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Rapid isolation method of soil bacilli and screening of their quorum quenching activity

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Abstract. In Gram-negative bacteria, quorum sensing is mediated by *N*-acylhomoserine lactones (AHL) to regulate different biological functions, including production of virulence factors. Quorum quenching refers to the interruption of quorum sensing, and the most attractive way is to degrade the AHL molecules. With the aim of isolating soil bacteria capable of blocking quorum sensing by inactivating AHL, a novel enrichment method has been designed. Bacteria degrading AHL were isolated from a Malaysian soil sample. We identified twelve isolates which possessed an *aiiA* homologue gene by using PCR approach and three strains were selected for further analysis. AHL-inactivation assay showed that these three isolates rapidly degraded synthetic 3-oxo-C6-HSL *in vitro*. Morphological phenotypes and 16S ribosomal DNA sequence analysis indicated that these isolates fell into the genus of *Bacillus*. Our data suggest that soil *Bacillus* spp. can be isolated using the selection method and a number of soil bacilli in Malaysian soil exhibited quorum quenching activity.

Keywords. AHL-inactivation assay, N-acylhomoserine lactones, quorum sensing, quorum quenching, 3-oxo-C6-HSL, 16S ribosomal DNA

INTRODUCTION

In Gram negative bacteria cell-to-cell communication (quorum sensing) is achieved via the production and sensing of signalling molecules called *N*-acylhomoserine lactones (AHLs). AHLs differ in the length and degree of saturation of the acyl chain (4 to 18 carbons; presence or absence of a double bond) and substituent at the 3-position of their *N*-acyl side chains (no substituent, keto or hydroxy; for review see Chhabra *et al.,* 2005). Arguably the most convenient way to detect AHLs is using a biosensor based on the AHL-dependent regulation of the purple pigment violacein by a mutant of *Chromobacterium violaceum* (CV026). This mutant is unable to make AHLs but responds to their presence by synthesising violacein (McClean *et al.*, 1997).

The potential for microbiological degradation of these signals is important for several reasons. Since AHL-mediated signalling mechanisms are widespread and highly conserved in many pathogenic bacteria, they can be attractive targets for novel anti-infective therapies (Williams *et al.*, 2002). Quorum quenching bacteria sharing the same habitat with quorum sensing bacteria could gain a competitive advantage by degrading AHL signal molecules. Enzymes that degrade AHLs might have commercial value as a means to manipulate cell-to-cell signalling. Since AHLs are stable under slightly acidic conditions but not in alkali media (Yates *et al.*, 2002), bacterial degradation of AHLs could play an important role in the environment such as in soil. Two types of AHL degrading enzymes have been documented: AHL lactonase (e.g. AiiA, from *Bacillus* sp., Dong *et al.*, 2002) and AHL-acylase (Lin *et al.*, 2003).

A medium for isolation of soil bacteria that can metabolise AHLs has been designed by Leadbetter and Greenberg (2000). However, to date, there is no reported method targeted isolation of quorum quenching soil bacilli. It has been reported that AHL lactonase is very common in *Bacillus* sp. (Dong *et al.*, 2002). In this paper, a simple, rapid and efficient method for isolation of quorum quenching soil bacilli is described.

MATERIALS AND METHODS

Bacterial strains, growth media and culture conditions. *Chromobacterium violaceum* strain CVO26 was used as a biosensor to detect exogenous AHLs (3-oxo-C6-HSL). Violacein is inducible by short chain AHL compounds with *N*-acyl

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side chains ranging from C_4 to C_8, with different sensitivity (McClean *et al*., 1997). Unless stated otherwise, soil bacteria and CVO26 were grown at 28 ºC.

Escherichia coli DH5α served as negative control in AHL inactivation assay and *E. coli* strain JM109 was used as a host for DNA cloning. For growth of *E. coli*, Luria–Bertani (LB) broth or agar was used and cultures incubated at 37 ºC.

All media used in this study (including LB) were buffered with 50 mM 3-[*N*-morpholino] propanesulfonic acid (MOPS) to pH 6.8, to prevent spontaneous degradation of AHLs. Where necessary, growth media were supplemented with ampicillin (100 μg/ml).

Soil sample processing and isolation of soil bacilli. Soil samples were collected from Rimba Ilmu (University Malaya) and sampling was done at the surface and subsurface using a sterile spatula. Soil samples were placed in sterile plastic bags. The soil was processed by removing all large particles and plant materials such as leaves. Each soil sample (20 g) was suspended with 20 ml of sterile distilled water in a sterile universal bottle. Soil suspensions were vortexed and placed in a water bath with temperature adjusted to 100ºC. Heat treatment of the soil suspensions was performed at 100ºC for 5 min with gentle shaking. After heat treatment, heat-treated soil suspensions were incubated at room temperature for 2 h and serially diluted prior to plating on LB agar for isolation of single colonies. Plates were incubated at 28 ºC for 16 h. Pure colonies were obtained by repetitive dilution streaking.

PCR amplification of aiiA homologue gene and 16S rDNA gene. Before PCR was carried out, PCR mix, *Taq* DNA polymerase, and water were subjected to treatment with RNase-free DNase I (0.1U) for 30 min at 37°C. The volume of the reaction mixture per tube was 50 μl. DNase I was inactivated by heating at 95°C for 30 min in an automated DNA Thermal cycler (Perkin Elmer GeneAmp PCR System 2400, Applied Biosystems).

Bacteria with distinct morphology were screened for presence of *aiiA* homologue gene by amplifying their *aiiA* homologue gene sequence using PCR. The thermal cycling programs for PCR consisted of initial denaturation at 94ºC for 10 min, 35 cycles of 94ºC (30 s), 52ºC (30 s), 72ºC (1 min); followed by primer extension at 72ºC for 5 min. The forward and reverse primers used were *aiiA*F (5'-ATGGGATCCATGACAGTAAAGAAGCTTTAT-3') and *aiiA*R (5'-GTCGAATTCCTCAAC AAGATACTCCTA-ATG-3'), respectively.

For PCR of 16S rDNA genes, PCR conditions were as follows: initial denaturation at 94 ºC for 5 min; followed by 30 cycles of denaturation (94 ºC for 30 s), annealing (59 ºC for 30 s) and extension (72 ºC for 1 min 30 s). Final extension was initiated at 72 ºC for 5 min. The forward and reverse primers used were 27F (5'-AGAGTTTGATC(M)TGGC-TCAG-3') and 1525R (5'-AAGGAGGTG(W)TCCA(R)-CC-3'), respectively. For all PCR, negative controls were included which purified chromosomal DNA or colony DNA is replaced by water.

Sequences of 16S rDNA products were determined using an ABI3700 automatic sequencer (Applied Biosystems), with M13 forward and M13 reverse primers. Routinely, PCR products of 16S rDNA showing correct size as determined by agarose electrophoresis were gel excised, column-cleaned and cloned into the pGEM® -T Easy vector (Promega, USA) according to the manufacture's instructions followed by transformation into *E. coli* JM109 (Sambrook *et al*., 1989.)

16S rDNA nucleotide sequence analysis. Sequences were edited to exclude the PCR primer binding sites and manually corrected with Chromas 2 (Chromas Version 2.22; www.technelysium.com.au/chromas.html). Sequence alignments were carried out using MEGA 3.1 software (http://www.megasoftware.net/). For identification of closest relatives, newly determined sequences were compared to those available in the GenBank (www.ncbi.nlm.nih.gov) databases using the standard nucleotide–nucleotide BLAST program (www.ncbi.nlm.nih.gov), followed by phylogenetic analysis with MEGA 3.1.

Whole-cell AHL inactivation assay. Bacterial cells grown overnight at 28°C with shaking (220 rpm) in LB medium were centrifuged for 10 min at 7000 x *g*. The cells were resuspended in 100 ml Tris-HCl (100 mM, pH6.5) and washed twice in the same buffer. Cell pellets were resuspended in 100μl 0.1M Tris-HCl (pH 6.5), equalised to an OD_{600} of 1.0 and used directly as a source of resting cells for *in vivo* AHL inactivation assays. The synthetic 3-oxo-C6-HSL was obtained from Sigma.

Aliquots of 3-oxo-C6-HSL (10 μg/μl) in absolute ethanol were dispensed into sterile tubes and the solvent evaporated to dryness under sterile conditions. Resting cells were used to rehydrate the 3-oxo-C6-HSL to a final concentration of 0.10 μg/μl. The mixtures were incubated at 37 ºC for 4.5 h with gentle shaking in a hybridization oven. Heat denatured suspension (10 μl) was inoculated onto LB agar seeded with the bioreporter CVO26 and incubated at 28 ºC. *E. coli* strain DH5α served as negative controls. Disappearance of 3-oxo-C6-HS from the mixture was assessed at t + 0 h, t + 1.5 h, t + 4.5 h using CVO26 as bioreporter. Degradation of 3-oxo-C6-HSL is evident by loss of purple pigmentation shown by CV026 and the results were digitally recorded.

RESULTS

Isolation of soil bacteria and detection of aiiA homologue gene. Using the heat-treatment method, approximately 10^3 to10^4 cfu/ml was observed and bacteria colonies were randomly selected for further studies. A total of 50 bacterial isolates were screened for presence of an *aiiA* homologue

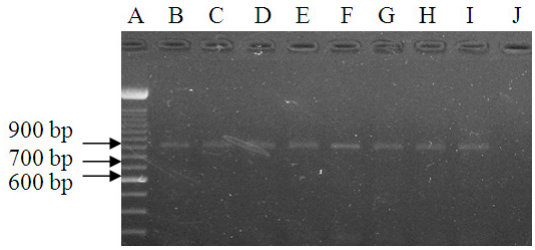

Figure1. PCR detection of aiiA homologue gene. Lane A: 100 bp Plus DNA ladder (Invitrogen), lane B: isolate 12; lane C: isolate 20; lane D: isolate 29; lane E: isolate 2; lane F: isolate 8; lane G: isolate 9; lane H: isolate 10; lane I: isolate 18; lane J: negative control. Arrows show the size of the DNA ladder. The expected amplicon size is approximately 800bp.

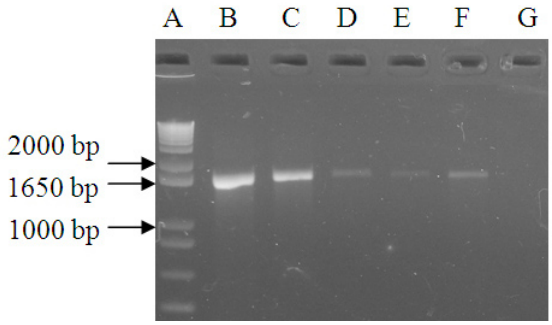

Figure 2. PCR amplification of soil bacterial isolates 16S rDNA genes. Lane A: 1 kb Plus DNA ladder (Invitrogen); lane B: isolate 8; lane C: isolate 12; lane D: positive control 1; lane E: isolate 29; lane F: positive control 2; lane G: negative control. Arrows show the size of the DNA ladder. The expected amplicon size is approximately 1500bp. Positive control: recombinant plasmid with 16S rDNA gene; negative control: PCR mixtures without DNA template

gene by PCR and 12 bacteria with an *aiiA* homologue gene amplified by PCR were observed. The expected amplicon size was approximately 800 bp (Figure 1).

Molecular identification of soil bacterial isolates. PCR amplification of the 16S rDNA gene produced the expected amplicon size of approximately 1.5 kb (Figure 2). 16S rDNA sequence analyses of soil bacterial isolate 12, isolate 8 and isolate 29 (Figure 2, lane B, F, and D, respectively) were essentially as described in Materials and Methods. Web-based similarity searches against the GenBank and database suggested that soil bacterial isolate 8 belongs to the genus *Bacillus*. The 16S rDNA (841 nucleotides) of bacterial isolate 8 share 99.0% sequence identity with the 16S rDNA of *Bacillus cereus* (GenBank accession number AM020677). Bacterial isolates 12 (861 nucleotides) and 29 (861 nucleotides) both share 99.0% 16S rDNA sequence identity with *Bacillus thuringiensis* (GenBank accession number AE017355) and *Bacillus cereus* strain G8639 (GenBank accession number AY138271), respectively.

Whole-cell AHL inactivation assay. Bacterial isolates that possessed *aiiA* homologue genes were randomly selected for AHL inactivation assay. A representative result is shown in Figure 3. Nearly all 3-oxo-C6-HSL was degraded after incubating with soil bacterial isolates for 1.5 h, indicating rapid AHL degradation. Only residual 3-oxo-C6-HSL was detected by CVO26 when the reaction was stopped after incubation for 1.5 h (Figure 3, left panel). *E. coli* DH5α served as negative controls, no apparent AHL degradation was observed (Figure 3, right panel).

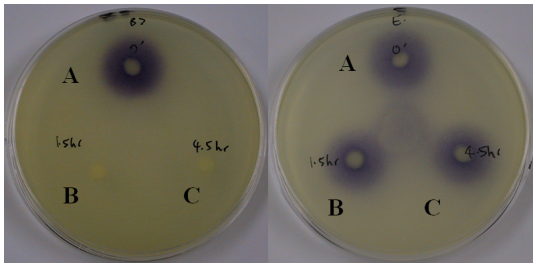

Figure 3. AHL inactivation assay. Soil bacterial isolate incubated with 3-oxo-C6-HSL for 0 h (A), 1.5 h (B) and 4.5 h (C). Left panel: Soil bacterial isolates; right panel: E. coli DH5α (negative control). A representative result is shown. Pigment formation indicates the presence of 3-oxo-C6-HSL, disappearance of 3-oxo-C6-HSL is evident by loss of pigment formation on the bioreporter lawn.

DISCUSSION

A simple heat treatment selective method that is efficient for the rapid isolation of soil bacilli is reported here. Non-sporulating microbes were minimized by heat treatment at 100ºC for 5 min thus avoiding the problem inherent in enrichment culture.

The DNase treatment of the PCR mixtures successfully eliminated all unwanted DNA. 16S rDNA PCR using universal primers can otherwise amplify the conserved regions of the eubacteria 16S rDNA genes giving unclear results. Our data suggest that this simple DNase treatment represents an effective step to eliminate unwanted DNA prior to the PCR amplification.

It has been reported that *Bacillus* sp. 240B1 is an AHL-degrading bacterium isolated from soil (Dong *et al.,* 2000). In a more recent study, it has been documented that *Bacillus* species are the main AHL-degrading bacteria that are readily isolated from soil samples (D'Angelo-Picard *et al.*, 2005). The present work is in agreement with Dong *et al.*, (2002) that bacilli are commonly found in the soil and putatively

that these bacteria possessed the *aiiA* homologue genes that confer AHL-degradation activity. The *aiiA* primers used in this study were designed based on identical sequences at the 5' ends of the $aiiA_{240B1}$ and $aiiA_{COTI}$ open reading frames and a conserved region of 119 nucleotides after the stop codon (Dong *et al*., 2002). A total of 50 bacteria were isolated and screened for the presence *aiiA* homologue gene. Among them, 12 bacterial isolates contained *aiiA* amplicons of the correct size.

The *aiiA* gene amplification by PCR is a preliminary screening for quorum quenching activity. A total of three bacterial isolates containing the *aiiA* gene were selected to confirm their QQ activity by AHL inactivation assay. Using the whole-cell AHL-inactivation assay, all of these three isolates showed rapid 3-oxo-C6-HSL degradation *in vitro*. The pH of the reaction mixture was buffered to pH 6.5 to avoid alkali lactonolysis because AHLs are easily hydrolyzed under alkaline conditions (Yates *et al*., 2002). AHL concentration of 0.1 μg/μl was determined empirically and selected as the most optimal 3-oxo-C6-HSL concentration under our experiment condition based on the pigment formation of the bioreporter (data not shown).

Analysis of the 16S rDNA sequences showed that the heat-treatment method used in this study will preferentially select bacilli. Furthermore, a majority of these isolates appeared to possess an *aiiA* homologous gene. The AHL-inactivation assay indicated that these isolates can rapidly degrade the 3-oxo-C6-HSL.

The method developed here coupling heat treatment and PCR techniques to screen for *aiiA* homologue genes followed by a rapid AHL inactivation assay, was readily isolate soil bacilli exhibiting quorum quenching activity from a tropic soil sample. This is the first report of selection of bacilli with quorum quenching activity in tropical soils using the technique described in the present work. This method should provide a powerful isolation and study of such organisms from a wide variety of soils.

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