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Quorum Quenching Bacteria Isolated from Rice and Tomato Rhizosphere Soil in Malaysia

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Abstract

A total of 45 bacterial isolates were obtained from rhizosphere soil of rice and tomato in Peninsular Malaysia, of which 6 have the ability to degrade quorum sensing signal molecules, N-acyl-homoserine lactones (AHLs), in vitro. The six isolates designated as SP17, SPB2, SPB9, CHB4, CHB18 and CHB19 were identified as *Bacillus thuringiensis*, *Bacillus sp*., *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus megaterium* and *Bacillus pumilus*, respectively, using 16S ribosomal RNA sequence-based identification. PCR amplification of AHL lactonase genes (*aiiA*) using *aiiA* primers revealed the presence of *aiiA* gene homologues in all of the isolates which may contribute to its AHL degradation ability. These AHL degrading bacteria have the potential for use as biological control agents against the papaya dieback disease pathogen, *Erwinia mallotivora*, in Malaysia, where their virulence and pathogenicity are regulated by AHL- mediated quorum sensing.

Keywords: *aiiA* genes, *Erwinia mallotivora*, quorum sensing, N-acyl-homoserine lactones, papaya dieback disease

INTRODUCTION

Simple unicellular organisms such as bacteria have a remarkable ability for social cooperation. In a competitive environment, teamwork and communication are fundamental for acquisition of food resources, niche colonisation and for disabling host defences. This cooperative behaviour relies upon a global level of regulation - "quorum sensing", where density-dependent diffusible signal molecules synchronize gene expression throughout the population [1-3].

Quorum sensing has been associated with several aspects of bacterial biology, including regulation of bioluminescence, regulation of antibiotic production, induction of virulence genes, Ti plasmid conjugal transfer, motility, swarming and biofilm formation. The signal compounds involved in quorum sensing in gram negative bacteria are N-acyl-L-homoserine lactones (AHLs) [4, 5] and they are produced by a multitude of different bacterial plant pathogens that use these compounds to regulate their virulence in a process termed "AHL-mediated quorum sensing" [5, 6]. "Quorum quenching", the method of disrupting or manipulating these AHLs, has therefore been proposed as a promising approach for control of plant-pathogenic bacteria [4, 7, 8].

Quorum quenching enzymes, such as N-acyl-homoserine lactonase found in rhizosphere soil bacteria, have the ability to degrade AHLs, thus can disrupt quorum sensing and in turn decrease bacterial virulence and symptoms of the associated infection [9]. In this study, we isolate and identify several bacterial isolates from rhizosphere soil of rice and tomato in Malaysia and demonstrate that they are capable of degrading AHLs in vitro as a first step for control of bacterial plant pathogens.

MATERIALS and METHODS

Bacterial strains, growth media and culture conditions

Chromobacterium violaceum strain CV026 was used as a biosensor in the detection of exogenous AHL (3-oxo-C6-HSL). *C. violaceum* CV026 and soil bacteria were cultivated at 28°C in Luria-Bertani (LB) medium. Escherichia coli strain TOP 10 was used in DNA cloning and cultivated in LB at 37 °C. The culture medium was supplemented with ampicillin (50µg/ml) for selection and X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) (Sigma Aldrich, USA) (40µg/ml) for detection of β-galactosidase activity when required.

Bacterial isolation

5 ml of sterile distilled water was used to suspend a 2.5 g soil sample, which was homogenised and serially diluted at ten-fold dilutions before plating on to LB agar and incubating at 28°C for 16 to 36 hours for isolation of single colonies. Pure cultures were acquired by repeatedly streaking the colonies on to fresh LB agar plates.

Bacteria screened for AHL degradation activity

AHL degradation activity of soil bacteria was assayed according to McLean et al. [10] with modifications. Briefly, 5µl of bacterial overnight culture was spotted onto LB agar supplemented with 10 µm of 3-oxo-C6-HSL (Fluka, USA) and incubated at 28°C overnight. The cultures were then overlain with 5 ml soft LB agar (LB broth with 0.5% w/v agar) containing 106 CFU/ml of the indicator strain *C. violaceum* CV026 which was cooled to 45°C. Plates were incubated at 28°C overnight awaiting observations of opaque zones.

16S rRNA gene amplification, cloning and sequence analysis

The Elute Bacterial Genomic DNA Extraction Kit (Sigma-Aldrich, USA) was used to isolate bacterial genomic DNA. 16S rRNA gene was amplified in a PCR reaction using thermostable DyNAzymeTM EXT DNA polymerase (Thermo Scientific, USA) with universal F8 and rP2 primer pair [11]. The PCR mixture was composed of: 1 × PCR buffer, 0.2 mM of dNTPs, 2.0 mM of MgCl2, 2.5 U of the enzyme mix, 2 µM of forward and reverse primers and 100 ng of bacterial genomic DNA in a 25 µl volume

reaction. The following cycling conditions were used: initial denaturation for 3 min at 95 °C, 30 cycles of 30 s at 94 °C, 1 min at 55 °C and 2 min at 72 °C, with a final extension of 10 min at 72 °C. PCR products were analyzed on 1 % agarose gels stained with ethidium bromide, purified using QIAquick Gel Extraction Kit (QIAGEN, Germany) and ligated into pGEM®T-Easy Vector (Promega, USA). QIAprep Spin Miniprep Kit (QIAGEN, Germany) was used to recover plasmids from recombinant *E. coli* clones, which were subsequently analyzed by restriction digestion and the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) search (http://www.ncbi. nlm.nih.gov/BLAST/).

AHL lactonase, *aiiA* homologue gene amplification

Bacteria with distinct colony morphologies were screened for the presence of *aiiA* homologues using PCR with a conserved primer pair for *Bacillus sp*ecies developed by Dong et al., 2002. PCR was performed using thermostable DyNAzyme™ EXT DNA polymerase (Thermo Scientific, USA) using similar conditions to those described for the 16S rRNA gene. PCR cycling conditions were initial denaturation for 3 min at 95 °C, with 30 cycles of 30 s at 94 °C, 45 sec at 55 °C and 1 min of 72 °C, and a final extension of 10 min at 72 °C. PCR products were subsequently analyzed on 1% agarose gels stained with ethidium bromide and visualized using U-Genius gel documentation system (Syngene, France).

RESULTS and DISCUSSION

C. violaceum CV026 is a mini Tn5 mutant of ATCC 31532 which lacks the ability to synthesize violacein, a purple pigment that is water-insoluble. The violacein production is restored in the presence of short chain AHLs (C4 to C6), that allow detection of AHLs using CV026 as a simple biosensor. A total of 45 bacterial colonies with distinct morphology were isolated on LB agar using serial dilution and spread plate method, of which 6 had the ability to antagonize violacein production by CV026 when 3-oxo-C6-HSL added as a medium supplement. After overnight incubation, white opaque zones were visible surrounding the spotted bacterial colonies in a purple background. The isolates were designated as SP17, SPB2, SPB9, CHB4, CHB18 and CHB19 and display significant AHL degradation activity as seen in Figure 1.

PCR analysis of the 16S rRNA gene from these six isolates using universal primers yielded PCR products of 1500 bp (Figure 2). Analysis of BLAST results for the 16S rRNA gene showed that SP17, SPB2, SPB9, CHB4, CHB18 and CHB19 belonged to the *Bacillus* genus and were identified as *Bacillus thuringiensis*, *Bacillus sp*., *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus megaterium* and *Bacillus pumilus*, respectively, with 99 to 100% nucleotide sequence identity. Members of the *Bacillus* genus are known to produce a variety of antimicrobial compounds including hydrolytic enzymes, peptide antibiotics [12, 13] and quorum quenching enzymes, such AHL lactonase, which are capable of degrading AHLs thus can control bacterial virulence [9, 14, 15]. The majority of AHL-degrading bacteria isolated from soil samples are from the *Bacillus* genus [16].

The six *Bacillus* isolates were further screened for the presence of *aiiA* homologues by PCR amplification of their genomic DNA with *aiiA* primers. The *aiiA* primers used in this study, developed by Dong et al. [9], were designed based on conserved regions at the 5' ends of the *aiiA* 240B1 and *aiiA* COT1 open reading frames (ORF) and the conserved sequences of 119 nucleotides in the 3' UTR. All six isolates produced PCR products of *aiiA* gene of the correct size, which is approximately 900 bp (Figure 3). This work is in accordance with Dong et al. [9, 14], where *Bacillus sp*ecies contained *aiiA* homologues and displayed AHL-degradation activity.

CONCLUSION

Bacteria which are capable of degrading AHL were successfully isolated from rhizosphere soil of rice and tomato in Malaysia and were identified as *Bacillus sp*ecies. These AHL degrading bacteria could potentially be used in biological control against the papaya dieback disease pathogen, *Erwinia mallotivora*, in which the virulence and pathogenicity are regulated by AHL-mediated quorum sensing. In addition, these locally-isolated AHL degrading bacteria which from the same habitat as the pathogen could use degradation of the AHL signal molecules to gain a competitive advantage. These findings also will increase the reliability of biological control using locally-isolated bacteria for degradation of pathogen signal molecules.

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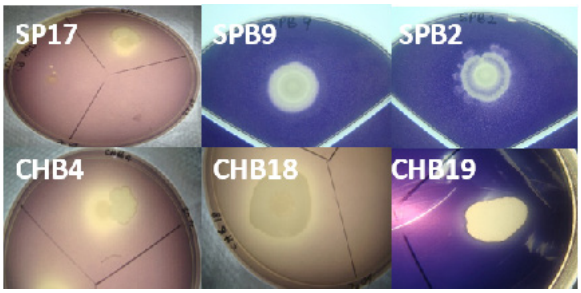

Figure 1: Inhibition of violacein synthesis in *C. violaceum* CV026 by rhizosphere soil bacteria in AHL plate assay.

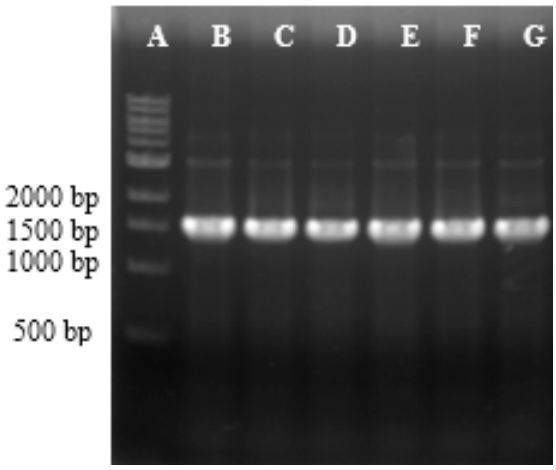

Figure 2: PCR amplification of soil bacterial isolates 16S rRNA gene. Lane A: 1 kb DNA ladder (New England Biolabs, USA); lane B: isolate SP17; lane C: isolate SPB2; lane D: isolate SPB9; lane E: isolate CHB4; lane F: isolate CHB18 and lane G: isolate CHB19. The amplicon size is approximately 1500 bp.

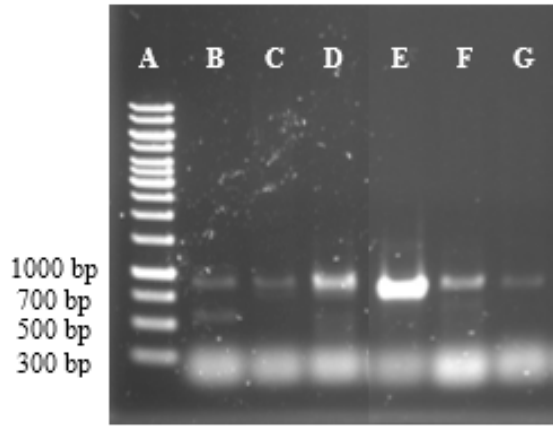

Figure 3: PCR detection of *aiiA* homologue gene from six *Bacillus* isolates. Lane A: HighRanger 1kb DNA ladder (Norgen Biotek Corporation, Canada); lane B: isolate SP17; lane C: isolate SPB2; lane D: isolate SPB9; lane E: isolate CHB4; lane F: isolate CHB18 and lane G: isolate CHB19. The amplicon size is approximately 900 bp.