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Acyl Homoserine Lactonase Genes from *Bacillus* Species Isolated from Tomato Rhizosphere Soil in Malaysia

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Abstract: Numerous gram negative bacterial plant pathogens use a cooperative cell to cell communication system known as quorum sensing to synchronize expression of genes essential for survival and virulence. N-Acyl-L-Homoserine Lactone (AHL) is the diffusible density-dependent signal molecule that is used in quorum sensing and is responsible for pathogenicity in most species of plant pathogenic bacteria including the papaya dieback disease pathogen, *Erwinia mallotivora*. Quorum quenching enzymes such as N-acyl-L-homoserine lactonase found in rhizosphere soil bacteria have the ability to degrade AHL and eliminate bacterial virulence therefore are key targets for disease control in plants. AHL inactivation genes coding for AHL lactonase were cloned from five *Bacillus* species isolated from tomato rhizosphere soil in Malaysia. Analysis of nucleotide sequences revealed an Open Reading Frame (ORF) of 753 bp which encodes a polypeptide of 250 amino acids with a predicted molecular weight of 28 kDa. BLAST analysis showed that the 753 bp nucleotide sequence was homologous with the AHL lactonase gene (*aiiA*) from *Bacillus* species in the GenBank database with 88-99% nucleotide sequence identity. The deduced AHL-lactonases have 90-100% amino acid identity with *Bacillus* aiiA proteins and belong to the group of metallo-beta-lactamase superfamily proteins.

Key words: AHL lactonase, *Bacillus* species, N-acyl-homoserine lactones, quorum quenching, quorum sensing

INTRODUCTION

Cell to cell communication mechanisms found in many bacterial pathogens are termed Quorum Sensing (QS) and play an essential role in the regulation of expression of genes essential for survival and host virulence. The QS bacteria use small diffusible molecules to synchronise expression of target genes throughout a population. N-Acyl-L-Homoserine Lactone (AHL) has been determined to be the main signal molecule involved in quorum sensing in gram-negative bacteria and is among the most well-characterized cell to cell communication molecules. Generally, mutants lacking AHL are non-pathogenic, therefore it is likely that the manipulation or disruption of genes coding for AHL will result in a loss of virulence due to the down-regulation of virulence genes.

In several fields, the interruption of quorum sensing, often referred to as quorum quenching has been proposed as one of the most promising disease management strategies due to its ability to decrease bacterial virulence (Hentzer and Givskov, 2003; Molina *et al*., 2003). Recently, many bacterial AHL-degrading enzyme producers have been isolated from soil and plant samples and identified from bacterial culture collections in laboratories (Dong *et al*., 2000; Morohoshi *et al*., 2009). This study describes the cloning and sequence analysis of AHL lactonase genes from five *Bacillus* species isolated from tomato rhizosphere soil in Malaysia.

MATERIALS AND METHODS

Bacterial strains and growth conditions: The bacterial strains used in this study (CH4, CH13, CHB10, CHB18 and

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CHB37) were isolated from rhizosphere soil of highland tomato in Malaysia and previously identified as *Bacillus thuringiensis*, *Bacillus* sp., *Bacillus acidiceler*, *Bacillus megaterium* and *Bacillus* cereus, respectively (Data not shown). All *Bacillus* isolates and *Escherichia coli* TOP 10 cultures were cultivated at 37°C in Luria-Bertani medium. The medium was supplemented with ampicillin (50 μg mL^{-1}) when required and X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) (Sigma Aldrich, USA) (of 40 μg mL^{-1}) was added to the medium for detection of β-galactosidase activity.

Genomic DNA extraction and PCR amplification of AHL lactonase gene (*aiiA*): Bacterial genomic DNA was isolated using GenElute Bacterial Genomic DNA Extraction kit (Sigma-Aldrich, USA) following the manufacturers protocol. PCR amplification was performed in a PTC-200 thermal cycler (MJ Research, USA) in a 25 μL reaction using thermostable DyNAzyme™ EXT DNA polymerase (Thermo Scientific, USA).

The PCR mixture consisted of 1×PCR buffer, 0.2 mM of dNTPs, 1.5 mM of $MgCl_2$, 1 μM of forward and reverse primers, 100 ng of bacterial genomic DNA as a template and 2.5 U of the enzyme mix. A primer pair from Dong *et al.* (2002) was used to amplify the AHL lactonase gene. The following cycling conditions were used in the amplification reaction: initial denaturation for 3 min at 95°C and then 30 cycles of 30 sec at 94°C, 45 sec at 55°C and 1 min of 72°C with a final extension of 10 min at 72°C. A 1% agarose gel was used to visualize PCR products. PCR products were subsequently purified using QIAquick Gel Extraction Kit (QIAGEN, Germany).

Cloning and sequence analysis of AHL lactonase gene: Purified PCR products were cloned directly into a vector using pGEM®T-Easy Vector System (Promega, USA). Plasmids of recombinant clones were recovered using QIAprep Spin Miniprep kit (QIAGEN, Germany) followed by analysis with restriction endonucleases to ascertain presence of vector insert. The clones were sequenced commercially and analyzed with a Basic Local Alignment Search Tool (BLAST) search on the non-redundant data bank of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). The AHL lactonase amino acid sequences were aligned using Clustal Wand were then analyzed with ExPASy: SIB Bioinformatics Resource Portal tools (http://www.expasy.org/tools/).

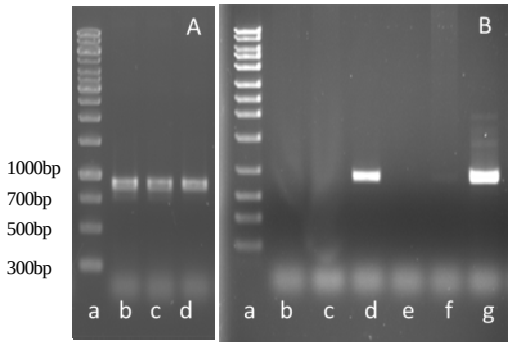

Fig. 1: PCR amplification of AHL lactonase genes (*aiiA*) from five *Bacillus* isolates. (A) Lane a: Highranger 1 kb DNA Ladder (Norgen Biotek Corporation, Canada); Lane b: CHB 10; Lane c: CHB18; Lane d: CHB37. (B) Lane A: Highranger 1kb DNA Ladder (Norgen Biotek Corporation, Canada); Lane d: CH4; Lane g: CH13. The expected amplicon size is ~900 bp

RESULTS AND DISCUSSION

PCR was used to clone the AHL lactonase genes (*aiiA*) from five *Bacillus* species isolated from tomato rhizosphere soil in Malaysia (Fig. 1). The sequences of five AHL lactonase genes from strains CH4, CH13, CHB10, CHB18 and CHB37 contained an Open Reading Frame (ORF) of 753 bp nucleotide which when translated, encodes a polypeptide of 250 amino acids with a predicted molecular mass of 28 kDa.

Sequence analysis of the five *aiiA* genes cloned from *Bacillus* species indicated high conservation with nucleotide sequence identities ranging from 88-99% when compared with the nucleotides of AHL lactonases in the GenBank database. The AHL lactonase-encoding genes are present in numerous species of *Bacillus*, especially the species in the *B. cereus* group including *B. cereus*, *B. thuringiensis*, *B. mycoides* and *B. anthracis* (Dong *et al*., 2002, 2004; Dong and Zhang, 2005; Lee *et al*., 2002).

The presence of thse *aiiA* gene in *B. subtilis* and *B. amyloliquefaciens* belonging to the *B. subtilis* group has been reported previously (Pan *et al*., 2008; Yin *et al*., 2010).

The deduced AHL-lactonases have 90-100% amino acid identity with *Bacillus* aiiA proteins. Based on a protein sequence search using the pfam protein family database at ExPASy proteomics server (http://br.expasy.org/tools/), the five AHL lactonases were found to be

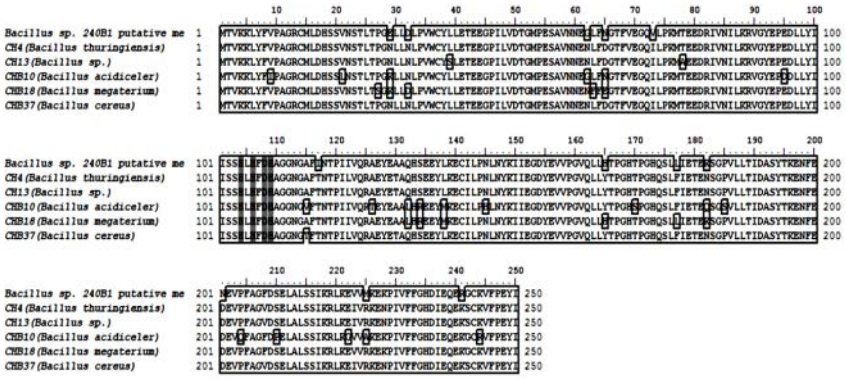

Fig. 2: Alignment of five AHL lactonase (*aiiA*) from *Bacillus* species isolated from tomato rhizosphere soil with AHL lactonase from *Bacillus* sp. 240B1. The conserved motif for metallo-beta-lactamase, HXHXDH is indicated by shading

grouped into the metallo-beta-lactamase superfamily of proteins. A conserved motif for metallo-beta-lactamase, HXHXDH was found in AHL lactonase of all of isolates (Fig. 2). A previous study has shown that several histidine and glutamate residues, i.e., H106, D108, H109, H169 and D221 of aiiA from *Bacillus* sp. 240B1 are conserved and required for AHL lactonase activity (Dong *et al*., 2000).

Similarly, these residues are also conserved in our newly identified putative AHL-inactivating enzymes. We also discovered amino acid diversity of AHL lactonases between the five *Bacillus* isolates (showed in boxes) and it would be interesting to investigate the roles of this sequence diversity in bacterial ecology and interaction. The discovery of AHL lactonases in this study suggests that these local *Bacillus* isolates could be used as a bio-control agent with quorum quenching properties in control of plant pathogenic bacteria.

CONCLUSION

As a conclusion, five genes encoding AHL-inactivating enzymes were successfully cloned from *Bacillus* strains isolated from rhizosphere soil of tomato in Malaysia. A high level of homology to the *aiiA* gene from *Bacillus* sp. that encodes AHL lactonase 240B1 was evident. Detailed information of AHL lactonase genes existing in our local *Bacillus* isolates will be helpful for elucidation of the biocontrol mechanism of *Bacillus* sp. as well as the practical applications of the strains. In addition, these AHL genes could potentially be used for the production of transgenic papaya resistant to papaya dieback disease caused by the bacterial pathogen *Erwinia mallotivora* in the near future.

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