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Molecular identification of *aiiA* homologous gene from endophytic *Enterobacter* species and in silico analysis of putative tertiary structure of AHL-lactonase

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ABSTRACT

The *aiiA* homologous gene known to encode AHL- lactonase enzyme which hydrolyze the *N*-acylhomoserine lactone (AHL) quorum sensing signaling molecules produced by Gram negative bacteria. In this study, the degradation of AHL molecules was determined by cell-free lysate of endophytic *Enterobacter* species. The percentage of quorum quenching was confirmed and quantified by HPLC method ($p < 0.0001$). Amplification and sequence BLAST analysis showed the presence of *aiiA* homologous gene in endophytic *Enterobacter asburiae* VT65, *Enterobacter aerogenes* VT66 and *Enterobacter ludwigii* VT70 strains. Sequence alignment analysis revealed the presence of two zinc binding sites, "HXHXDH" motif as well as tyrosine residue at the position 194. Based on known template available at Swiss-Model, putative tertiary structure of AHL-lactonase was constructed. The result showed that novel endophytic strains of *Enterobacter* genera encode the novel *aiiA* homologous gene and its structural importance for future study.

1. Introduction

Bacteria produce small diffusible signaling molecules and sensing signals to communicate in the environment, which is known as Quorum Sensing (QS) [1]. Acyl homoserine lactones (AHLs) are the major quorum sensing signaling molecules in Gram negative bacteria. AHLs have conserved regions at lactone moiety and varied in their acyl-chains [7]. These AHL molecules play a major role in expression of virulence factors as well as quorum sensing regulated biofilm formation [13]. Degradation of AHLs has proven the efficient control of bacterial infections in transgenic plants were virulence factors are regulated by QS [34].

The inactivation/degradation of AHL molecules are observed in quorum quenching enzymes. The first quorum quenching enzyme identified from a soil bacterial isolate belonging to Gram positive *Bacillus* species encode by *aiiA* gene [36]. The homologous *aiiA* was also identified in many species of *Bacillus,* like *Bacillus thuringiensis*, *Bacillus cereus*, *Bacillus mycoides* [35], *Bacillus anthracis* [21] and *Bacillus weihenstephanensis* [16]. These *aiiA* genes expressed as AHL-lactonase and mediate the cleavage of lactone ring. The existence of lactonase can attenuates AHL mediated quorum sensing in microbes, leading to blocking of virulence and pathogenic phenotypes [37].

Introduction of cloned genes of AHL-lactonase into bacterial cells caused the degradation of AHL. The expression of lactonase is known to attenuate the virulence factors in *Erwinia carotovora* [36], *Aeromonas hydrophila* and *Pseudomonas aeruginosa* (encoded by AiiM gene) [33]. Many subspecies of *B. thuringiensis* like *galleriae*, *kurstaki*, *aizawai*, *ostriniae* and *subtoxicus* were shown to degrade AHL encoded by *aiiA* gene [28]. Recently, *Bacillus amyloliquefaciens* showed the presence of *aiiA* gene with high identity to its homologous gene present in different *Bacillus* species and strain exhibited quorum quenching property on bacterial disease control [32].

Most of the AHL-lactonase are belongs to the metallo-β-lactamase super family because it show significant sequence and structural homology. A study on evolutionary history between structural and chemical similarities between AHL molecules and β-lactams as well as structural and substrate binding similarities between metallo-β-lactamase and AHL-lactonase suggest the role of chemical ecology in microbial systems [8]. The catalytic activity of AHL lactonase purely depends on presence of two zinc ions, which facilitate the ring opening hydrolysis of lactones. Coordination of these zinc ions with single oxygen of a bridging carboxylate and a bridging hydroxide ion facilitates the nucleophilic attack on AHLs molecules [5].

The endophytic bacteria that live in plant tissues without harming the host plant except residency. These endophytic Gram positive and Gram negative bacteria can be isolated from surface-sterilized plant tissue or extracted from internal plant tissue.

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Endophytes primarily get entry through root zone, which can reside within the cells, in the intercellular spaces, or in the vascular systems and also reaches to the aerial parts of plant [4]. Bacterial endophytes reported to produce a number of metabolites including plant growth promoting agents and biological control agents [25]. The plant *Ventilago madraspatana* (Family: *Rhamnaceae*), is a woody climber growing on hills, foothills and scrub jungles of Peninsular India, Sri Lanka and Malay Peninsula to Malaysia. Chemical constituents of *V. madraspatana* have been well-documented and used as folk remedy for vitiated conditions of gout, dyspepsia, erysipelas, leprosy, scabies and skin problems [24]. So far, no reports are available on *aiiA* homologous gene identification and putative tertiary structure of AHL lactonase from endophytic bacteria. Therefore, endophytic bacterial species of *Enterobacter* were isolated from *V. madraspatana* and *aiiA* homologous gene was identified by amplification of conserved regions; elucidated with putative tertiary structure of AHL-lactonase.

2. Materials and methods

2.1. Endophytic bacterial isolates

The *V. madraspatana* plant sample was collected from Western Ghat forest of Karnataka, India [18]. The leaves, bark and roots samples were surface sterilized and bacterial endophytes are isolated from the plant. The samples were cut into small pieces, each piece put on a plate of Luria-Bertani (LB) agar medium and the plates were cultivated at 30 °C for 2 days to promote endophytic bacterial growth. Individual colonies were selected randomly and inoculated onto another LB plate, and incubated at 30 °C for 24 h. Each bacterial culture was checked for purity and the purified endophytic isolates were numbered, transferred separately to LB slants, and stored at 4 °C [19].

2.2. Bacterial strains and chemicals

AHL biosensor *Chromobacterium violaceum* CV026 (Mutant, which respond to short chain of AHLs) was maintained in Luria-Bertani (LB) broth at 30 °C in rotary shaker incubator (Labline Industries, Cochin, India) for 24 h [12]. *N*-Butanyl-L-homoserine lactone (C_4-HSL, Sigma–Aldrich, Bangalore, India) and *N*-Hexanoyl-homoserine-L-lactone (C_6-HSL, Sigma–Aldrich, Bangalore, India) were used as substrate for *C. violaceum* CV026.

2.3. Preparation of cell-free lysate

The endophytic bacterial isolates were grown in the minimal medium contained (per litre) NaCl, 1 g; KCl, 0.5 g; $MgCl_2$, 0.4 g; $CaCl_2$, 0.1 g; Na_2SO_4, 0.15 g; KH_2PO_4, 2 g; Na_2HPO_4, 2.25 g and trace elements were added to final concentrations of 1 mg $FeCl_3$, 0.1 g $MnCl_2$ and 46 mg $ZnCl_2$ per litre [27], at 30 °C with shaking for 48 h and then harvested by centrifugation at 12,000 rpm for 10 min. The cell-free lysate was extracted with 10 ml of potassium phosphate buffer (PPB, 100 mM; pH 7.0) and centrifuged; supernatant was filtered through 0.45 μm filter, the filtrate was stored at −20 °C until use. All assays were performed with assay control (PPB).

2.4. AHL Degradation by Agar overlay assay

The LB broth was taken in microcentrifuge tubes loaded with 50 μl of cell-free lysate supplemented with 200 μM of C_4-HSL and C_6-HSL to get 1.5 ml final concentration in separate tubes. Then tubes were incubated at 30 °C for 2 h. After incubation, it was heated at 95 °C for 10 min. The LB agar plates (10 ml of LB, 1.5% agar) were overlaid with *C. violaceum* CV026 biosensor (0.8% agar) and the wells were loaded with 0.45 μm filter sterilized 50 μl of the reaction mixture and incubated at 30 °C for 24 h along assay controls. The presence of clear zone (inhibition of violacein production) around the wells is the indication of AHL degradation [30]. To confirm the enzymatic activity, the cell-free lysate was heat treated at 95 °C for 10 min and above agar overlay assay was repeated with heat treated cell-free lysate by using *C. violaceum* CV026 biosensor. The relative AHL-degradation activity was calculated as nmol/hour/ml, based on initial concentration of AHLs with leftover amount from cell-free lysate digestion.

2.5. Extraction of total DNA, PCR analysis and sequencing

To identify the bacterial species, the DNA was extracted according to the previously reported method [31]. Amplification of 16s rRNA gene of endophytic bacteria was performed by universal primers for set of forward and reverse primers (27F-5′-AGAGTTT GATCCTGGCTCAG-3′ and 1492R-5′ACGGCTACCTTGTTACGC TT-3′, Bangalore Genie, India) respectively. The thermocycling conditions (Eppendroff Mastercycler, Germany) maintained as initial denaturation at 94 °C for 4 min, 35 amplification cycles of 94 °C for 45 s, 54 °C for 45 s, 72 °C for 1 min and final polymerization step of 94 °C for 8 min. The final PCR product was resolved in 1.5% agarose gel and purified using GenElute gel elution kit (Sigma Aldrich, USA). DNA sequencing was performed and sequence subjected to BLAST analysis and Nucleotide sequence similarities were determined by NCBI (National Center for Biotechnology Information databases). The identified isolates sequences were deposited and assigned with accession numbers.

2.6. Confirmation of Quorum quenching activity by HPLC

The cell-free lysate (suspended in 100 mM phosphate buffer, pH 7.0) was incubated with 100 μM C_6-HSL for 12 h at 30 °C. Then, the reaction was stopped by addition of equal amount of dichloromethane and the solvent part was evaporated. The residue resuspended in acetonitrile (100 μl) and the amount of residual AHLs determined by AHL biosensors and HPLC. Reverse phase HPLC analysis were performed by using C18 column, Waters system coupled with photodiode array detector (210 nm) and eluted with gradient (1–100%) of water: formic acid (0.1%, pump A) and acetonitrile: formic acid (0.1%, pump B) at the flow rate of 0.5 ml/min. The percentages of AHL degradation were determined by estimation of AHL with respect to known loaded concentration. The uninoculated preparation used as control.

2.7. PCR amplification of aiiA homologous gene

The endophytic bacteria which degrade AHLs were confirmed for the presence of AHL-lactonase enzyme by amplification of *aiiA* homologous gene by previously reported method [35] with some modification. Briefly, genomic DNA was amplified using forward and reverse primers, *aiiAF2* (5′-CGGAATTCATGACAGTAAAG AAGCTTTA-3′) and *aiiAR2* (5′-CGCTCGAGTATATATTCAGGGAACA CTT-3′) [9]. The thermal cycling conditions were maintained as initial denaturation at 94 °C for 5 min, 5 cycles of 94 °C (45 s), 44 °C (45 s), 72 °C (1 min); 30 cycles of 94 °C (45 s), 53 °C (45 s), 72 °C (1 min); followed by primer extension at 72 °C for 8 min. The amplicons was resolved by 2% agarose gel.

2.8. DNA sequencing and alignment of lactonase gene

The amplicons of *aiiA* gene was purified using GenElute gel elution kit (Sigma Aldrich, USA) and sequenced. The obtained sequence was analyzed, and assembled using CAP3 sequence

assembly program. The open reading frame (ORF) was detected using Geneious 6.1 software and the nucleotide sequence was submitted to GenBank database.

2.9. Analysis of in silico tertiary structure of the AHL-lactonase enzyme

The putative tertiary structure of the AHL-lactonase enzyme was predicted using Swiss-Model Software (http://swissmodel.expasy.org/) [11]. The tertiary structure of AHL lactonase enzyme was analyzed for presence of metal ligands for dinuclear zinc binding.

2.10. Statistical analysis

Statistical significance of variance for the AHLs degradation by agar overlay assay and HPLC method were quantified based on difference between amount of left over AHL molecules, which were analyzed by one way ANOVA ($p < 0.0001$) followed by Tukeys test. Statistics was performed using Graphpad Prism 5.03 software (Graph Pad Software Inc., La Jolla, CA, USA).

3. Results and discussion

3.1. Isolation of endophytic bacterial isolates

The morphologically different bacterial colonies were selected for screening of quorum quenching activity and totally 98 isolates were obtained from *V. madraspatana* as endophytes. Endophytes are mutualistic to their host and prevent the host from successful attacks of pathogens, pests and mammals by producing many secondary metabolites [2]. The interesting lifestyle of endophytes and application of these organisms in many fields [29] allows the speculations in utilization of Quorum quenching (QQ) enzymes from endophytes for disruption of quorum sensing in pathogens.

3.2. 16S rRNA sequence analysis

The isolates degrading synthetic AHLs were identified by amplifying the genomic DNA followed by sequence of 16S rRNA gene. The isolates tentatively identified by BLAST analysis for obtained sequence of 16S rRNA. Identified endophytic bacteria were deposited at NCBI (GenBank database) with accession number (Table 1). Sequence analysis 16S rRNA of endophytic isolates were identified as *E. asburiae* VT65, *E. aerogenes* VT66 and *E. ludwigii* VT70. *E. asburiae* previously isolated from peanut as endophyte plant growth promoting bacteria [26], *E. aerogenes* as endophyte from maize seedlings [14] and *E. ludwigii* as endophyte from strawberry fruit [8]. Furthermore, to find difference/relationship between endophytes of our isolates and isolates of pathogen, environment origin was determined by generating phylogenetic tree. The phylogenetic tree was generated by taking 16S rRNA sequences of closest hits in BLAST analysis with above said criteria. Phylogram showed an unique subclade of isolated endophytic bacteria related to an uncharacterized genomic system and it suggests novel strains as endophytes (Fig. 1).

3.3. Screening of endophytic bacteria for AHL degradation

The quorum quenching activity of cell-free lysate of each endophytic bacterium was screened with synthetic C_4-HSL and C_6-HSL molecules by agar overlay assay method. Inhibition of violacein production was considered as positive for degradation of AHL molecules using *C. violaceum* CV026 biosensor. The isolate VT65, VT66 and VT70 were showed the degradation of AHLs by agar overlay assay (Fig. S1). Further, to confirm the enzymatic activity in cell-free lysate, it was heat killed at 95 °C for 10 min results in complete loss of activity. The loss of AHL degradation upon heat treatment shows that possibility of quorum quenching enzymes in cell-free lysate of isolated endophytic bacteria (data not shown). All the positive isolates were significantly ($p < 0.0001$) degraded AHL molecules and quantitatively *E. aerogenes* VT66 possessed highest degradation activity for C_6-HSL molecule at 357.4 ± 1.3 nmol/h/ml (Table 1). Endophytic *Streptomyces* has shown the HSL-acylase activity with highest HSL-degrading activity of 151.3 ± 3.1 nmol/h/ml on C_{10}-HSL as most favorable substrate and *in vitro* pathogenicity assay efficiently reduced the soft rot of potato in *Pectobacterium carotovorum* ssp. *carotovorum* [23].

3.4. HPLC analysis

The product of C_6-HSL degradation by cell-free lysate of endophytic bacteria was analyzed by HPLC. *E. asburiae* VT65, *E. aerogenes* VT66 and *E. ludwigii* VT70 exhibited significant ($p < 0.0001$) hydrolysis of C_6-HSL compared to control (Fig. S2). These three endophytic bacteria showed about 95% degradation of the respective QS molecule under test condition. Therefore, the degradation of QS molecules further confirms the presence of quorum quenching enzymes, which uses the given C_6-HSL as substrate. Many Gram negative bacteria have shown the QQ activity including *P. aeruginosa* PAO1 [10], *Pseudomonas syringae* [22], *Ralstonia solanacearum* [3] etc.

3.5. Analysis of aiiA homologous gene

Based on Agar overlay assay, inactivation of degradation activity upon heat treatment and HPLC analysis suggests the presence of quorum quenching genes in reported endophytic bacterial isolates. Using the extracted genomic DNA, an amplicons of about 800 bp was detected using 2% agarose gel electrophoresis (Fig. 2). The previous reports showed that, the detection of about 800 bp as AHL-lactonase using specific primers for *aiiA* gene [35,16].

3.6. DNA sequencing and alignment of lactonase gene

The amplicon of *aiiA* homologous gene was sequenced and using NCBI database closest BLAST hit was showed the *aiiA* homologous gene indicating the presence of AHL lactonase as QS degrading enzyme in cell-free lysate of endophytic bacteria. Furthermore, multiple alignment analysis using NCBI database and ClustralW for *aiiA* homologous gene of our sample with AHL lactonases from other species revealed the presence of the motif "HXHXDH" as well as tyrosine (Y) residue at the position 194 (Fig. 3) [16]. Thomas

Table 1
AHL degradation of endophytic *Enterobacter* species by bioassay and HPLC method.

Endophytic isolate	Identified bacteria (GenBank accession No.)	AHL degradation by *C. violaceum* (nmole/ml)		AHL degradation (HPLC method, %)	*aiiA* gene accession No. (GenBank)
		C_4-HSL	C_6-HSL		
VT65	*Enterobacter asburiae* VT65 (KC818122)	184.4 ± 3.8	210.0 ± 2.2	98.5 ± 3.2	KF768740
VT66	*Enterobacter aerogenes* VT66 (KC818121)	242.7 ± 2.8	357.4 ± 1.3	99.6 ± 1.1	KF768741
VT70	*Enterobacter ludwigii* VT70 (KC818123)	237.0 ± 1.4	303.1 ± 2.3	97.2 ± 2.3	KF768742

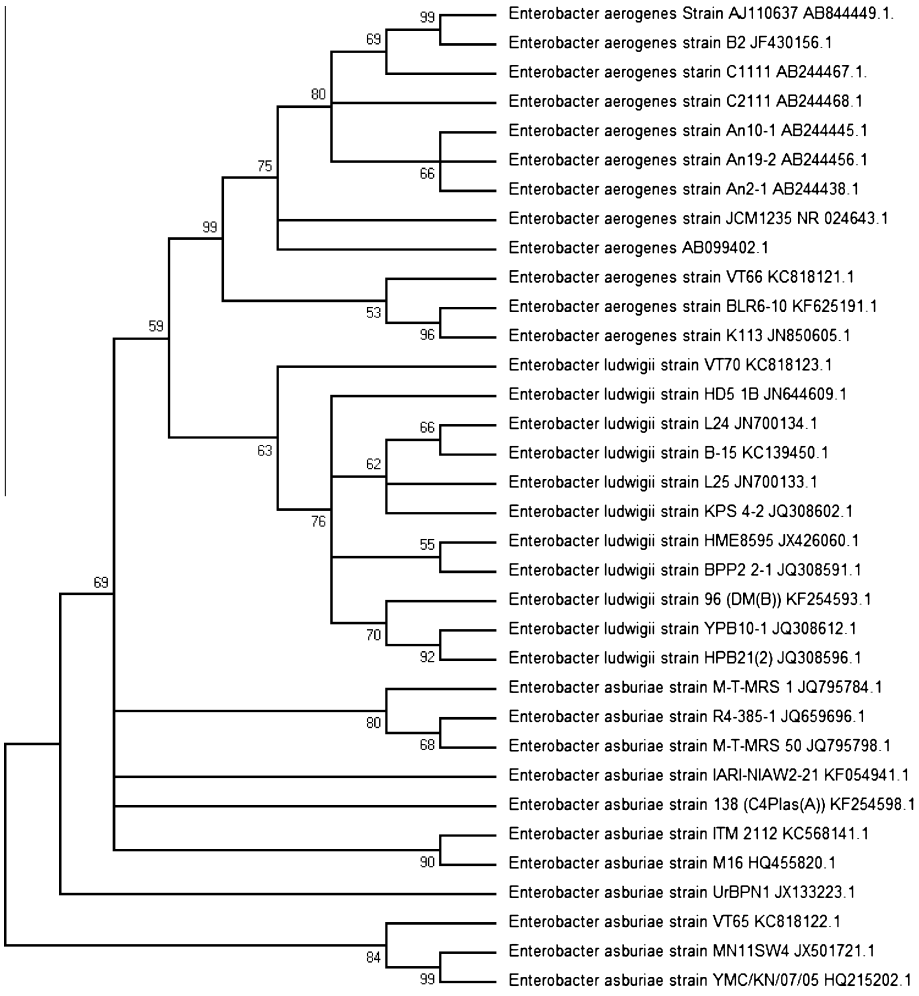

Fig. 1. Unrooted phylogenetic tree deriving from neighbor-joining showing the evolutionary relationship of endophytic *Enterobacter asburiae* VT65, *Enterobacter aerogenes* VT66 and *Enterobacter ludwigii* VT70 with its closest BLAST hits. The neighbor-joining tree was maintained by 1000 boot straps using the MEGA-5 software.

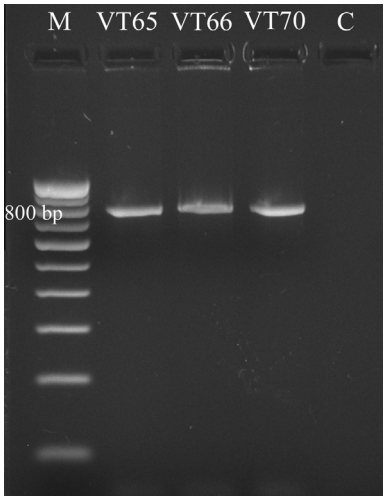

Fig. 2. Amplicons of *aiiA* homologous gene (about 800 bp) from endophytic isolates. Lane M; 100 bp molecular marker, Lane VT65; *Enterobacter asburiae* VT65, Lane VT66; *Enterobacter aerogenes* VT66, Lane VT70; *Enterobacter ludwigii* VT70, Lane C; PCR control (without DNA template).

et al. proposed the possible model representing the zinc binding residue which is totally conserved in all known AHL-lactonase; His104, His 106, and His 169 for the metal-1 site and Asp 108, His 109, and His 235 for the metal-2 site also observed in our sequences [20].

3.7. Analysis of in silico tertiary structure of the AHL-lactonase enzyme

A putative tertiary structure of AHL-lactonase was predicted by using Swiss-Model using *N*-acyl homoserine lactone hydrolase of *B. thuringiensis* as a template [6] accession No. 3dh.1 for *E. asburiae* VT65, 2br6.1 for *E. aerogenes* VT66 and 2a7m.1 for *E. ludwigii* VT70 along with two Zn^{++} binding sites respectively (Fig. 4). The model showed a QMEAN4 score of 0.866 for *E. asburiae* VT65, 0.844 for *E. aerogenes* VT66 and 0.851 for *E. ludwigii* VT70 [17]. These putative tertiary structural models of AHL-lactonase hypothesized to contain binding sites for two zinc atoms as represented by metalloenzymes, which play a major role in positioning of lactone ring of the substrate [15]. In our study, AHL-lactonase from reported endophytic bacteria revealed similarity with proposed conserved regions and metal ions required for the enzymatic activity.

The study demonstrates that novel endophytic isolate of *Enterobacter* species with *aiiA* homologous gene which specifically encode for the AHL lactonase enzyme. The alignment of *aiiA* gene from endophytes and other know sources revealed the proposed enzymatic activity relation and its tertiary structure of AHL-lactonase. Further studies are recommended for the study of enzyme

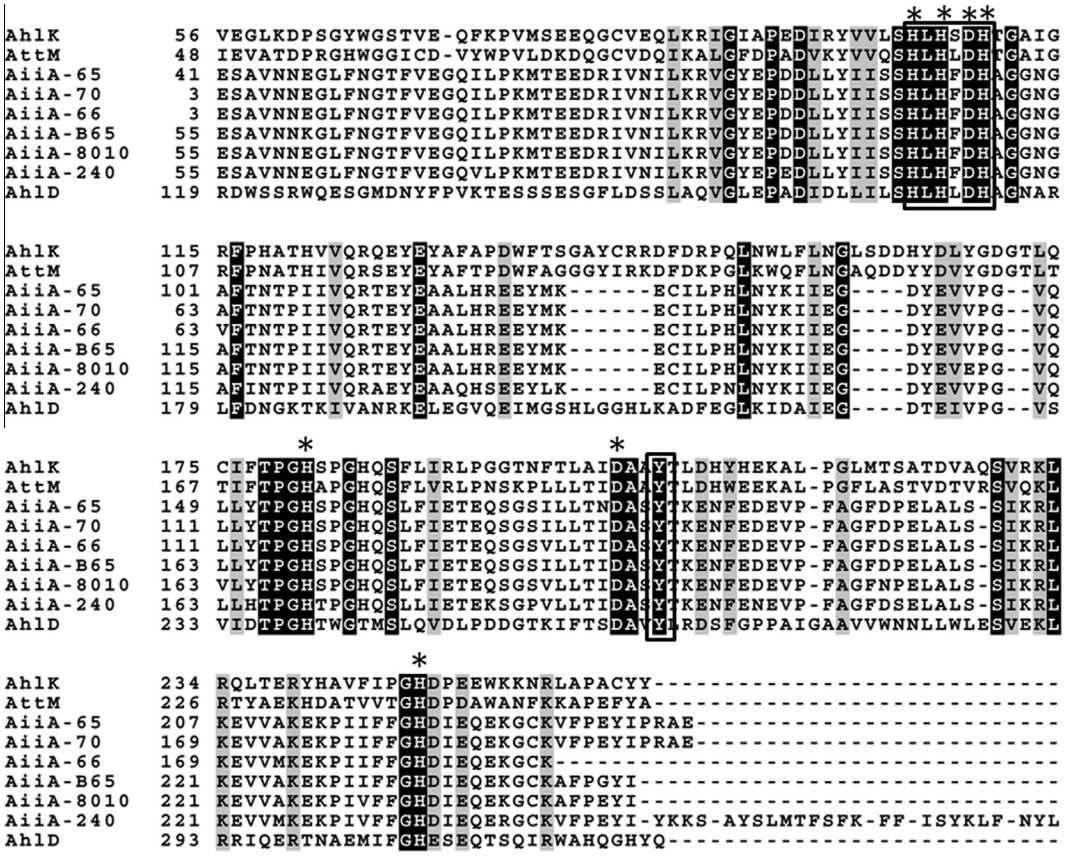

Fig. 3. Multiple alignment of amino acid sequence of lactonase from *Enterobacter asburiae* VT65 (AiiA-65), *Enterobacter aerogenes* VT66 (AiiA-66), *Enterobacter ludwigii* VT70 (AiiA-70) with other known sequences. AHL lactonase of AiiA-65, AiiA-66 and AiiA-70 was aligned with *Klebsiella pneumoniae* (AhlK, accession number: AY222324.1), *Bacillus* sp. 240B1 (AiiA 240, accession number: AF196486.1), *Arthrobacter* sp. IBN110 (AhlD, accession number: AF525800.1), *Bacillus thuringiensis serovar kurstaki* strain 8010 (AiiA 8010. accession number: AY943832.1), *Bacillus weihenstephanensis* strain B65 (AiiA B65, accession number: KC823046.1), *Agrobacterium tumefaciens* (AttM, accession number: U59485.2). ClustralW2 was used for alignment of sequences and BoxShade server was used to shade identical residue with black and conserved residues with gray. "HXHXDH" motif and Tyr (194) were boxed based on the proposed metal ligands according to Thomas et al. (2005) along with the dinuclear zinc form of AHL lactonase (asterisks).

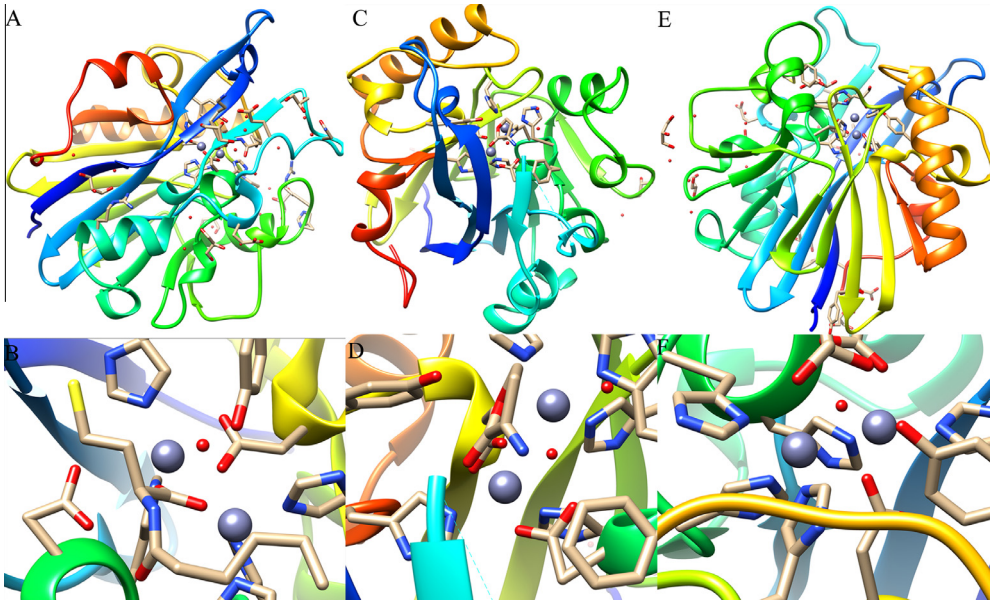

Fig. 4. The predicted putative tertiary structure of AHL-lactonase from *Enterobacter asburiae* VT65 (A), *Enterobacter aerogenes* VT66 (C), *Enterobacter ludwigii* VT70 (E) and metal ligands for dinuclear zinc binding (B, D and F respectively).

stability and *in vivo* tool for quorum quenching in control of virulence factors in pathogens.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.101.

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