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VIBRIO SP., A POTENTIAL PLANT GROWTH-PROMOTING BACTERIUM FROM MANGROVE ROOTS, PRODUCES FOUR TYPES OF ACYL HOMOSERINE LACTONE

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ABSTRACT

Gram-negative bacteria communicate with each other mainly through acyl homoserine lactones (AHLs), which after reaching a certain concentration, activate diverse bacterial processes including antibiotics synthesis, interbacterial conjugation, and swarming motility. It has been shown that AHLs are produced in the rhizosphere at concentrations sufficient to alter gene expression of other bacteria. However, the participation of AHLs in plant growth-promoting bacterial mechanisms has not been

elucidated, except for phenazine production by the biocontrol strain *Pseudomonas aureofaciens* and pea nodulation by *Rhizobium leguminosarum*.

Two AHL monitor systems, *Agrobacterium tumefaciens* NTL4 and *Pseudomonas aureofaciens* 30-841, were used to test for AHL synthesis by mangrove rhizosphere bacteria, which demonstrated that AHL production is a common phenomenon. One isolate, identified by 16S rRNA as *Vibrio* sp. strain LR6HC and a potential PGPB due to its phosphate-solubilizing activity and high production of IAA, responded to the two AHL monitor systems, suggesting that different types of AHLs were produced. HPLC and ESI MS/MS analysis of the ethyl acetate extract of *Vibrio* sp. strain LR6HC culture supernatant revealed C4-HSL, C6-HSL, C8-HSL, and 3-O-C8-HSL. There was at least 100 times more C4-HSL than C6-HSL, and about 10 times more C6-HSL than C8-HSL or 3-O-C8-HSL. The roles of these AHLs in the ability of *Vibrio* sp. strain LR6HC to solubilize phosphate and to synthesize IAA are currently being explored.

INTRODUCTION

The mangroves of the tropics are among the most productive ecosystems in the world, providing not only habitat but also nutrients in the form of detritus for a large number of organisms (Holguin et al., 2001). In spite of being highly productive, mangrove ecosystems from semiarid areas are generally nutrient-deficient, especially in N and P. This apparent paradox can be explained by the high rate of N_2 fixation associated with the roots and sediments, and by the presence of inorganic phosphate-solubilizing bacteria, which make phosphate available to the plants (Holguin et al., 2001).

Gram-negative bacteria typically communicate through *N*-acyl-L-homoserine lactones (AHLs), signal molecules that activate diverse bacterial processes including antibiotics synthesis, interbacterial conjugation, virulence, biofilm formation, and swarming motility, once they reach a certain concentration (Dunny and Winans, 1999; Winans and Bassler 2002).

The participation of AHLs in plant growth-promoting bacterial mechanisms is not known, except for two cases: in *Pseudomonas aureofaciens* 30-84, synthesis of the antibiotic phenazine against the fungal pathogen *Gaeumannomyces graminis* is regulated by the AHL molecule *N*-hexanoyl-L-homoserine lactone (Pierson et al., 1999), and in *Rhizobium leguminosarum*, AHLs are involved in the ability of the bacterium to nodulate peas (Rodelas et al., 1999).

In this paper we report on isolation from mangrove roots of AHL-producing bacteria with PGPB properties, including the PGPB *Vibrio* sp. strain LR6HC, with the ability to synthesize four types of AHL, of which the most abundant was *N*-butanoyl-L-homoserine lactone.

MATERIALS AND METHODS

Isolation and characterization of PGPB from the mangrove rhizosphere

The PGPB isolation strategy was based on N_2 fixers as described by (Holguin et al., 1992). N_2-fixation was tested through acetylene reduction assay (ARA) as described by Holguin et al. (1992). All cultures, including those that rendered

negative AR, were plated on solid N_2-free HGB medium (Holguin et al., 1992) until pure cultures were obtained. The bacteria were identified by ACCULAB (Newark, DE, USA) by 500 bp sequencing of the 16S RNA gene. Indole-3-acetic acid (IAA) was detected colorimetrically using the R1 reagent (Glickmann and Dessaux, 1995) in the supernatants of bacteria grown at 30°C in marine broth 2216, or liquid HGB with 0.5% final concentration glucose, with and without tryptophan (0.5 mg/ml). Phosphate-solubilizing activity was determined by plating on SRSM1 medium, as described by Vazquez et al., (2000).

Screening for AHL production in mangrove root bacterial isolates

Two AHL monitor systems were used to screen for AHL-producing isolates: i) *Agrobacterium tumefaciens* NTL4 (Luo et al., 2001), and ii) *Pseudomonas aureofaciens* 30-84I (Wood and Pierson, 1996). *A. tumefaciens* NTL4 and *P. aureofaciens* 30-84I were grown in AT minimal medium (Tempé et al., 1977) supplemented with 4.5 µg/ml tetracycline and 50 µg/ml streptomycin, and Luria Broth (LB), respectively. AHL production was tested with *A. tumefaciens* NTL4 by streaking a test isolate in parallel with the monitor strain on a plate of HGB agar supplemented with NH_4Cl (0.1%) and glucose. Tests with *P. aureofaciens* 30-84I were done as described by Pierson (2000), but in modified marine 2216 medium (1% peptone, 0.5% yeast extract, and 2% salts).

Detection, isolation, purification, and chemical characterization of AHLs

Supernatants from 72-hr isolate cultures grown in HGB medium with glucose, or in AT medium in the case of *A. tumefaciens* KYC6, were extracted twice with 400 ml acidified ethyl acetate (0.1 ml glacial acetic acid/L). The two ethyl acetate fractions were combined, evaporated dry with N_2 flow, reconstituted in ethyl acetate, and identified by TLC using *A. tumefaciens* NTL4 monitor strain, as described by Shaw et al. (1997).

For further characterization, the extract was evaporated and suspended in 150 µL acetonitrile (MeCN) and 50 µL water. The sample was centrifuged to remove insoluble material, and injected into a HPLC. A 1 x 25 cm Phenomenex Ultracarb 5 C-8 column was run at 3 ml/min with a gradient of 2 min at 10 % MeCN/ 90 % H_2O (v/v), then ramped to 100 % MeCN over 20 min and held at 100 % MeCN for another 15 min. Fractions (6 ml) were collected, evaporated dry with a centrifuging vacuum concentrator, redissolved in 10 µl MeCN. Then 0.2 µl samples were used for gas chromatography/mass spectrometric (GC/MS) analysis in a Hewlett-Packard (HP) model 5890 fitted with an HP model 5971A mass selective detector and an HP 6 m x 0.2 mm x 0.33 µm thick column of 5 % PH ME siloxane. All fractions were scanned by both fragmentation patterns and single ion monitoring (SIM) for fragments of masses 143, 172, and 185. Subsequently, the same fractions were treated with 150 µL MeCN and 50 µL 1 % formic acid in water, and subjected to electrospray ionization tandem mass spectrometry (ESI MS/MS), as described by Marketon et al. (2002).

RESULTS

Isolation of PGPB that synthesize AHLs

Ten of twenty isolates from mangrove roots synthesized AHLs. All isolates synthesized AHLs that were recognized by the *A. tumefaciens* NTL4 monitor system, while only two of them produced AHLs recognized by *P. aureofaciens* 30-84I. Most

isolates presented plant growth promoting properties such as N_2 fixation, inorganic phosphate solubilization, and IAA synthesis. Four of the AHL-producing isolates reduced acetylene when grown in N-free HGB medium.

Table 1. PGPB that synthesize AHLs

Isolate code	Name of closest match	PGPB properties	AHL recognized by
AG1HC	*Vibrio furnissii* 5.24*	IAA synthesis PO_4 sol.	*A. tumefaciens*, *P.aureofaciens*
LR6HC	*Vibrio fluviali* 4.28	IAA synthesis PO_4 sol.	*A. tumefaciens*, *P.aureofaciens*
AG1MCB	*Vibrio tubiashii* 2.79	IAA synthesis	*A. tumefaciens*
AG8AA	*Pseudoalteromonas nigrifaciens* 4.20	PO_4 sol.	*A. tumefaciens*
AG8AB	Pseudoaltermonas nigrifaciens 5.08	PO_4 sol.	*A. tumefaciens*
LR6B	*Pseudomonas stanieri* 7.74	N_2 fixation	*A. tumefaciens*
LR6A	*Pseudomonas stanieri* 7.22	N_2 fixation	*A. tumefaciens*
LR7YC	*Aeromonas enteropelogenes* 4.26	IAA synthesis N_2 fixation	*A. tumefaciens*
AG4BC	*Paracoccus marcusii* 2.12	IAA synthesis N_2 fixation	*A. tumefaciens*
AG1SC	*Labrys monachus* 7.25	IAA synthesis	*A. tumefaciens*

*The number refers to the percentage genetic distance to the closest match

Detection, isolation, purification, and chemical characterization of AHLs produced by *Vibrio* sp. strain LR6HC

Vibrio sp. strain LR6HC produced a compound that activated *A. tumefaciens* NTL4 (Fig. 1). Since the compound migrated on the TLC plate like the standard 3-O-C8-HSL and the extract of *A. tumefaciens* KYC6, it was identified tentatively as 3-O-C8-HSL.

Fig. 1. Acyl homoserine lactones (AHLs) chromatographed on a C18 reversed phase TLC plate developed with methanol/water (60:40). The plate was visualized using *A. tumefaciens* NTL4. Lanes are (1) *Vibrio* sp. strain AG1HC, (2) *A. tumefaciens* KYC6, (3) *Vibrio* sp. strain LR6HC, (4) 3-O-8C-HSL, and (5) 3-O-6C-HSL.

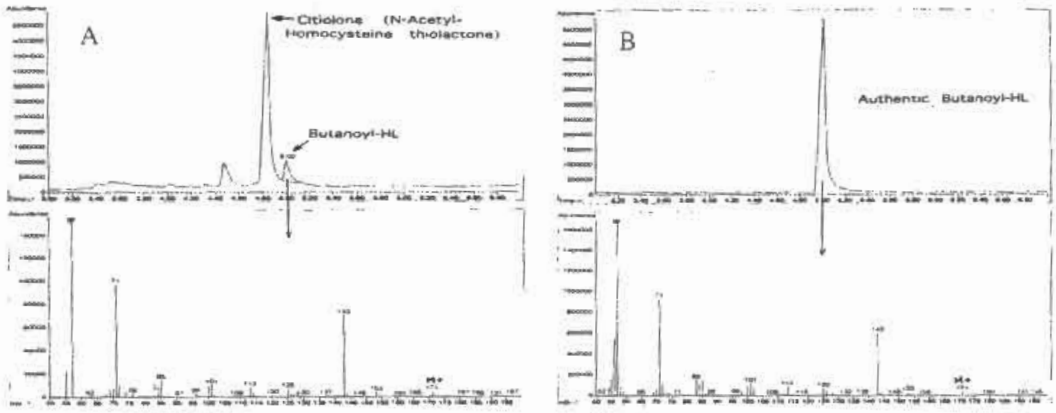

Fig. 2. HPLC chromatograms and GC/MS profiles of the peaks at 171 identified in A) *Vibrio* sp. strain LR6HC supernatant and in B) synthetic 4C-HSL.

A HPLC fraction having the same retention time as authentic 4C-HSL showed a peak in the GC/MS at the retention time, and had the fragmentation pattern expected for 4C-HSL (Fig. 2A, B). Similarly, the ESI MS/MS gave the fragments expected for 4C-HSL (Fig. 3). In addition, the ESI MS/MS clearly showed the presence of 6C-HSL, and suggested the presence of small amounts of 8C-HSL and 3-O-C8-HSL. There was at least 100 times more 4C-HSL than 6C-HSL, and about 10 times more 6C-HSL than 8C-HSL or 3-O-C8-HSL.

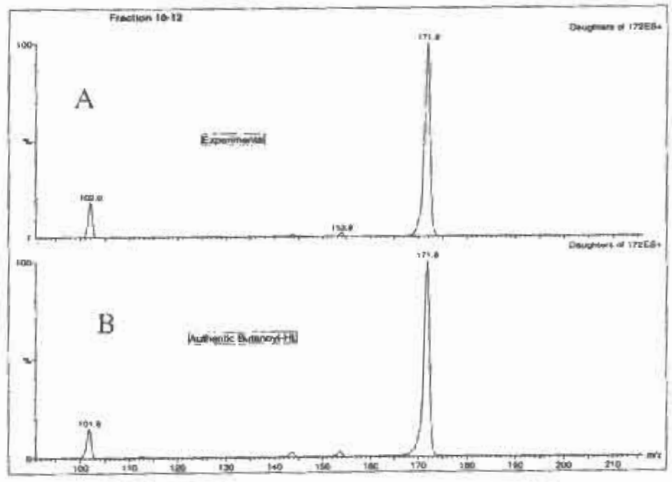

Fig. 3. ESI MS/MS spectra in A) the natural sample, and in B) synthetic 4C-HSL.

DISCUSSION

The PGPB isolation strategy rendered not only N_2-fixing isolates, but also bacteria with one or more plant growth-promoting properties. Our work is the first report of IAA-producing bacteria from the roots of plants growing in a marine environment. Screening of isolate AHL production using *A. tumefaciens* NTL4 and *P. aeruginosa* 30-84I monitor systems revealed that 50 % of isolates produced AHLs. In contrast to the high percentage (50 %) of isolates that produced AHLs recognized by

A. tumefaciens NTL4, only two (10 %) produced AHLs recognized by *P. aeruginosa* 30-84I.

One of the isolates, named *Vibrio* sp. strain LR6HC, with PO_4-solubilizing and IAA-synthesizing abilities, responded strongly to the *A. tumefaciens* NTL4 monitor system on agar and TLC, as well as to the *P. aurefaciens* 30-84I monitor system on agar, suggesting that the strain produced different types of AHLs. HPLC and ESI MS/MS analysis of the ethyl acetate extract of *Vibrio* sp. strain LR6HC culture supernatant revealed four types of AHLs: 4C-HSL, HHL, 8C-HSL, and 3-O-8C-HSL. There was at least 100 times more 4C-HSL than HHL, and about 10 times more HHL than 8C-HSL or 3-O-8C-HSL. 4C-HSL has been identified as a quorum-sensing signal molecule in four gram-negative bacteria: *P. aeruginosa*, (Pearson et al., 1997; Winson et al., 1995), *Serratia* sp. strain ATCC 39006 (Thomson et al., 2000), *S liquefaciens* MG1 (Eberl et al., 1999), and *Aeromonas hydrophila* and *A. salmonicida* (Swift et al., 1999).

The tidal exchange that mangrove trees experience makes the mangrove rhizosphere an extremely unstable environment, exposed to continuous changes in salinity, pH, nutrient availability, O_2 concentration, and temperature (Holguin et al. 2001). The high occurrence of PGPB that produce AHLs in mangroves suggests that these molecules probably play a major role in orchestrating physiological and genetic changes required for proliferating in such a changing environment.

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