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ORIGINAL PAPER

Inhibition of *Vibrio cholerae* biofilm by AiiA enzyme produced from *Bacillus* spp.

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Abstract *Vibrio cholerae* is the causative agent of water-borne diarrheal disease, cholera. The formation of biofilm favors survival and persistence of *V. cholerae* in the aquatic environment and also inside the host. AHL lactonase (AiiA), a metallo-beta-lactamase produced by *Bacillus* spp., blocks quorum sensing in Gram-negative bacteria by hydrolyzing N-acyl-homoserine lactones (AHLs). In the present investigation, AiiA-mediated inhibition of *V. cholerae* biofilm was studied. Two novel alleles of *aiiA*-encoding genes from *Bacillus* spp. were expressed in *E. coli,* and the results demonstrated that AiiA enzyme is a potent inhibitor of *V. cholerae* biofilm.

Keywords AiiA enzyme · Biofilm · Quorum sensing · *V. cholerae*

Introduction

V. cholerae is the causative agent of the water-borne disease cholera that still threatens a large proportion of world's population. Cholera is characterized by severe diarrhea and sometimes even death. *V. cholerae* has the ability to survive in diverse aquatic environments as well as in human host. Biofilm development plays an important role for the survival as well as sustenance of *V. cholerae* during and after epidemic period (Kierek and Watnick 2003; Gutierrez et al. 2009). Several studies have shown that the expression of various virulence factors as well as genes responsible for biofilm development is controlled by quorum sensing in *V. cholerae* (Zhu et al. 2002; Hammer and Bassler 2003; Zhu and Mekalanos 2003; Kamruzzaman et al. 2010). Quorum sensing is a process by which bacteria detect their local cell density, coordinate gene expression and regulate processes beneficial to bacterial community. Quorum sensing requires production, secretion and detection of certain extracellular signal molecules called autoinducers. The most common signal molecule in Gram-negative bacteria is acyl-homoserine lactones (AHL), which is synthesized by the family of LuxI homologue proteins (Rasmussen and Givskov 2006). *Vibrio* spp. use AHL molecules to coordinate virulence gene expression in response to the surrounding bacterial population. Bai et al. (2008) reported that many *Bacillus* spp. are capable of secreting an enzyme AiiA that cleaves the lactone rings from acyl moieties and makes it inactive in signal transduction and also showed the quorum sensing inhibitory activity of AiiA enzyme against *V. harveyi*. But till date, there are no reports of biofilm inhibition in *V. cholerae* mediated by AiiA enzyme. In this context, we isolated *Bacillus* spp. from soil samples to evaluate their biofilm inhibition property. In the present study, we report the cloning and expression of AHL lactonase gene (*aiiA*) from two strains of *Bacillus*. The effect of AiiA protein on biofilm formation of *V. cholerae* was examined. The results showed that AiiA protein from isolated *Bacillus* sp. could significantly reduce the biofilm formation capacity of *V. cholerae.*

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Materials and methods

Biofilm assay

Twenty *Bacillus* spp. were isolated from the soil samples collected from Kannur and Wayanad districts of Kerala, South India. *V. cholerae* O110 (PL91) strain was used for biofilm inhibition assay. All *Bacillus* spp. were first cultured in LB broth and then subjected to ultrasonication followed by centrifugation at 13,000 rpm. The supernatant obtained was stored at −20°C till further use. The biofilm assay was done by the method previously described by Pratt and Kolter (1998). Briefly, 10 µl of overnight culture of *V. cholerae* was inoculated in 96-well microtitre plates (polystyrene) containing 100 µl LB medium and 100 µl cell extract followed by incubation for 24 h at room temperature. Planktonic cells and spent medium were discarded, and adherent cells were gently rinsed twice with deionised water and allowed to air dry before being stained. The biofilms were stained with 210 µl of 0.1% crystal violet solution (w/v) for 10 min, after which the dye was discarded, and the wells were rinsed twice with deionized water. The wells were allowed to air dry before solubilization of the crystal violet with 210 µl of dimethyl sulfoxide. The optical density was determined at 595 nm in an enzyme-linked immunosorbent assay reader (Bio-Rad).

Cloning, sequencing and expression of *aiiA* gene in *E. coli*

Total genomic DNA of selected *Bacillus* spp. (BC6 and BC10) was isolated using commercially available Wizard® Genomic DNA Purification Kit (Promega). The identification of the test strains was done by partial sequencing of conserved 16S rRNA gene as described earlier (Weisburg et al. 1991). The full-length *aiiA* gene was amplified by following the method of Pan et al. (2008). The PCR was done by using Phusion High-Fidelity DNA Polymerase (NEB). The PCR products were purified by using Ilustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). The purified product was first 'A' tailed by incubating with 1 unit of taq polymerase (Sigma) for 10 min at 72°C and subsequently ligated to the pGEM-T easy vector (Promega) to obtain the recombinant plasmid and transformed into competent *E. coli* MACH1 cells with ampicillin and blue/white screening in accordance with the manufacturer's instructions. The DNA sequencing of selected clones was done on both strands using T7 and SP6 primers on an ABI PRISM 3100 DNA sequencer system using the Big Dye Terminator kit (Applied Biosystems). The sequence alignment was performed on the *aiiA* gene sequences and the deduced amino acid sequences using clustalW multiple alignment program in Bioedit sequence alignment editor version 7.0.9.0 and BLAST network. For expression, the *aiiA* gene was amplified with primers AIF1 (5′-GGGAA TTC**CATATG**ACAGTAAAAAAGCTTTATTTC-3′) and AIR1 (5′-CCG**GAATTC**CGGCTATATATACTCCGGGA ACTC-3′) with restriction site for NdeI and EcoRI, respectively. The amplified products were first purified, double digested with NdeI and EcoRI (NEB) and subsequently cloned into the corresponding sites of the plasmid pET-32a and transformed with chemically competent *E. coli* BL21 (DE3) pLysS cells. Plasmids were isolated from recombinant clones by using Wizard® plus SV Minipreps DNA Purification Kit (Promega). Orientation of *aiiA* gene was verified by sequencing. The recombinant *E. coli* BL21 (DE3) pLysS cells were induced with 0.5 mM IPTG at 28°C for 2–6 h. Overexpression of protein was analyzed using SDS–PAGE on a 10% polyacrylamide separating gel. The biofilm assay was repeated with recombinant *E. coli* extracts as mentioned earlier.

Air–liquid interphase coverslip assay

V. cholerae was incubated with a cover glass in a 50-ml Erlenmeyer flask containing 2 ml of LB medium at 28°C for 24 h. To evaluate biofilm inhibition, the biofilm was treated with extracts of recombinant *E. coli* possessing *aiiA* gene of BC6 and BC10. After staining with 0.1% crystal violet solution (w/v) for 10 min, the cover slips were washed with water and air dried and observed under inverted microscope with 63× oil immersion objective (Nikon). We also examined the biofilm inhibition of *V. cholerae* in borosilicate glass tubes when treated with extracts of native *Bacillus* sp. and recombinant *E. coli.*

Results and discussion

Of 20 *Bacillus* strains screened for biofilm inhibition, only two strains (BC6 and BC10) showed significant biofilm inhibitory activity. Both BC6 and BC10 were identified as *Bacillus* sp. belonging to *B. cereus* group on the basis of 16S rRNA gene sequence and BLAST analysis (Accession No: HM196279 and HM196280, respectively). The *B. cereus* group includes four species: *B. cereus, B. thuringiensis, B. anthracis* and *B. mycoides* (Chang et al. 2003). However, we could not differentiate it as *B. cereus* or *B. thuringiensis* as these two are very closely related species and cannot be identified on the basis of 16S rRNA sequencing (Manzano et al. 2003; Park et al. 2007). The *aiiA* gene was amplified from BC6 and BC10 by using Phusion High-Fidelity DNA Polymerase so as to reduce the errors during PCR amplification. The 753-bp *aiiA* gene from BC6 and BC10 was sequenced and submitted to the Genbank with accession number HM196281 and HM196282, respectively. Both *aiiA* genes encoded a predicted protein of 250 amino acid

residue. BLAST analysis and pairwise alignment revealed that *aiiA* gene of BC6 and BC10 was 99.2% similar to that of *aiiA* of *B. cereus* strain KM1S (Accession No: FJ960449) and *B. cereus* B4264 (Accession No: CP001176), respectively. The comparison at predicted protein level showed that there is substitution of 2 amino acids at position 61 (glutamic acid to alanine) and 245 (glutamic acid to valine) in BC6 and at position 21 (valine to leucine) and 245 (glutamic acid to valine) in BC10 (data not shown). Hence, the results indicated that the two alleles of *aiiA* genes sequenced are novel.

To confirm that the biofilm inhibition in BC6 and BC10 was due to AiiA enzyme, we cloned the gene into an expression vector (pET-32a). As anticipated, 28 kDa over-expressed protein was detected in SDS–PAGE. (Fig. 1). The results also indicated that there was no considerable variation in the level of expressed protein at different time intervals. Use of NdeI enzyme for digestion of pET-32a ensured that the recombinant protein expressed carried no tags. Results obtained from the biofilm assay and air–liquid cover slip assay with extracts of recombinant *E. coli* showed significant inhibition of biofilm formation (Figs. 2 and 3). However, no inhibition of biofilm activity was detected in untreated *V. cholerae* (Figs. 2 and 3). The results obtained in the present study also indicated that the biofilm-forming property of *V. cholerae* was reduced by ~80% when treated with extracts of recombinant *E. coli* BL21 (data not shown). The genes encoding the AiiA enzyme are widespread among strains of *B. cereus* group (Lee et al. 2002; Wang et al. 2004). The human airway epithelial cells are also capable of inactivating AHLs (Rasmussen and Givskov 2006). Production of AHL lactonase may also be a bacterial strategy to compete with AHL-producing strains in the environment and is a prokaryote to prokaryote quorum quenching approach. Homologues of AiiA have been reported in *Agrobacterium tumefaciens, Arthrobacter* sp., *Klebsielle pneumoniae, Rhodococcus* sp. (Carlier et al. 2003; Park et al. 2003). From this study, we have clearly demonstrated that AiiA enzyme of BC6 and BC10 is involved in biofilm inhibition of *V. cholerae*. The inhibition of *V. cholerae* biofilm on AiiA treatment may be due to its AHL degrading activity as it is reported that AiiA enzyme hydrolyzes the lactone bond within the AHL moiety, thus changing the structural conformation of the signaling molecule and also demonstrated the disruption of quorum sensing pathways by AiiA enzyme in *V. harveyi*, a fish pathogen (Bai et al. 2008). So, targeting quorum sensing circuits of Vibrios in aquaculture farms is extremely significant and may prevent huge economic loss. The AHL lactonase group of enzymes has potent autoinducer degradation activity; however, its application is limited due to the problems faced in delivering proteinaceous agents (Rasmussen and Givskov 2006). So, there is need of further research to generate new methods for efficient delivery of AHL-degrading enzyme in aquatic settings. Biofilm formation is important for the life cycle of *V. cholerae*, facilitating environmental persistence within natural aquatic habitats during interepidemic periods (Reidl and Klose

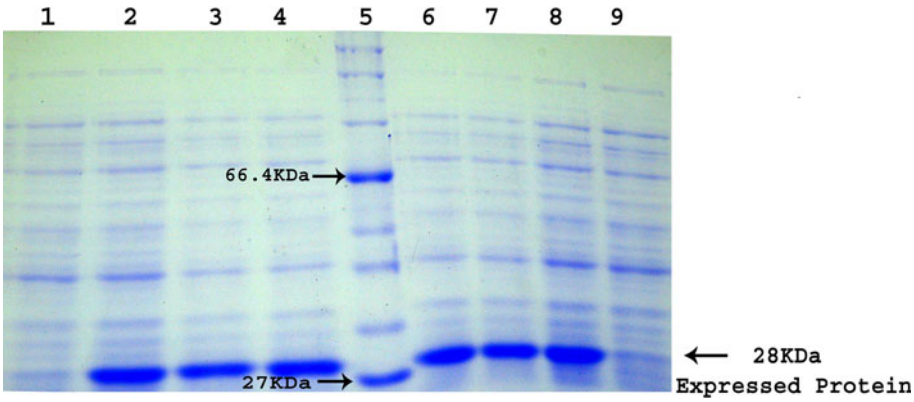

Fig. 1 SDS–PAGE analysis of over expressed AiiA protein in *E. coli* BL21 DE3 pLysS. *Lane 1 and 9*, Cell lysates of recombinant *E. coli* (harboring *aiiA* gene of BC6 and BC10, respectively) in absence of IPTG. *Lane 2–4 and 6–8*, Cell lysate of recombinant *E. coli* carrying *aiiA* gene of BC6 and BC10 in different time intervals (2, 4 and 6 h, respectively). *Lane 5*, Broad-range protein marker (NEB)

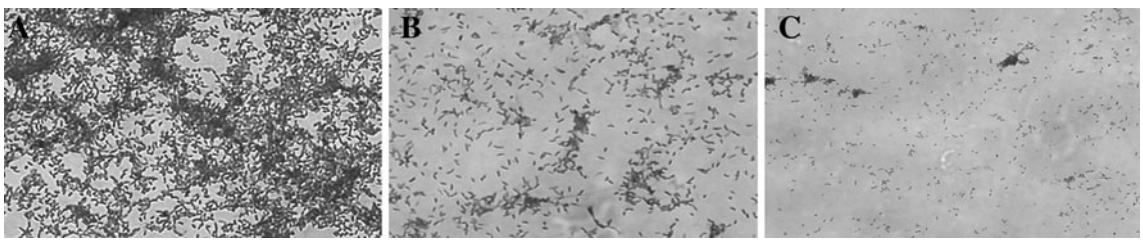

Fig. 2 Air–Liquid interphase coverslip assay. **a** *V. cholerae* biofilm (positive control). **b** and **c** Biofilm treated with extract of recombinant *E. coli* (AiiA of BC6 and BC10, respectively)

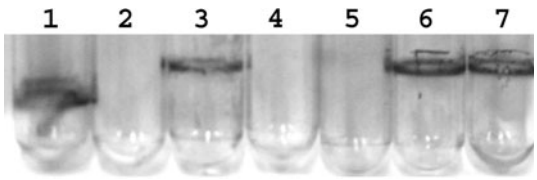

Fig. 3 Biofilm inhibition assay in test tube. *1 V. cholerae* biofilm (positive control), *2* LB broth (negative control) *3* Biofilm treated with extract from the *Bacillus* spp strain (BC6). *4* and *5* Biofilm treated with extract of recombinant *E. coli* (*aiiA* of BC6 and BC10, respectively. *6* Biofilm treated with *E. coli* BL21 cell extract (without pET-32a). *7* Biofilm treated with extract of *E. coli* BL21 transformed with pET-32a (without *aiiA* gene)

2002). To prevent the circulation of viable toxigenic *V. cholerae* in the environment, it is essential to control its quorum-mediated regulatory pathways. As multidrug resistance is rapidly emerging in *V. cholerae* and related pathogens, there is an urgent need for novel compounds that will interfere with quorum sensing. In this context, AiiA enzyme identified in this study may be a potent candidate. To our knowledge, this is the first report of biofilm inhibition of *V. cholerae* by AiiA enzyme.

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