSL	FRA32 from *E. psidii* IBSBF435^T	FRA32 + (±)-*N*-h exanoyl -HSL	
enantiomers	R	S	S	S	R	S
retention times (min)	56.72	56.89	56.93	56.97	56.79	56.96
relative abundances (%)	∼50.00	∼50.00	96.34	>99.00	42.16	57.83

[(±)-*N*-hexanoyl-HSL, *S*-(−)-*N*-hexanoyl-HSL, and (±)-*N*-heptanoyl-HSL], ethyl acetate extract from *E. psidii* cultivation medium, and fraction FRA32 in ethanol (2 mg/mL). The blank was performed with ethanol (20 μL).

***N*-Hexanoyl-HSL Absolute Configuration.** The optimum analytical conditions for the enantiomeric discrimination were established using synthetic (±)-*N*-hexanoyl-HSL (two peaks in an approximately 1:1 ratio), according to the conditions described above. The identification of the *R*- and *S*-*N*-hexanoyl-HSL retention times was obtained by analysis of the synthetic *S*-(−)-*N*-hexanoyl-HSL. The absolute configuration of the natural product was determined by comparing the retention times and relative abundances of the *R*- and *S*-(−)-*N*-hexanoyl-HSL in the synthetic racemic standard, in FRA32, and by co-injection of both samples (**Table 1**).

RESULTS AND DISCUSSION

Initially, the production of acyl-homoserine lactones in *E. psidii* was evaluated using *Agrobacterium tumefaciens* NTL4(pZLR4) as reporter, by a methodology adapted from the literature (*13*, *14*). This bioassay has been extensively employed in screenings of acyl-HSL producers, mainly because of its simplicity, quickness, and high sensitivity. This mutant cannot produce its own acyl-HSL by deletion of the *traI* gene. The detection system was inserted by the pZLR4 plasmid, which contains the *traR* gene and the fusion *traG::lacZ*. Exogenous acyl-HSL bind to the *TraR* receptor, and this complex regulates the expression of *lacZ* gene, which codifies the synthesis of β-galactosidase enzymes. Finally, the reagent X-Gal is metabolized by the β-galactosidase enzymes, yielding a blue color. Bioassays with *E. psidii* and *P. aeruginosa* (used as positive control) (*15*) exhibited blue coloration, furnishing evidence of the presence of acyl-HSL in both strains.

As previously reported (*15*, *16*), acyl-HSL are usually synthesized in low concentrations, requiring therefore large amounts of culture medium for spectroscopic investigations. Consequently, *E. psidii* extracts from 8 L of culture medium were purified by column chromatography, and fractions were analyzed by GC-MS. *N*-Hexanoyl-HSL was detected in 7% of relative abundance in fraction FRA32 (a mixture of 2.0 mg,) and in 1% in FRA33-35 (a mixture of 2.5 mg), and trace amounts of *N*-heptanoyl-HSL were identified in fraction FRA33-35 (**Figure 1**). To improve the detection, some analyses were carried out in the SIM mode. These substances were not detected in the blank analyses with NB medium (data not shown).

The final structural confirmation of both acyl-HSL was carried out by co-injection and fragmentation pattern comparison of the natural products with the synthetic ones in GC-MS. These synthetic standards were fully characterized by FTIR, GC-MS, ^{1}H, and ^{13}C NMR spectroscopy in one- and two-dimensions (^{1}H, ^{1}H gCOSY; ^{1}H, ^{13}C ^1J HSQC; ^{1}H, ^{13}C ^nJ gHMBC, $n = 2$ and 3; ^{13}C NMR DEPT-135 and DEPT-90; data not shown). The synthetic substances, ethyl acetate extract, and fraction FRA32 from *E. psidii* cultivation medium also exhibited positive bioassays with *A. tumefaciens* NTL4(pZLR4) reporter, corroborating the biological activity observed with the strain *E. psidii* IBSBF 435^T in vivo.

The absolute configuration of acyl-homoserine lactones plays an important role in communication systems (*11*). However, these substances are produced in low amounts and their analysis requires appropriate methodologies; in the present case, the chiral configuration of *N*-hexanoyl-HSL was determined using gas chromatographic analyses with chiral stationary phase and flame ionization detection. The optimum analytical conditions for the enantiomeric separation were obtained using a synthetic racemic mixture (*R* stereoisomer at 56.72 min and *S* stereoisomer at 56.89 min). Elution of synthetic stereoisomer *S*-(−)-*N*-hexanoyl-HSL (92% ee) under the same conditions furnished the retention time (56.93 min) for the *S* stereoisomer. Analysis of fraction FRA32, obtained from *E. psidii* culture medium, displayed a signal with retention time (56.97 min) very close to that of the synthetic *S* stereoisomer. Co-injection of the natural product and of the synthetic racemic mixture resulted in perfect signal overlap, with increase in the relative abundance of the *S* stereoisomer (56.96 min) in comparison with that of the *R* stereoisomer (56.79 min). Thus, the natural product was the *S*-(−)-*N*-hexanoyl-HSL (**Table 1**). The *N*-heptanoyl-HSL is a minor constituent (trace amounts), and the same procedure could not be applied. The methodology proved to be very efficient in situations where the amount of substance is small or when they are present in complex mixtures. Furthermore, chiral GC-FID is applied to determine the absolute configuration of a natural acyl-homoserine lactone for the first time, providing an additional tool in quorum-sensing researches.

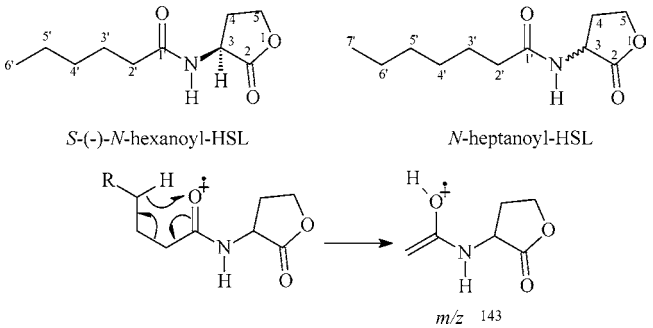

Figure 1. Acyl-homoserine lactones identified in extracts from *Erwinia psidii* IBSBF 435^T culture medium and origin of the diagnostic base peak fragments in MS spectra.

In conclusion, it was demonstrated that *Erwinia psidii* IBSBF 435^T produces acyl-HSL, which are well-recognized substances of the bacterial intercellular communication systems. *N*-hexanoyl-HSL is relatively common in quorum-sensing of Gram-negative bacteria, for example, controlling the violacein production (*17*) in *Chromobacterium violaceum* B., and is reported in trace concentrations in the phytopathogen *Pectobacterium chrysanthemi* S. (*18*). However, *N*-heptanoyl-HSL, with an odd carbon number acyl side chain, is an autoinducer of rare occurrence, previously reported in *Serratia marcescens* B. (*19*) and in *Rhizobium leguminosarum* (*20*). Therefore, this is the first report of this substance in *Erwinia* genus. Odd-chain fatty acids are biosynthesized by a route that exploits propionyl-CoA and malonyl-CoA as starter and extender of the acyl chain, respectively. Possibly, this biosynthetic route is explored by these microorganisms in the production of *N*-heptanoyl-HSL (*21*).

Further work should be done to understand the role of these substances in the intercellular communication system in *E. psidii*. As interferences in quorum-sensing mechanisms are promising avenues for bacterial control, it is expected that these studies could provide an alternative method to detain this guava phytopathogen.

ABBREVIATIONS USED

HSL, homoserine lactone; X-Gal, 5-bromine-4-chloro-3-indolyl-β-D-galactopyranoside.

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