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THERMOSTABLE ACYL HOMOSERINE LACTONASE *FROM Bacillus cereus* SM01 AND ITS QUORUM-QUENCHING ACTIVITIES ON *Burkholderia pseudomallei* AND METHICILLIN RESISTANT *Staphylococcus aureus*

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Abstract

N-acyl homoserine lactone (AHL) plays an important role in bacterial quorum sensing. Bacterial cells use AHL molecules to coordinate the expression of various virulence determinants and biofilm development within the population. Therefore, quenching the activity of these molecules offers an alternative strategy to control bacterial infection. In this study, isolate *Bacillus cereus* SM01 was found to possess enzymes that could inactivate AHL molecules, rendering them unfit for quorum sensing. This study describes the characterization and quorum quenching activities of recombinant AHL-lactonase (AiiA) cloned from *B. cereus* SM01. Using gene-specific primers, a 753 base pair *aii*A homologue that codes for AHL-lactonase were successfully amplified from *B. cereus* SM01 genomic DNA and cloned into the pCOLD expression system. Homology comparison of the deduced amino acid of the cloned DNA sequence showed that it was highly identical with known AHL-lactonases from the *Bacillus* group and belonged to the metallo-beta-lactamase superfamily of proteins. The resultant 29kD recombinant AHL lactonase protein possessed broad pH tolerance (from pH 5 up to 11) and good thermal stability. Prolonged exposure of the recombinant AHL-lactonase to high temperatures (65°C or 85°C, 1 hour) did not diminish the AHL-inactivating activity. It successfully quenched the activity of the quorum-sensing molecules of *Burkholderia pseudomallei* (*B. pseudomallei)* UKMS01. The recombinant AHL lactonase also significantly reduced biofilm development in methicillin-resistant *Staphylococcus aureus* and *B. pseudomallei* by 3.4 folds and 4.8 folds, respectively.

INTRODUCTION

Continuous use of antimicrobials to control bacterial infections has led to the emergence of multidrug-resistant microbes. An alternative strategy to control bacterial infection is to minimize the expression of virulence determinants by the bacterial pathogen. Bacteria have evolved complex cell-to-cell communication mechanisms in which quorum sensing (QS) signaling molecules are released into the extracellular environment and consequently detected by a sensor protein [1]. In bacteria, QS is mediated by diffusible small molecules of various structural classes. N-acyl homoserine lactones (AHL) are among the best characterized of these signaling molecules [2]. They share a very similar basic structure, consisting of a homoserine lactone ring and an acyl chain that can vary in length and degree of saturation [1].

Interference of QS can occur by enzymatic degradation of the AHLs [3]. These quorum-quenching enzymes termed AHL-lactonases, catalyze the hydrolysis of the homoserine lactone ring of AHL [4]. Inactivation of these AHL molecules would consequently lead to dysfunctional QS systems and crippling of QS-related activities such as bacterial virulence, biofilm formation, and swarming motility [5]. Thus, disruption or inhibition of the signaling molecules in the QS systems of bacterial pathogens could

potentially attenuate expressions of their virulence traits [6].

Unlike antibiotic therapy that acts upon individual cells, management of bacterial infection through quorum-quenching strategy has the advantage in that it does not affect the viability of the individual cells, but rather the virulence of the population as a whole [7]. Adopting quorum-quenching approach in the management of bacterial infections could be an alternative solution to the management of emerging multi-resistant bacteria strains that evolved as a consequence of prolonged exposure to antibiotics [8, 9]. Enzymes such as AHL-lactonases and AHL-acylases that could quench the QS activity, therefore, offers a promising strategy to manage bacterial infections in which their virulence is regulated by autoinducers such as AHL.

In lieu of the attractive potential of AHL lactonases and AHL acylases as candidates for future drug development, an attempt to isolate and identify bacteria capable of interfering with AHLs-mediated quorum sensing systems from biodiversity rich tropical soils was initiated. The search for lactonase producing bacteria was targeted towards Gram-positive sporulating Bacilli since the presence of AHL lactonases and acylases are reported to be quite common amongst this group of bacteria.

MATERIALS AND METHODS

Bacillus cereus sp. SM01 and *Chromobacterium violaceum* CV026 were maintained at room temperature in Luria Bertani (LB) and Nutrient Broth (NB) media, respectively. All *Escherichia coli* strains, *Staphyloccus aureus* ATCC 33591 and *Burkholderia pseudomallei* UKMS01 were grown at 37ºC in LB. For *E. coli* strains harboring pCold I plasmids, ampicillin (100 µg/ml) was added to their growth medium. Kanamycin (25 µg/ml) was added when growing *C. violaceum* CV026.

Isolation and Screening of Soil Bacilli for AHL-inactivating Activity

Qualitative detection of AHL-inactivating activity was carried out according to [11] with *C. violaceum* CV026 [12] as the biosensor strain. Prior to use, *C. violaceum* CV026 was grown overnight at room temperature in NB/kanamycin (25 µg/mL).

About 20 g of subsurface soil samples were resuspended with 20 mL of sterile distilled water. The soil sample was heat-treated at 100ºC, for 10 min and then left to sit at room temperature for 3 h. Subsequently 1 mL of the suspension was transferred into sterile 9 mL distilled water. This mixture was then serially diluted before being inoculated on LB agar and incubated at room temperature for 24 h. For initial screening, each bacterial isolate was inoculated in 1 mL of LB and incubated overnight at 28ºC. Subsequently, 50 µL of 10 µM N-octanoyl homoserine lactone, C8-AHL (Sigma Biochemicals, USA) was added to the culture and further incubated at 28ºC for 6 h. The bacterial cells were centrifuged at 4 000 g for 10 min and the supernatant was transferred to a sterile tube. After exposure to ultra-violet (UV) light for 30 min, 5 µL of the supernatant was spotted onto *C. violaceum* CV026 lawn. The *C. violaceum* CV026 plates were incubated for 24 h at 28ºC and then inspected to detect the expression of purple pigment by CV026. One isolate, identified as SM01, was detected to possess AHL-inactivating activity and used for subsequent studies.

Determining the Nature of the AHL-inactivating Enzyme

To determine the nature of AHL-inactivating enzyme produced by SM01, acid test was performed [11]. For this test, two reaction tubes, each holding 100 µl of overnight SM01 supernatant previously incubated in the presence of C8-AHL were used. An aliquot of 10 µl of 1M HCL was added to the first tube (acid reaction tube) while the second tube (control) was added with 10 µl of distilled water. Both tubes were incubated for 3 h, at room temperature. Subsequently, 5 µl of reaction mixture from each of the tubes were spotted onto CV026 lawn grown on NA plates and incubated for 24 h at room temperature.

Identification of AHL-lactonase Positive Soil Isolate SM01

The identity of SM01 was determined by 16S rDNA sequencing. The genomic DNA of SM01 was prepared according to [13]. PCR amplification was carried out using primers 16F (5'-CAGGCCTAACACATGCAAGTC-3') and 16R (5'-GGGCGGwGTGTACAAGGC-3'). The PCR mixture contained 5 µL 10X reaction buffer, 4 µL 25 mM $MgCl_2$, 2 µL of 10mM dNTP mix, 1 µM/µl of forward and reverse primers, 100 ng of témplate DNA, 1 µL *Taq* DNA Polymerase (5 U/µL) and topped up to a final volume of 50 µL with deionized water. PCR was performed in Veriti® 96-Well Fast Thermal Cycler (Applied Biosystems, USA) using this thermal profile; an initial denaturation at 95ºC for 2 min, followed by 29 cycles of template denaturation at 95ºC for 15 s, primer annealing at 55ºC for 30 s and DNA extension at 72ºC. The PCR mixtures were then subjected to a final extension at 72ºC for 7 min. The PCR product was purified and sequenced. Analyses of DNA sequences were done using BLAST software at http://www.ncbi.nim.nih.gov/BLAST/ [14].

Cloning and Expression of AHL-lactonase Gene in pCOLD1 DNA Vector

The structural gene of the AHL homolog was amplified from SM01 genomic DNA using primers BCLF (5'- CTACGGGCATATGACAGTAAAGAAGCT -3')

and BCLR (5'-CATGCAAAGGATCCTATATATATTCCGGG-3'). These primers were designed based on *Bacillus cereus* AHL-lactonase gene (Accession Number ACX55099). The PCR was carried out as previously described, using AccuPrime ™ *Pfx* DNA Polymerase, with primer annealing conducted at 50ºC and the DNA extension at 68ºC. The PCR fragment was cloned into the *Nde*I-*Bam*HI site of pCOLD I (Takara Bio. Inc.) and transformed into *E. coli* BL21 (DE){F–, *omp*T, *hsd*S_B (r_B–, m_B–), *dcm*, *gal*, λ(DE3)} cells. The DNA inserts carried by recombinant pCOLD I DNA clones were sequenced using pCold I DNA standard primers.

An *E. coli* clone, BCL10, which had been transformed with the recombinant plasmid, pBcl10 carrying the AHL-lactonase gene was used for gene expression studies. A colony of BCL10 was inoculated into 5 mL of LB/ampicillin (100 µg/µL), incubated at 37°C with 200 rpm agitation, overnight. About 200 µL of the overnight culture was transferred into fresh 20 mL of LB/ampicillin (100 µg/µL) medium, incubated at 37°C, with 200 rpm agitation. Upon reaching OD_{600} of ~ 0.5, the culture was shifted to 15ºC, allowed to acclimatize at 15ºC for 30 min before being induced with 1 mM IPTG. The induced cells were further incubated at 15ºC, with 200 rpm agitation, overnight. The protein profile of the cells was analyzed using 12% SDS-PAGE [15]. The expressed recombinant AHL-lactonase was purified using TALON IMAC (Clontech Laboratories, Inc). The concentration of the purified protein was determined using Bradford Assay. The final concentration of the protein solution was adjusted to 1 mg/mL.

Effects of pH and Temperature on Recombinant AHL-lactonase Activity

The stability of the recombinant AHL-lactonase over a broad range of pH and temperature was evaluated. C8-AHL was used as the substrate in all assays performed. The pH-enzyme activity profile was determined by preincubating 10 µL (0.01 µg/µL) of AHL-lactonase in 1 mL of phosphate buffered saline (PBS) of different pH values (pH 5, pH 8 and pH 11), respectively, at 30ºC for 1 h. After the initial incubation, 50 µL of 10 µM C8-AHL was added to the AHL lactonase/PBS solution and further incubated at 30ºC for 1 h.

The temperature-enzyme activity relationship was determined by preincubating 10 µL (0.01 µg/µL) of enzyme in 1 mL of PBS buffer (pH 8) set at different temperatures (30ºC, 65ºC and 85ºC) for 1 h. Subsequently, 50 µL of 10 µM C8-AHL was added to the enzyme/PBS mixture and further incubated at the selected temperatures for another hour. The pH-activity and temperature-activity relationship profiles were both assessed using the AHL migration bioassay [11] with CV026 as the biosensor strain.

Quorum-quenching Activity of Recombinant AHL-lactonase

The ability of the recombinant AHL-lactonase to quench AHL produced by *B. pseudomallei* UKMS01 was assessed. A volume of 20 µL (0.01 mg/mL) of purified AHL-lactonase enzyme was added to 1 mL of an overnight *B. pseudomallei* UKMS01 culture and incubated at 30ºC for 6 hr. Subsequently, the bacterial cells were pelleted by centrifugation at 12 000 rpm for 2 min. An aliquot of 20 µL of the cell supernatant was spotted onto CV026 lawn plates. The CV026 plates were then incubated at room temperature for 24 hr.

Effect of Recombinant AHL-lactonase on Biofilm Development in *B. pseudomallei* UKMS01 and *S. aureus* ATCC 33591

Methicillin-resistant *S. aureus* ATCC33591 (MRSA) and *B. pseudomallei* UKMS01 were used to evaluate the ability of the recombinant AHL-lactonase to interfere with biofilm development in bacteria. Briefly, 10 mL overnight cultures of *B. pseudomallei* UKMS01 and *S. aureus* ATCC 33591 were diluted to the ratio of 1:100 using fresh LB. An aliquot of 200 µL of the diluted culture was transferred into the wells of sterile microtitre plates. With the exception of the experimental controls, 10 µL (1 mg/mL) of purified AHL-lactonase enzyme was added into each of the well containing the diluted bacterial cultures. After 24 hours incubation at 37ºC (without any agitation), the liquid cultures were carefully drained from the wells of the microtitre plates. The attached cells lining the plate wells were then stained with 200 µL of 0.1% crystal violet solution for 30 min at room temperature. Subsequently, the crystal violet solution was discarded with care to minimize unintentional dislodging of the attached cells. The plates were washed with distilled water and air-dried for 10 min. The retained crystal violet stained-cells were resuspended in 200 µL dimethyl sulphoxide (DMSO) and the A_{595} was recorded using the microtiter plate reader (Model 680, Bio-Rad Laboratories Inc, Berkeley, CA, USA).

RESULTS AND DISCUSSION

Isolation and Identification of Bacteria with AHL-inactivating Activities

It has been recognized that bacterial pathogens depend on their QS mechanisms to synchronize activities essential for infection and survival in the host. In contrast, it has also been observed that certain bacterial populations have the ability to inactivate or metabolize the QS signaling molecules, primarily N-acyl homoserine lactones (AHL) that lead to quenching of the microbial QS mechanisms. Since interference or inhibition of QS signaling can minimize the expression of virulence traits in bacteria,

quorum quenching seems to be an attractive strategy for the management of bacterial infections. In nature, bacteria are known to produce AHL-lactonases and AHL- acylases, enzymes that can degrade or metabolize homoserine lactones.

The production of AHL-lactonases in particular is known to be widespread in many species of *Bacillus.* Hence, to facilitate and minimize the number of bacterial isolates needed to be screened and tested for AHL-inactivating activity, an approach that would selectively promote the isolation of these endospore-forming Bacilli was adopted. Pre-treatment of the soil samples at 100ºC for 10 min has facilitated the elimination of non-sporulating bacteria and favoured the isolation of spore-forming Bacilli.

Screening of AHL-inactivating activity amongst the soil isolates was done using the biosensor strain *C. violaceum* CV026. *Chromobacterium violaceum* CV026 is a mutant strain in which the production of the purple pigment violacein is triggered in response to exogenous AHL [12]. If the AHL molecules were successfully inactivated by any of the respective soil isolates, the expression of violacein would not be triggered in CV026 and therefore, the bacterial lawn would not exhibit purple color. As depicted in Figure 1, the area where CV026 lawn was spotted with supernatant pooled from isolate SM01 did not turn purple. This indicated that SM01 possesses AHL-inactivating activity that is capable of degrading the lactone molecules, C8-AHL. Based on the 16s rDNA sequence analysis, isolate SM01 is identified as a *Bacillus cereus* strain.

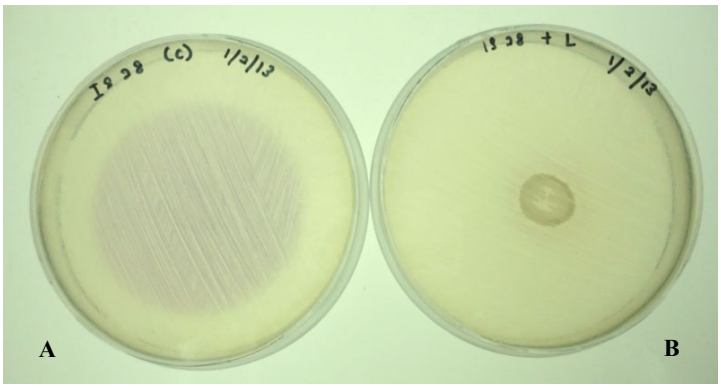

Figure 1. AHL-inactivating activity of isolate SM01. (A) Control of the experiment where C8-AHL was incubated in Luria broth and the mixture spotted on CV026 lawn. (B). Supernatant of SM01 that was pre-incubated with the commercial lactone, C8-AHL at room temperature and spotted on CV026 lawn.

Enzymatic Classification and Characterization of the Gene Responsible for AHL-inactivating Activity in *B. cereus* SM01

Enzymatic inactivation of AHL molecules could either be due to AHL-lactonases, AHL-acylases or oxireductases [16]. Inactivation of AHL molecules by lactonases happens by hydrolysis of the ester bond of the homoserine lactone ring. Hydrolysis of the ester bond by lactonases is a reversible process, in which restoration of the ester bond could be promoted under acidic conditions [17]. In contrast, AHL-inactivation by an acylase through hydrolysis of the acyl-amide bond between the acyl tail and lactone ring of AHLs is a nonreversible process [18]. Hence, to determine if the AHL-inactivating activity exhibited by SM01 was due to a lactonase or acylase, an acid test was conducted. It has been demonstrated that co-incubation of C8-AHL molecules with the supernatant of SM01 cells led to the inactivation of these lactones, making them ineffective as signaling molecules for CV026. However, when this supernatant/C8-AHL mixture was allowed to undergo acidification by the addition of HCl, these lactone molecules regained their ability to induce the production of purple pigment, violacein in CV026 (Data not shown). This observation is a strong indication that the AHL-inactivating activity exhibited by SM01 cells could be due to the enzyme lactonase.

AHL-inactivating enzymes, especially AHL-lactonases are known to be widely expressed by various *Bacillus* species and the genes encoding these lactonases are highly conserved [10]. Based on the available databases, a pair of lactonase gene-specific primers, BCLF and BCLR were designed and the targeted 753 bp gene fragment was successfully amplified from the genomic DNA of *B. cereus* SM01. The amplified fragment was successfully cloned into the *Nde*I-*Bam*HI site of pCOLD I DNA expression vector, to generate the recombinant plasmid, pBcl10.

Sequencing of the insert carried by pBcl10 confirmed the presence of the targeted fragment, which included the structural gene of AHL-lactonase (GenBank accession number MT856957). Homology comparison of the deduced amino acid sequences of the cloned gene exhibited high identity (E value = 0.0) with previously described AHL-lactonases within the *Bacillus* group (Table 1). An analysis

of the predicted 250 amino acid sequence revealed the presence of two conserved regions ([103]SHLHFDH[109] and [166]TPGHSPGH[173]), which are characteristics of the metallo-beta-lactamase superfamily of proteins (Figure 2). The histidine and glutamate residues within this short sequence motif are essential for the zinc-binding and enzyme activity of metallohydrolases [19, 20].

Table 1. The tBlastx analysis for the gene sequence carried by recombinant pCOLD vector, pBcl10

Accession	Description	Max Score	E value	% Identity
WP_171484358.1	MBL fold metallo-hydrolase *Bacillus paranthracis*	513	0.0	99.60
WP_000216606.1	Multispecies N-acyl homoserine lactonase family protein (*Bacillus*)	513	0.0	100.00
WP_046198343.1	Multispecies N-acyl homoserine lactonase family protein (Bacillus)	512	0.0	99.60
AT153688.1	N-acyl homoserine lactonase *Bacillus cereus*	511	0.0	98.80
WP_085782473.1	N-acyl homoserine lactonase family protein *Bacillus cereus*	510	0.0	98.80
WP_000216591.1	Multispecies N-acyl homoserine lactonase family protein (*Bacillus*)	510	0.0	99.20

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    atgacagtaaagaagctttatttcgttccagcaggtcgttgtatg
  1 M  T  V  K  K  L  Y  F  V  P  A  G  R  C  M
    ttagatcattcttctgttaatagtacactcgcgccggggaattta
 16 L  D  H  S  S  V  N  S  T  L  A  P  G  N  L
    ttgaacttacctgtatggtgttatcttttggagacagaagagggg
 31 L  N  L  P  V  W  C  Y  L  L  E  T  E  E  G
    cctattttagtagatacaggtatgccagaaagtgcagttaataat
 46 P  I  L  V  D  T  G  M  P  E  S  A  V  N  N
    gaagggctttttaacggtacatttgttgaaggacagattttaccg
 61 E  G  L  F  N  G  T  F  V  E  G  Q  I  L  P
    aaaatgactgaagaagatagaatcgtgaatatattaaaacgtgta
 76 K  M  T  E  E  D  R  I  V  N  I  L  K  R  V
    gggtatgagccggacgacctttatatattattagttctcactta
 91 G  Y  E  P  D  D  L  L  Y  I  I  S  S  H  L
    cattttgatcatgcaggaggaaacggtgcttttacaaatacaccg
106 H  F  D  H  A  G  G  N  G  A  F  T  N  T  P
    attattgtgcaacgaacggaatatgaggcagcacttcatagagaa
121 I  I  V  Q  R  T  E  Y  E  A  A  L  H  R  E
    gaatatatgaaagaatgtatattaccgcatttgaactacaaaatt
136 E  Y  M  K  E  C  I  L  P  H  L  N  Y  K  I
    attgaaggggattatgaagtggtaccaggtgtgcaattattgtat
151 I  E  G  D  Y  E  V  V  P  G  V  Q  L  L  Y
    acgccaggccattctccaggccatcagtcgctattaattgagaca
166 T  P  G  H  S  P  G  H  Q  S  L  L  I  E  T
    gaaaaatccggtcctgtattattaacgattgatgcatcttatacg
181 E  K  S  G  P  V  L  L  T  I  D  A  S  Y  T
    aaagaaaattttgaagatgaagtgccgttcgcgggatttgattcg
196 K  E  N  F  E  D  E  V  P  F  A  G  F  D  S
    gaattagctttatcttccattaaacgtttaaaagaagttgtggcg
211 E  L  A  L  S  S  I  K  R  L  K  E  V  V  A
    aaagagaaaccaattattttctttggtcatgatatagagcaggaa
226 K  E  K  P  I  I  F  F  G  H  D  I  E  Q  E
    aagggttgtaaagtgttcccggaatatatatag
241 K  G  C  K  V  F  P  E  Y  I  *
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Figure 2. The presence of two small conserved regions ([103]SHLHFDH[109] and [166]TPGHSPGH[173]) indicating that this protein belongs to the metallo-beta-lactamase superfamily. These regions represent the signature Zn2+-binding consensus motif of [104]HXHXDH[109] of metallohydrolases.

Heterologous Production and Functional Characterization of Recombinant AHL-Lactonase from *B. cereus* SM01

Heterologous recombinant protein production can be difficult. Problems such as leaky expression, protein instability, insoluble and toxic protein products are among the issues that need to be addressed. Hence, appropriate choice of expression vectors and host strains plays a critical role in protein expression, solubility, and yield. Expression of SMO1 *aii*A gene cloned into pCOLD I DNA vector was successfully achieved in *E. coli* BL21 (DE) cells. BL21 (DE) strain was chosen as a host because it possesses several traits that could enhance the production of a functional recombinant protein product. This strain is deficient in Lon and OmpT proteases, enzymes that mediate the degradation of abnormal or foreign proteins [21-22]. This is an important attribute since degradation of recombinant protein could be minimized, thereby increasing product stability and yield.

To obtain a tighter control of the gene expression and further enhance the yield and solubility of the recombinant protein, a low-temperature expression system, pCOLD I DNA was used. Expression of cloned genes in pCOLD I DNA vector is regulated by the cold shock Protein A, *cps*A promoter and the *lac* operator. The application of a low-temperature expression system such as pCOLD I DNA would allow selective induction of target protein synthesis at low temperature while suppressing the expression of host proteins and reduce protease activity [23]. pCOLD I DNA vector is also equipped with a translation enhancing element at the 5' untranslated region preceding the initiation codon, ATG, of the cloned gene. Translation enhancing elements are known to have a positive impact on the post-transcriptional regulation of gene expression and eventually determine the amount of protein synthesized [24].

The induction and heterologous expression of AHL lactonase by *E. coli* Bcl10 clone harboring the recombinant pCOLD vector pBcl10, was successfully carried out at 15^{O}C using IPTG as the inducer. As shown in Figure 3, SDS-PAGE analysis of total protein preparations from the IPTG-induced *E. coli* Bcl10 cell culture confirmed the presence of an extra protein band of approximately 29 kDa. This particular protein was predominantly abundant in comparison to the other proteins produced by the *E. coli* clone. This is an indication that the IPTG/cold shock induction had significantly enhanced the expression of the cloned *aii*A gene in the *E. coli* Bcl10 cells. The relative abundance of the target protein also suggests that there is no problem with protein solubility. Purification of the induced *E. coli* Bcl10 cell lysate by column affinity chromatography yielded an intact single protein band of 29kDa (Figure 3).

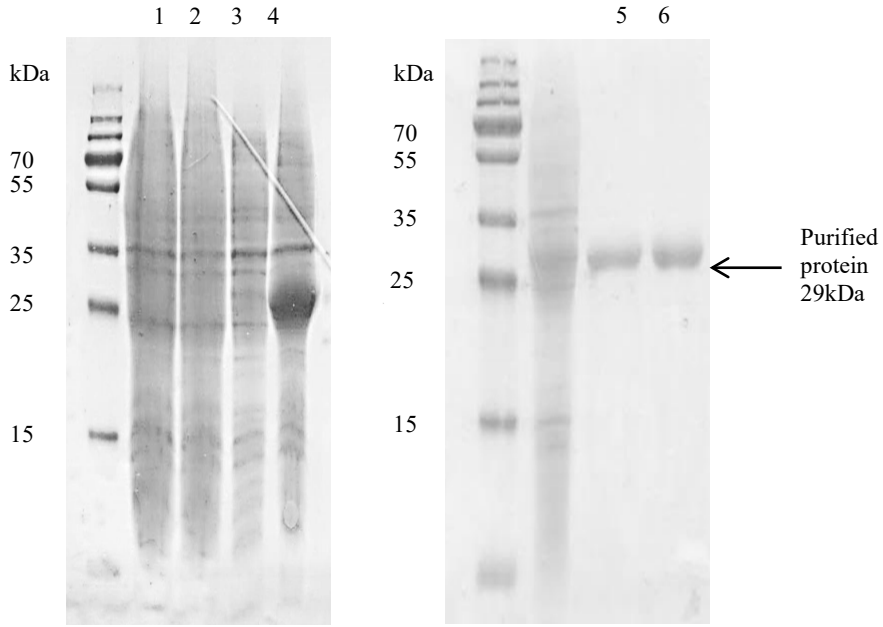

Figure 3. An SDS-PAGE analysis of proteins from uninduced and induced *E. coli* cells. Lane 1: Uninduced *E. coli* BL21 harboring pCold I DNA; Lane 2: Induced *E. coli* BL21 harbouring pCold I DNA; Lane 3: Uninduced *E. coli* BL21 harbouring pBcl10 (BL-Bcl); Lane 4: Induced *E. coli* BL21 harboring pBcl10 (BL-Bcl); Lanes 5 & 6: Purified protein

The stability of the purified recombinant AHL-lactonase was evaluated by incubating this enzyme in PBS buffer with varying pH values and temperature. As observed in the AHL migration bioassay, no distinguishable differences in AHL-inactivating activity were observed when this AHL-lactonase was maintained in either acidic (pH 5) or alkaline pH (pH 8 and 11) buffering conditions. The recombinant AHL-lactonase was also thermally stable and retained its AHL-inactivating activity after one hour of exposure to high temperatures of 65ºC or 85ºC (Figure 4).

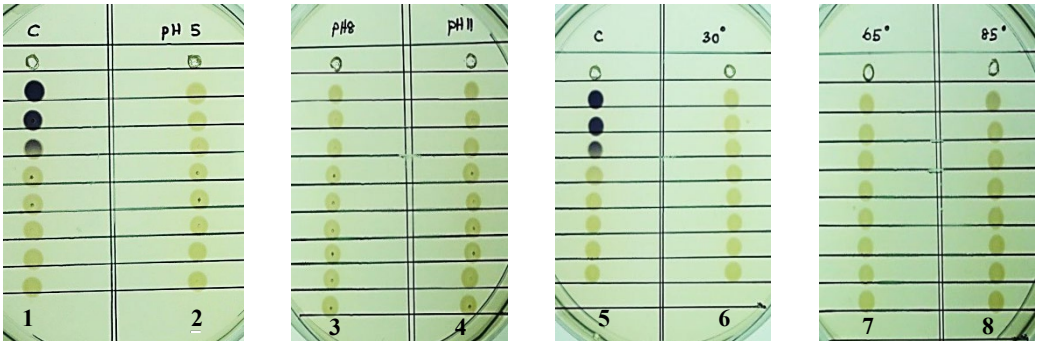

Figure 4. Effects of pH and temperature on recombinant AHL-lactonase activity**.** Lane1: Control (C8-HSL), Lane 2: AHL-lactonase/pH 5, Lane 3: AHL-lactonase/pH 8, Lane 4: AHL-lactonase/pH 11 PBS, Lane 5: Control (C8-HSL), Lane 6: AHL-lactonase/30ºC, Lane 7: AHL-lactonase/65ºC, Lane 4: AHL-lactonase/85ºC.

Based on the AHL migration bioassay, it was demonstrated that this recombinant AHL-lactonase possesses broad pH tolerance and good thermal stability and still maintained its AHL-inactivating activity even after being exposed to high pH and temperature. The integrity of most enzymes would have been jeopardized when subjected to such harsh physical condition. The majority of previously reported AHL-lactonases belonging to *B. thuringiensis* serovar shandongiensis ($AiiA_{SS10}$), *Bacillus sp. B546* ($AiiA_{B546}$) and *Bacillus* sp. Strain 240B1 ($AiiA_{240BI}$) were mostly active between 20ºC to 30ºC [3, 25, 26].Another AHL-lactonase homologue from *Bacillus* sp. A196 ($AiiA_{196}$) displayed optimum activity at 40ºC, retained 20% of its activity at 60ºC, and stable at 80ºC for 10 minutes [6]. Thus, the AHL-lactonase coded by the *aii*A_{SM01} homologue of *Bacillus cereus* SM01 is a unique lactonase that is stable at high temperature and maintained its catalytic activity even after exposure to a high temperature for a prolonged period of time.

The effectiveness of this recombinant AHL-lactonase in quenching the quorum-sensing signalling molecules produced by bacteria was evaluated using *Burkholderia pseudomallei* UKMS01. An established AHL producer, *B. pseudomallei* has been reported to produce several types of AHL molecules, ranging from C8HSL to C12HSL depending on the strain [27-28]. The addition of purified recombinant AHL-lactonase to overnight *B. pseudomallei* culture led to the hydrolysis of the AHL produced by this bacterium. This was evident by the reduced effectiveness of the supernatant collected from *B. pseudomallei* that had been co-incubated with recombinant AHL-lactonase to trigger violacein production in *C. violaceum*, CV026 (Figure 5). The production of the purple-pigment violacein by CV026 strain is inducible by exogenous AHL compounds with N-acyl side chains ranging from C4 to C8 [12]. This observation indicated that the majority of the lactones produced by *B. pseudomallei* had been hydrolysed or quenched by the recombinant AHL-lactonase thereby, reducing the efficiency of the *B. pseudomallei* AHL in inducing the production of violacein molecules in CV026.

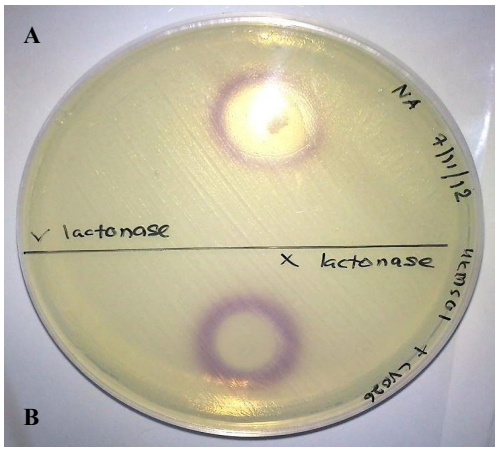

Figure 5. Quenching of *B. pseudomallei* AHL molecules by recombinant AHL-lactonase. Development of the purple zone was observed to be much less intense for CV026 induced with the *B. pseudomallei* supernatant that was co-incubated with AHL-lactonase (A), compared to the one grown in media without AHL-lactonase (B).

The purified recombinant AHL-lactonase has been shown to possess good AHL-inactivating activity, rendering the *B.pseudomallei* AHL molecules inefficient as an inducer for the biosensor strain CV026. Since quorum-sensing signaling molecules or auto-inducers play a major role in the development and establishment of bacterial biofilms, the impact of this AHL-lactonase on biofilm

development in both *B. pseudomallei* (Gram-negative) and methicillin-resistant *Staphylococcus aureus*, MRSA (Gram-positive) was also evaluated. Both *B. pseudomallei* and MRSA are established biofilm producers. *B. pseudomallei* use AHL as their signalling molecule while MRSA use auto-inducing peptide (AIP), as their auto-inducers. The AIP molecules of *S. aureus* are seven to nine amino acids in length with the five of the C-terminal residues presented as a thiolactone ring [29].

After 24 hours of growth in an unagitated environment, both *B. pseudomallei* and MRSA had established a decent amount of biofilm, as indicated by the absorbance values ($O.D_{590nm}$ value 1.367 $\pm$ 0.208 for *B. pseudomallei and* $O.D_{590nm}$ value 1.664 $\pm$ 0.323 for MRSA). However, in comparison, co-incubation of *B. pseudomallei* UKMS01 and MRSA in the presence of recombinant AHL-lactonase enzyme led to a significant reduction of biofilm development in both bacteria. This is evidenced by the much lower absorbance values for both *B. pseudomallei* UKMS01 ($O.D_{590nm}$ value 0.686 $\pm$ 0.180) and MRSA ($O.D_{590nm}$ 1.129 $\pm$ 0.251) cells that were grown in a culture medium supplemented with the recombinant AHL-lactonase. In the presence of the AHL-lactonase, the biofilm development in MRSA was reduced by approximately 3.4-fold while the reduction in *B. pseudomallei* was approximately 4.8-fold. A significant disruption of biofilm development in MRSA and *B. pseudomallei* suggests that this particular AHL-lactonase can hydrolyze both AHL and thiolactones, rendering these molecules unfit as QS inducers.

In conclusion, the AHL-lactonase from *B. cereus* SM01 has some interesting attributes that potentiate its suitability as a lead candidate for therapeutics. This recombinant AHL-lactonase has broad pH tolerance, displayed good thermal stability and can disrupt biofilm development in both Gram negative and Gram positive bacteria.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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