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Quorum-quenching activity of the AHL-lactonase from *Bacillus licheniformis* DAHB1 inhibits vibrio biofilm-formation *in vitro* and reduces shrimp intestinal colonisation and mortality

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Running Head: Quorum-quenching activity of a novel AHL-lactonase

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Abstract

Vibrio parahaemolyticus is a significant cause of gastroenteritis resulting from the consumption of undercooked sea foods and often cause significant infections in shrimp aquaculture. Vibrio virulence is associated with biofilm-formation and is regulated by *N*-acylated homoserine lactone (AHL) - mediated quorum sensing. In an attempt to reduce vibrio colonisation of shrimps and mortality, we screened native intestinal bacilli from Indian white shrimps (*Fenneropenaeus indicus*) for an isolate which showed biofilm-inhibitory activity (quorum-quenching) against the pathogen *V. parahaemolyticus* DAHP1. The AHL-lactonase (AiiA) expressed by one of these, *Bacillus licheniformis* DAHB1, was characterised as having a broad-spectrum AHL substrate specificity and intrinsic resistance to the acid conditions of the shrimp intestine. Purified recombinant AiiA inhibited vibrio biofilm development in a cover slip assay and significantly attenuated infection and mortality in shrimps reared in a recirculation aquaculture system. Investigation of intestinal samples also showed that AiiA-treatment also reduced vibrio viable counts and biofilm development as determined by CLSM imaging. These findings suggest that the *B. licheniformis* DAHB1quorum quenching AiiA might be developed for use as a prophylactic treatment to inhibit or reduce vibrio colonisation and mortality of shrimps in aquaculture.

Keywords: AHL-lactonase, AiiA, *Bacillus*, biofilm, quorum-quenching, *Vibrio parahaemolyticus.*

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Introduction

Vibrio parahaemolyticus is a major cause of gastroenteritis world-wide, and contaminates fresh and seawater produce, including fish, molluscs, shrimps and prawns, and can be found in lakes, rivers and coastal seas (Sarkar et al. 1985; Thompson et al. 2004; Yeung et al. 2004; Su et al. 2007). Vibrio virulence involves arrange of factors (Johnson et al. 2013), including biofilm-formation, which is regulated by quorum signalling (QS) using *N*-acylated homoserine lactones (AHLs) including C6-HSL (*N*-hexanoyl-L-homoserine lactone, HHL) (Fuqua et al. 2001; Kamruzzaman et al. 2010; Defoirdt et al. 2011; Ng et al. 2011; Fast et al. 2012). Disruption of QS by quorum-quenching has been suggested as an anti-infective strategy to control pathogenic bacteria through the interference of the colonisation processes, including biofilm-formation and the invasion of host tissues (Rasmussen et al. 2006; Defoirdt et al. 2008; Czajkowski et al. 2009). This might be achieved by treatments with exogenous AHL-lactonases that degrade AHLs, though these would need to have broad substrate specificity, as AHLs vary in chain length (4 – 16 carbons) and oxo or hydroxyl substitutions. Some bacteria, especially *Bacillus* spp., may use AHL-lactonases in quorum-quenching to boost competitive strength in soil, and a number of AHL-degrading AiiA metallo-β-lactamase super family (MBL) type enzymes have been characterised from a variety of soil-associated *Bacillus* spp. and other bacteria (AHL-lactonases are reviewed by Roche et al. 2004; Rasmussen et al. 2008; Czajkowski et al. 2009; Fast et al. 2012). *Bacillus* spp. also naturally colonise the intestines of shrimps and prawns, suggesting that some isolates might be developed into bio-control agents or exogenous AiiA-based treatments (Rasmussen et al. 2006; Ninawe et al. 2009). In the latter case, the activity profile and intrinsic acid and protease resistance of AiiAs suggest that they would function effectively in shrimp intestines. In this study, we have isolated bacilli from healthy Indian white shrimps and characterised the

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AHL-lactonase (AiiA) produced by one as a potential quorum quenching agent against a pathogenic *V. parahaemolyticus* strain, using *in vitro* biofilm assays and a virulence assay using shrimps maintained in an aquaculture system.

Material and Methods

Bacteria

Bacteria were isolated from the dissected gut samples of Indian white shrimp (*Fenneropenaeus indicus*) collected from the Bay of Bengal on the south-east coast of India, about 250 km south of Chennai (11°27'9 N, 79° 47' E) in April 2010. Bacterial samples were incubated on minimal media agar plates (1 g NaCl, 0.5 g KCl, 0.4 g $MgCl_2.6H_2O$, 0.1 g $CaCl_2.2H_2O$, 0.2 g KH_2PO_4, 0.15 g Na_2SO_2, and 1 g MES per litre, with trace elements and vitamins also added after Leadbetter and Greenberg (2000)) containing 5 mg l^{-1} C6-HSL as the sole carbon and nitrogen source for 48 h at 30 °C to isolate bacteria capable of utilising C6-HSL. A total of 50 *Bacillus* spp. strains were isolated and stored at -80 °C (the four key strains investigated here were subsequently labelled DAHB1 – DAHB4 and species were determined subsequently by 16S analysis, listed in Table 1). *Chromobacterium violaceum* CV026 (McClean et al. 1997) was used for the AHL-lactonase activity bioassay. *Vibrio parahaemolyticus* DAHP1, previously isolated from the haemolymph of an Indian white shrimp (Vinoj and Vaseeharan, *unpublished research*), was used for biofilm inhibition and virulence assays. *V. parahaemolyticus* DAHP1-GFP was produced by conjugal transfer of pVSV102 (Km^R) (Dunn et al. 2006) and GFP expression was confirmed by confocal laser scanning microscopy (CLSM). *Escherichia coli* JM109 (Promega) was used for cloning purposes and *E. coli* BL21 (DE3) pLysS (Promega) for protein expression.

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Screening of bacillus for the ability to inhibit biofilm-formation using the microtitre plate (MTP) assay

Cell extracts from *Bacillus* spp. were tested for their ability to inhibit *V. parahaemolyticus* DAHP1 biofilm-formation in microtitre plates (Pratt et al. 1998). Bacilli were cultured in Zobell marine broth (ZMB) (Himedia Laboratories, India) for 24 h at 30 °C. Cultures were sonicated using a UP100H ultrasonic processor (Hielscher, Germany) for 1 min at 100 W, and then centrifuged at 800 x*g* for 10 min before the supernatant was passed through a 0.2 µm pore-sized filter and storage at -20 °C. An overnight culture of *V. parahaemolyticus* DAHP1 (10 µl, ~10^6 cells ml^{-1}) was used to inoculate 96-well polystyrene microtitre plates containing 100 µl Luria-Bertani (LB) medium and 50 µl of extract which were then incubated statically for 24 h at 30 °C. The cultures were discarded and the wells gently rinsed twice with deionised water and allowed to air dry before staining with Crystal violet (CV).The wells were stained with 210 µl of 0.1% (w/v) CV for 10 min before rinsing twice with deionized water and air drying. CV was then eluted with 210 µl dimethyl sulfoxide and the absorbance (A_{595}) determined using a Bio-Rad enzyme-linked immunosorbent assay reader.

AHL-lactonase activity bioassay

The AHL-lactonase activity of cell-free culture samples and purified AiiA protein was determined using *C. violaceum* CV026 as the reporter strain in a well-diffusion C6-HSL bioassay (Chen et al. 2010). Agar plates were prepared by mixing 3 ml overnight culture of *C. violaceum* CV026 in 20 ml LB agar which was then poured into petridishes. After setting, a 5 mm diameter plug was removed from each plate to form a well for the assay. The AHL-lactonase reactions contained 10 µl sample plus 190 µl reaction mixture containing 24 nM C6-HSL in 50 mM phosphate buffer (pH 8.0) and were incubated at 25 °C for 45 min before

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termination by the addition of 50 µl 10% (w/v) SDS. The reaction mixture was then transferred into plate wells, and the radius of the *C. violaceum* CV026 non-pigmented zone after 2 days was used to determine residual C6-HSL levels. AHL-lactonase activity is reported here as the degradation rate of AHL (mg l^{-1} h^{-1}) or as the units (U) of AHL-degrading activity mg^{-1} protein where 1 U is defined as the amount of enzyme required to hydrolyse 1 nM C6-HSL per minute under the conditions described.

Cell-free culture supernatants

Residual C6-HSL concentrations were determined for supernatants obtained from *Bacillus* spp. cultures inoculated with 10^8 cells ml^{-1}, incubated in LB medium containing 20 g l^{-1} synthetic sea salt (LB20) medium (pH 6.5) (Himedia Laboratories) and supplemented with 5 mg l^{-1} C6-HSL, for 48 h at 30 °C with shaking. The cultures were periodically sampled, and 1 ml aliquots were passed through a 0.2 µm filter to produce cell-free supernatants which were then tested for residual C6-HSL by the AHL-lactonase activity bioassay.

Characterization of AiiA activity in vitro

Activity was determined for the AHL-lactonase activity bioassay using C6-HSL as the substrate at pH 8.0 and 30 °C for 30 min unless otherwise indicated. The activity-temperature profile was determined over 0 – 80 °C at pH 8.0 for 30 min. pH stability was determined after pre-incubation in McIlvaine (pH 3.0 – 6.0), PBS (pH 6.0 – 8.0), 0.05 M Tris-HCl (pH 8.0 – 9.0), and 0.1 M glycine-NaOH (pH 9.0 – 12.0) buffers for 30 min at 30 °C. Thermal stability was determined after pre-incubation at 70 °C, 80 °C and 90 °C at pH 8.0 for 0 – 60 min, and resistance to proteolysis determined after pre-incubation of a 1:10 (w/w) mixture of chymotrypsin, proteinase K, subtilisin A, or trypsin (Sigma Aldrich) and AiiA, at pH 8.0 at 30 °C for 60 min. Resistance to prawn intestinal juice supernatant was also evaluated. The full-

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length intestine of a shrimp that had been anesthetized with tricaine methane sulfonate 1 h after feeding was gently washed three times with PBS (pH 7.4) before the intestinal mucus was washed out with PBS (pH 7.4) and centrifuged at 8,000 x*g* for 20 min at 4 °C. Residual activity was determined after pre-incubation of a 1:10 (v/v) mixture of 0.6 U AiiA and supernatant for 60 min at 30 °C. Substrate specificity was tested with the AHLs C4-HSL, C6-HSL, C8-HSL, C10-HSL, C12-HSL, C14-HSL, 3-oxo-C6-HSL, and 3-oxo-C8-HSL (Sigma Aldrich).

AiiA inhibition of Vibrio biofilm formation using the cover slip assay

A cover-slip system was used to investigate the impact of sonicated *Bacillus* spp. culture extracts and purified AiiA on *V. parahaemolyticus* DAHP1-GFP biofilm formation *in vitro*. *V. parahaemolyticus* DAHP1-GFP was incubated statically in replicate flasks containing a standard microscope cover-slip with 2 ml LB medium or LB medium with 50 µl sonicated extract or 120 U ml^{-1} AiiA at 30 °C for 24 h. The cover-slips were then recovered and washed twice with PBS before microscopy. Cover-slips were stained with CV as before and examined using a Nikon inverted research microscope ECLIPSE Ti at 40x magnification. A Carl Zeiss LSM 710 confocal laser scanning microscope (CLSM) using a 488 nm argon laser and BP 500-640 band pass emission filter and running Zen 2009 software (Carl Zeiss, Germany) was also used to image the GFP signal from cover-slip samples. Biofilms were assessed using COMSAT software (Heydorn et al. 2000).

Virulence of vibrio in shrimps and inhibition with AiiA

Healthy shrimps (10 ± 0.5 g) were raised in an indoor recirculation aquaculture system maintained at 30 ± 1°C. The LD_{50} of *V. parahaemolyticus* DAHP1-GFP was determined by the injection-infection method. Shrimps were injected into the abdominal cavity with 50 µl of *V. parahaemolyticus* DAHP1-GFP cell suspension prepared from an overnight ZMB culture in

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PBS at 5 x 10^3 – 5 x 10^8 CFU ml^{-1}. Mortalities were recorded daily and LD_{50} values calculated. The impact of AiiA treatment on *V. parahaemolyticus* DAHP1-GFP virulence was investigated using triplicate groups of 30 shrimps injected in the abdominal cavity with 50 µl aliquots of saline, a *V. parahaemolyticus* DAHP1-GFP suspension of 1.8 x 10^5 CFU, *V. parahaemolyticus* DAHP1-GFP suspension plus 0.6 U AiiA, or AiiA alone. The shrimps were then maintained in separate tanks and the cumulative mortality of each treatment was recorded for ten days. Shrimp guts were dissected and imaged by CLSM to follow *V. parahaemolyticus* DAHP1-GFP infection. Selective plating was also used to quantify the number of cultivable *V. parahaemolyticus* DAHP1-GFP in gut samples. Dissected guts were homogenizing in 1mL sterilized sea water and plated in Zobell marine agar (ZMA) (Himedia Laboratories) supplemented with 1% (w/v) NaCl and 100 µg mL^{-1}kanamycin overlaid on *Vibrio parahaemolyticus* agar (Himedia Laboratories). CFU were determined after 48 h incubation at 30 °C and further confirmation that these were *V. parahaemolyticus* DAHP1-GFP was obtained by CLSM.

Cloning and sequencing

Bacillus spp. DAHB1 – DAHB4 16S rDNA and *B. licheniformis* DAHB1*aiiA* were amplified by PCR from genomic DNA prepared using a Wizard Genomic DNA Purification Kit (Promega). The universal Fp and Rp primers were used to amplify16S sequences as previously described (Pan et al. 2008). The *B. licheniformis* DAHB1*aiiA* gene was amplified using the Forward and AI Reverse primers as previously described (Dong et al. 2002). PCR products were purified using a PCR DNA and Gel Band Purification Kit (GE Healthcare) and cloned using the pGEM-T Easy Vector System (Promega) following standard procedures. Plasmids in *E. coli* JM109 were isolated using the alkaline lysis method and were examined on agarose gels

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to confirm the presence of the inserted fragments. Sequences were determined by the Dye-termination method (Applied Biosystems) using vector-specific primers and a LI-COR 4200 DNA sequencer.

Sequence analysis of AiiA

The coding region of the *B. licheniformis* DAHB1*aiiA* gene was determined using ORF finder (ncbi.nlm.nih.gov/), and the identity of the gene confirmed by TBLASTN homology analysis (ncbi.nlm.nih.gov/) and comparisons of protein sequence alignments using Vector NTI (Invitrogen) and tertiary structures using SWISS-MODEL (swissmodel.expasy.org) and Swiss-PdbViewer DeepView (spdbv.vital-it.ch). SignalP (cbs.dtu.dk/services/SignalP/) was also used to test for the presence of a signal peptide sequence.

Cloning, purification and western analysis of AiiA

The *B. licheniformis* DAHB1 *aiiA* gene was amplified by PCR from genomic DNA using modified primers incorporating *Nde*I and *Eco*RI restriction sites (5' – 3', AIF1: GGG AAT TCC ATA TGA CAG TAA AAA AGC TTT ATT TC, and AIR1: CCG GAA TTC CGG CTA TAT ATA CTC CGG GAA CTC) with 40 cycles (of 5 min at 95°C, 1 min at 50 °C and 2 min at 72 °C), and a final 10 min incubation at 72°C. The PCR DNA was purified directly using a Wizard SV Gel and PCR Clean-Up System (Promega), digested with *Nde*I and *Eco*RI (NEB), ligated into the expression plasmid pET-32a (Novogene) and then used to transform *E. coli* BL21 cells. Plasmid DNA was isolated from recombinant clones using a Wizard SV Miniprep DNA Purification Kit (Promega) and the insertion in one (pET-AiiA) verified by sequencing as described above. AiiA was purified from IPTG-induced *E. coli* BL21–pET-AiiA cultures using Ni-NTA columns (Qiagen) following manufacturer's specifications. Expression and isolation

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was monitored by SDS-PAGE, and protein concentrations determined by the Bradford method. *B. licheniformis* DAHB1 AiiA was lyophilized and stored at 4 °C.

Dissected Indian white shrimp gut samples were homogenized and suspended in buffer (10 mM HEPES, 1 mM EDTA, 150 mM NaCl, 0.6% (v/v) Nonidet P-40, 1 mM PMSF, 1 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin, 1 mM orthovanadate, pH 7.9) at approximately 200 mg ml^{-1}. Samples were separated by SDS-PAGE and then transferred to a BioTrace NT nitrocellulose membrane (Pall Corporation) according to the manufacturer's specifications. *B. licheniformis* DAHB1 AiiA was detected using the diaminobenzidine (DAB) system (GeNei, Bangalore), rabbit anti-AiiA serum obtained from a New Zealand white rabbit diluted 1:100 in PBS, and goat anti-rabbit HRP conjugate (Imgenex).

Statistics

All assays were repeated at least three times, and means ± standard deviations (SD) are provided. Means were compared by one-way ANOVA using SPSS (IBM Corporation).

Results

Isolation of *Bacillus* spp. that inhibit vibrio biofilm formation

A total of 50 bacteria capable of growing on C6-HSL as the sole carbon and nitrogen source were isolated from the guts of Indian white shrimps collected from the east coast of India. These were screened for their ability to inhibit *V. parahaemolyticus* DAHP1 QS-mediated biofilm formation using sonicated cell extracts and Crystal violet staining in a MTP-based assay. Four strains, DAHB1 – DAHB4, were found to significantly inhibit biofilm-formation, and cultures supplemented with C6-HSL and tested by AHL-lactonase bioassay demonstrated that each also degraded this quorum signal (Table 1). The strains were subsequently identified

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as *Bacillus* spp. by 16S sequence analysis, and their ability to degrade C6-HSL in a quorum quenching manner strongly suggested that each expressed an AHL-lactonase (AiiA). Sonicated cell extracts from all four strains were also found to significantly inhibit biofilm formation by a GFP-labelled vibrio, *V. parahaemolyticus* DAHP1-GFP, in a cover slip assay in which maximum biofilm depth and volumes were determined by CLSM (Table 1). In order to obtain a novel AiiA suitable for treating vibrio shrimp infections *in situ*, one of the four strains, *Bacillus licheniformis* DAHB1, was selected for further study on the basis of its high C6-HSL degradation rate.

Identification of the AiiA gene from *Bacillus licheniformis* DAHB1

We used degenerate *Bacillus* spp. primers to amplify the suspected *aiiA* gene in *B. licheniformis* DAHB1. The resulting 753-bp sequence (Genbank accession number: JQ284448.1) encodes a protein of 250 amino acids with a calculated MW of 28 kDa and a pI of 5.08. It shares 95% amino acid sequence similarity with *Bacillus sp.* DMS133 AiiA (JF970259) and ~85% similarity to AHL-lactonases from the *Bacillus* spp. *B. amyloliquefaciens* (ACX55096), *B. cereus* (ZP04290275), *B. subtilis* (AAY51610), and other bacteria including *Agrobacterium tumefaciens* (AAK91031 and AAD43990), *Arthrobacter sp*. (AAP57766), and *Klebsiella variicola* (003439519). No signal peptide sequence was identified in *B. licheniformis* DAHB1 AiiA, and homology modelling using *B. thuringiensis* AiiA (AAM92140/PDB3DHA) as a template suggests that it has a structure typical of metallo-lactamase (MBL) family proteins.

Characterisation of AiiA AHL-lactonase activity in vitro

The AHL-lactonase activity of the purified *B. licheniformis* DAHB 128 kDa His-tagged AiiA (hereafter referred to as AiiA) was found to have abroad-spectrum substrate specificity, with

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activities of 95.1 – 99.5 U ml^{-1} against C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL, 3-oxo-C8-HSL, C10-HSL, C12-HSL and C14-HSL. AiiA had a specific activity of 10 U C6-HSL–degrading activity mg^{-1} protein under standard conditions, and the temperature and pH activity profiles using C6-HSL are shown in Figure 1 where maximal activity was found at 30 – 50°C and pH 7.0 – 8.0. AiiA was stable, retaining more than 80% of the initial activity after incubation at pH 6.0 - 12.0 for 60 min, as well as 90% activity after incubation at 60 - 80 °C for 60 min. It was also resistant to the acid intestinal juices of shrimps and a number of proteases, retaining ≥ 98% of activity after 60 min exposure.

AiiA was also found to be sufficiently stable to inhibit *V. parahaemolyticus* DAHP1-GFP biofilm formation in a cover slip assay over a period of 24 h. AiiA significantly reduced biofilm thickness from 33 ± 1.7 µm in untreated samples to 14.1 ± 0.7 µm in treated samples exposed to 21 U ml^{-1} AiiA (Supplementary Figure 1).

Treatment of Indian white shrimps with AiiA reduces *vibrio* colonisation and mortality

The virulence of *V. parahaemolyticus* DAHP1-GFP in Indian white shrimps reared under standard aquaculture conditions was confirmed by abdominal injections, with individuals showing signs of anorexia, inactivity, muscle erosion, abnormal haemolymph coagulation, and eventual death, with an LD_{50} of 6 x 10^6 CFU ml^{-1}. The impact of AiiA treatment on *V. parahaemolyticus* DAHP1-GFP virulence was then investigated using triplicate groups of shrimps injected abdominally with *V. parahaemolyticus* DAHP1-GFP, *V. parahaemolyticus* DAHP1-GFP plus AiiA, and AiiA alone, and following mortality over a period of 5 days. AiiA could survive in shrimp intestines for this period as confirmed by preliminary experiments in which AiiA in homogenized gut samples could be detected by Western analysis (Figure 2). The accumulated mortality at 5 days was significantly reduced in the *V. parahaemolyticus*DAHP1-

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GFP plus AiiA treatment (23.2 ± 1.2%) compared to the *V. parahaemolyticus*DAHP1-GFP treatment (80.2 ± 4.0%) ($P< 0.05$), whilst injection with AiiA protein alone had no significant impact on mortality (Figure 3). Sampling of shrimp intestines also showed that the numbers of viable *V. parahaemolyticus* DAHP1-GFP were lower in the *V. parahaemolyticus* DAHP1-GFP plus AiiA treatment (1.1×10^2 CFU ml^{-1}) compared to the *V. parahaemolyticus* DAHP1-GFP treatment (5.4×10^6 CFU ml^{-1}). Investigation of the intestines by CLSM also confirmed the drop in viable *V. parahaemolyticus* DAHP1-GFP numbers, with a reduction in fluorescent signal observed for the *V. parahaemolyticus* DAHP1-GFP plus AiiA treatment samples compared to the *V. parahaemolyticus* DAHP1-GFP treatment samples (Figure 4). These findings strongly suggest that exogenously-added AiiA protein from *B. licheniformis DAHB1* was able to reduce vibrio virulence and biofilm-formation in shrimps reared under standard aquaculture conditions.

Discussion

Although antibiotics can be used to control vibrio infections in aquaculture, the prevalence of antibiotic-resistance strains and regulatory controls make this increasingly problematic. An alternative approach to controlling these and other pathogens is to challenge the colonisation process itself, rather than to attempt to reduce pathogen numbers directly using biocidal agents. In the case of pathogens which rely on QS to regulate colonisation and virulence, interdiction of the AHL signal molecules through quorum quenching may be possible through the expression of AHL-lactonases that degrade AHLs by bio-control bacteria, or through the supply of purified enzymes in treatment regimes (bacteria also express AHL-acylases and amidases that degrade AHLs, but these lack the broad substrate activity of the AHL-lactonases) (Dong et al. 2002 & 2005; Defoirdt et al. 2007, 2008 & 2011; Czajkowski et al. 2009).

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Quorum quenching has been demonstrated *in vitro* using the AHL-lactonase (AiiA) produced by the soil bacterium, *Bacillus thuringiensis*, against the plant pathogen *Erwinia carotovora* and the fish pathogen *V. harveyi* (Dong et al. 2004; Bai et al. 2008). AiiA produced by the pond sediment isolate, *Bacillus* sp. AI96, has recently been demonstrated to treat *Aeromonas hydrophila* infections in zebra fish in a practical manner when added to fish feed (Cao et al. 2012).

In this work, we have isolated from healthy Indian white shrimps *Bacillus* spp. strains capable of expressing AHL-lactonase activity against a range of AHLs. C6-HSL was used as a test compound because it is the major QS signal of significant aquatic animal pathogens such as *Aeromonas hydrophila, A. salmonicida, Edwardsiella tarda* and *Vibrio* spp., many of which are also opportunistic human pathogens (Swift et al. 1997; Morohoshi et al. 2004; Bruhn et al. 2005). Four of our *Bacillus* spp. isolates showed significant C6-HSL degradation rates and could inhibit vibrio biofilm-formation in microtitre plate and coverslip assays. We used *Bacillus AiiA* - based primers to identify, clone and then express AiiA from one isolate, *B. licheniformis* DAHB1, and showed that this AHL-lactonase had broad substrate specificity, good temperature and pH profile characteristics, and was resistant to the acid conditions found in shrimp intestines as well as to a variety of proteases.

Although AiiA proteins have been isolated from other *Bacillus* spp. with similar properties, most have been isolated from soil species. *B. licheniformis* DAHB1 AiiA is one of the first AHL-lactonases to be purified and characterised from a *Bacillus* spp. colonising the intestines of a marine animal, though recently *B. anthracis*–like strains isolated from Pacific white shrimp have been shown to degrade C6-HSL *in vitro* (Defoirdt et al. 2011) and presumably also express AiiA proteins (these degrade C6-HSL more slowly than DAHB1: 0.7 – 0.9 mg l^{-1} hr^{-1} *cf.*

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1.3 mg l^{-1} hr^{-1}, though this does depend on enzyme concentrations). This higher activity might be explained by small sequence differences between *B. licheniformis* DAHB1 AiiA and other AiiA proteins that might affect structure and function.

In this work, we have also demonstrated that exogenous *B. licheniformis* DAHB1 AiiA protein is effective in reducing the mortality of shrimps injected abdominally with *V. parahaemolyticus* DAHP1-GFP. CLSM imaging and viable CFU counts suggest that this was achieved by reducing vibrio numbers in the intestines by inhibiting biofilm development through quorum quenching. Treatment with *B. licheniformis* DAHB1AiiA protein alone showed no measurable impact on shrimp health or mortality, suggesting that the use of AiiA as a quorum quenching anti-pathogenic agent has significant advantages over alternatives such as halogenated furanones which are toxic to both brine shrimp and rotifers (Defoirdt et al. 2007).

Bacillus spp. are natural colonisers of shrimps and would compete with pathogenic vibrio to colonise their intestines. Although only some shrimp-associated *Bacillus* spp. might express broad-spectrum AHL substrate degrading activity, strains such as *B. licheniformis* DAHB1 isolated in this work may make good bio-control agents. These could be added to feedstock to prevent the establishment of vibrios in shrimp aquaculture systems (Nhan et al. 2010), and may also have additional positive probiotic effects on survival and yield (Ninawe et al. 2009).

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References

Bai F, Han Y, Chen J, Zhang XH(2008) Disruption of quorum sensing in *Vibrio harveyi* by the AiiA protein of *Bacillus thuringiensis*. Aquaculture 27:436–40

Bruhn JB, Dalsgaard I, Nielsen KF, Buchholtz C, Larsen JL, Gram L (2005) Quorum sensing signal molecules (acylated homoserine lactones) in gram-negative fish pathogenic bacteria. Dis Aquat Organ 65:43–52

CaoY, He S, Zhou Z, Zhang M, Mao W, Zhang H, Yao B (2012) Orally administered thermostable N-Acyl homoserine lactonase from *Bacillus* sp. strain AI96 attenuates *Aeromonas hydrophila* infection in Zebrafish. Appl Environ Microbiol 78:899–1908

Chen R, Zhou Z, Cao Y, Bai Y, Yao B (2010) High yield expression of an AHL lactonase from *Bacillus* sp. B546 in *Pichia pastoris* and its application to reduce *Aeromonas hydrophila* mortality in aquaculture. Microb Cell Fact 9:39

Czajkowski R, Jafra S (2009) Quenching of acyl-homoserine lactone-dependent quorum sensing by enzymatic disruption of signal molecules. Acta Biochim Pol. 56:1–16

Defoirdt T, Boon N, Sorgeloos P, Verstraete W, Bossier P (2008) Quorum sensing andquorum quenching in *Vibrio harveyi*: lessons learned from *in vivo* work. ISME J 2: 19–2

Manuscript in publication

Defoirdt T, Boon N, Sorgeloos P, Verstraete W, Bossier P (2007) Alternatives to antibiotics to control bacterial infections: luminescent vibriosis in aquaculture as an example. Trends Biotechnol 25:472–479

Defoirdt T, Thanh LD, Van Delsen B, De Schryver P, Sorgeloos P, Boon N, Bossier P (2011) N-acylhomoserine lactone-degrading Bacillus strains isolated from aquaculture animals. Aquaculture 311:258–260

Dong YH, Gusti AR, ZhangQ, Xu JL, Zhang LH (2002) Identification of quorum-quenching N-acyl homoserine lactonases from *Bacillus* species. Appl Environ Microbiol 68: 1754–1759

Dong YH, Zhang LH (2005) Quorum sensing and quorum quenching enzymes. J Microbiol 43:101–109

Dong YH, Zhang XF, Xu JL, Zhang LH (2004) Insecticidal *Bacillus thuringiensis* silences *Erwinia carotovora* virulence by a new form of microbial antagonism, signal interference. Appl Environ Microbiol 70:954–960

Dunn AK, Millikan DS, Adin DM, Bose JL,Stabb EV (2006) New *rfp*- and pES213-derived tools for analysing symbiotic *Vibrio fischeri* reveal patterns of infection and *lux* expression *in situ*. Appl Environ Microbiol 72:802–810

Fast W, Tipton PA (2012) The enzymes of bacterial census and censorship. Trends Biochem Sci. 37:7–14

Fuqua C, Parsek MR (2001) Greenberg EP Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. Annu Rev Genet 35:439–468

Manscript in publication

Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersbøll BK, Molin S (2000) Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology 146:2395–2407

Johnson CN (2013) Fitness factors in Vibrios: a mini-review. Microb Ecol. (Epub ahead of print)

Kamruzzaman M, Udden SM, Cameron DE, Calderwood SB et al (2010) Quorum-regulated biofilms enhance the development of conditionally viable, environmental *Vibrio cholera.* Proc Natl Acad Sci USA 107:1588–1593

Leadbetter JR, Greenberg EP (2000) Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus.* J Bacteriology 182:6921–6926

McClean KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW, Stewart GS, Williams P (1997) Quorum sensing and *Chromobacterium violaceum*, exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiology 143:3703–3711

Morohoshi T, Inaba T, Kato N, Kanai K, Ikeda T (2004) Identification of quorum-sensing signal molecules and the LuxRI homologs in fish pathogen *Edwardsiella tarda*. J Biosci Bioeng 98:274–281

Ng WL, Basslern BL (2009) Bacterial quorum-sensing network architectures. Annu Rev Genet. 43:197–222

Manuscript in publication

Nhan DT, Cam V, Wille M, Defoirdt T, Bossier P, Sorgeloos P (2010) Quorum quenching bacteria protects *Macrobrachium rosenbergii* larvae from *Vibrio harveyi* infection. J Appl Microbiol 109:1007-1016

Ninawe AS, Selvin J (2009) Probiotics in shrimp aquaculture: avenues and challenges. Crit Rev Microbiol 35:43–66

Pan J, Huang T, Yao F, Huang Z, Powell CA, Qiu S, Guan X (2008) Expression and characterization of *aiiA* gene from *Bacillus subtilis* BS-1. Microbiol Res 163:711–716

Pratt LA, Kolter R (1998) Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol Microbiol 30:305–293

Rasmussen TB, Givskov M (2006) Quorum sensing inhibitors: a bargain of effects. Microbiology 152:895–904

Roche DM, Byers JT, Smith DS, Glansdorp FG, Spring DR, Welch M (2004) Communications blackout? Do N-acylhomoserinelactone-degrading enzymes have any role in quorum sensing. Microbiology 150:2023–2028

Sarkar BL, Nair GB, Banerjee AK, Pal SC (1985) Seasonal distribution of *Vibrio parahaemolyticus* in freshwater environs and in association with freshwater fishes in Calcutta. Appl Environ Microbiol 49:132–136

Su YC, Liu C (2007) *Vibrio parahaemolyticus*: a concern of seafood safety. Food Microbiol 24:549–558

Manuscript in publication

Swift S,. Karlyshev AV, Fish L, Durant EL, Winson MK, Chhabra SR, Williams P, Macintyre S, Stewart GS (1997) Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the LuxRI homologs AhyRI and AsaRI and their cognate N-acylhomoserine lactone signal molecules. J Bacteriol 179:5271–5281

Thompson FL, Lida T, Swings J (2004) Biodiversity of Vibrios. Microbiol Mol Rev 68: 403–431

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703

Yeung PS, Boor KJ (2004) Epidemiology, pathogenesis, and prevention of foodborne *Vibrio parahaemolyticus* infections. Foodborne Pathog Dis 1:74–88

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FIGURE LEGENDS

Figure 1. **Temperature and pH activity profiles of the AHL-lactonase (AiiA) from *B. lichinoformis* DAHB1.** The activity profiles of purified recombinant AiiA protein were determined by AHL-lactonase assay using C6-HSL as the substrate over a range of temperatures (A) and pH (B). Relative activity is shown with mean ± SD indicated (with 100% activity at 40°C and pH 7.0).

Figure 2. **Exogenous AiiA survives in the shrimp intestine.** The presence of exogenous AiiA in the intestine of treated shrimps was confirmed by Western analysis. Shown is the purified AiiA protein (lane 1), and samples recovered from two shrimp (lanes 2 & 3). AiiA is ~28 kDa.

Figure 3. **Shrimp mortality caused by vibrio infection is reduced by exogenous AiiA protein.** Abdominal injections of *V. parahaemolyticus* DAHP1-GFP result in significant levels of mortality in shrimps reared in a closed aquaculture system. However, co-injection with exogenous AiiA significantly reduces mortality over a period of 124 hr. Shown are the cumulative mortalities for replicate groups of shrimps injected with *V. parahaemolyticus* DAHP1-GFP (triangles), *V. parahaemolyticus* DAHP1-GFP plus AiiA (circles), and AiiA alone (squares). Mean mortality ± SD are indicated. Those time points marked by '*' indicate a significant difference between treatments (one-way ANOVA; $P < 0.05$).

Figure 4. **Exogenous AiiA limits vibrio colonisation of shrimp intestines.** A clear difference in the colonisation of the luminal surface of shrimp intestines by *V. parahaemolyticus* DAHP1-GFP can be observed by CLSM between individuals

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infected with *V. parahaemolyticus* DAHP1-GFP alone or a mixture of *V. parahaemolyticus* DAHP1-GFP and AiiA protein. Shown are representative CLSM images of intestine samples from an uninfected shrimp (A), an infected shrimp (B), and a shrimp co-infected with *V. parahaemolyticus* DAHP1-GFP plus AiiA protein five days after injection (C). Image 'A' is shown with increased fluorescence intensity to high-light the lack of GFP activity; the fluorescence observed is therefore the natural background activity.

Supp. Figure 1. AiiA protein *in vitro* biofilm inhibition. Shown here are CLSM and light microscopic (40x) images demonstrating the impact of AiiA on biofilm-formation by *V. parahaemolyticus* DAHP1-GFP after 24 h; control (A & D), with AiiA (C & B).

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