Biocatalysis and Agricultural Biotechnology 18 (2019) 101009

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Biofilm inhibitory activity of metallo-protein AHL-lactonase from cell-free lysate of endophytic *Enterobacter* species isolated from *Coscinium fenestratum* Gaertn.

Rajesh P. Shastry[a,b,*], P.D. Rekha[b], V. Ravishankar Rai[a]

[a] Department of Studies in Microbiology, University of Mysore, Manasagangotri, Mysore, 570006, India
[b] Yenepoya Research Centre, Yenepoya University, University Road, Deralakatte, Mangalore, 575018, India

ARTICLE INFO

Keywords:
Quorum quenching
Endophytic bacteria
Enterobacter
Molecular docking
AHL-Lactonase
Aeromonas hydrophila

ABSTRACT

The quorum sensing mechanism is widely distributed in microorganisms to regulate the threshold population and controls the QS regulated genes. The aim of the present study was to evaluate the ability of metalloprotein AHL-lactonase which is homologous to *aiiA* gene to reduce the biofilm formation in *Aeromonas hydrophila*. Quorum quenching activity of endophytic bacteria associated with *Coscinium fenestratum* was explored using biosensor bioassay. Based on the screening, the genes coding for metalloprotein AHL-lactonase was amplified. Multiple alignment and in silico structural analysis was performed to test the presence of AHL-lactonase. The enzyme was purified by column chromatography and applied against biofilm formation of *A. hydrophila.* The endophytic bacterial strain isolates belonged to genera *Enterobacter* and exhibited significance quorum quenching activity. Endophytic bacteria from *Coscinium fenestratum* exhibited significantly ($p < 0.001$) high N-Acyl homoserine lactones (AHLs) degrading activity. Multiple sequence alignment suggested presence of AHL-lactonase gene and presence of conserved motif for dinuclear zinc binding sites as shown by metallo-β-lactamase superfamily. Molecular docking and stimulated interactions between predicted AHL-lactonase and AHLs revealed the possible mechanisms of enzyme-substrate interactions and active sites responsible for degradation of AHLs. The metallo-protein AHL-lactonase significantly inhibited formation of biofilm by *A. hydrophila*, suggesting possible biotechnological application.

1. Introduction

Gram negative bacteria use quorum sensing to monitor their local cell population and coordinately activate gene expression during the growth by utilizing *N*-acyl-homoserine lactone (AHL) signals (Miao et al., 2012). A variety of biological processes are regulated by quorum sensing including bioluminescence, biofilm formation, plasmid transfer, virulence gene expression, production of exoenzymes and antibiotics, and surface motility (Zheng et al., 2012). The degradation/inactivation of AHL molecules has proven the efficient way of control of bacterial infections which is regulated by quorum sensing mechanism (Rajesh and Rai, 2014a,b).

Inactivation/degradation of AHL is achieved by means of quorum sensing inhibitors or quorum quenching enzymes. For example, AHL-lactonase encoded by *aiiA* gene (Dong et al., 2000), *AhlD* gene (Park et al., 2003), *aiiT* gene (Morohoshi et al., 2014), *QsdH* gene (Huang et al., 2012) and many as AHL-acylase (Koch et al., 2014; Sio et al., 2006) have been established to have quorum quenching activities. AHL-lactonase catalyzes the ring opening hydrolysis of lactone ring and AHL-acylases hydrolyze amide bond between the acyl chain and homoserine moiety irreversibly (Chen et al., 2013).

AHL-lactonase belongs to metallo-β-lactamase enzyme superfamily and relies on a dinuclear zinc site for catalysis and enzyme stability. Different activities within these superfamily are reported, including oxygen and nitrogen reduction as well as cleavage of C-N, C-O, S-O, C-S, P-O, and possibly P-N bonds (Momb et al., 2008). In addition, X-ray crystal structure of AHL lactonase showed first-shell coordination sphere of the dinuclear zinc center which includes five histidines and an aspartate, and a second shell containing Tyr194 for enzymatic activity (Liao et al., 2009).

The quorum quenching enzymes have been found to be applicable in the control of infections associated with quorum sensing regulated genes. Expression of *aiiA* gene in phytopathogen *Erwinia carotovora* resulted in reduced decay in Chinese cabbage (Dong et al., 2000). The

* Corresponding author. Yenepoya Research Centre, Yenepoya University, University Road, Deralakatte, Mangalore, 575018, India.
E-mail address: rpshastry@gmail.com (R.P. Shastry).

https://doi.org/10.1016/j.bcab.2019.01.047
Received 30 November 2018; Received in revised form 23 January 2019; Accepted 24 January 2019
Available online 29 January 2019
1878-8181/

Abbreviations

AHL	Acyl homoserine lactone
C_4-HSL	N-Butanoyl-L-homoserine
C_6-HSL	N-Hexanoyl-L-homoserine lactone
3-oxo-C_6-HSL	N-3-oxo-Hexanoyl-L-homoserine lactone
C_8-HSL	N-Octanoyl-L-homoserine lactone
C_{10}-HSL	N-Decanoyl-L-homoserine lactone
C_{12}-HSL	N-Dodecanoyl-L-homoserine lactone
3-oxo-C_{12}-HSL	N-3-oxo-Dodecanoyl-L-homoserine lactone

oral administration of thermostable AHL-lactonase significantly attenuated *Aeromonas hydrophila* infection in zebrafish (Cao et al., 2012). These reports prove that application of quorum quenching enzyme represents a novel general antibacterial therapy and supports the potential values in bacterial infection.

We investigated the quorum quenching enzymes from endophytic bacteria associated with the plant *Coscinium fenestratum* Gaertn. The plant belong to family Menispermaceae and grows extensively in the Western Ghats of India and Sri Lanka (Rai et al., 2012). The isolated endophytes were screened for the presence of *aiiA* homologous gene and were analyzed for the putative tertiary structure and substrate binding complex by molecular interaction approach, further effect of AHL-lactonase on biofilm formation by *A. hydrohila* was analyzed. The Gram negative bacteria, *Aeromonas hydrophila* capable of infecting a wide variety of hosts, including humans, terrestrial and aquatic animals (Ponce-Rossi et al., 2016) was used as test organism. The pathogen uses conventional quorum sensing system regulated by predominantly *N*-(butyryl)-L-homoserine lactone (C_4-HSL) which controls expression of many virulence factors pigment production, serine protease and metalloprotease production (Garde et al., 2010).

2. Methods

2.1. Bacterial strains and substrates

Chromobacterium violaceum CV026 (mini-Tn5 mutant) was cultured in Luria-Bertani (LB) broth at 30 °C in a rotary shaker incubator for 24 h (Eppendrof-New Brunswisk Scientific, USA). C_4-HSL (Provided by Dr. Sang Sun Yoon, Korea), C_6-HSL and C_8-HSL (Sigma-Aldrich, Bangalore, India) were used as autoinducers for *C. violaceum* CV026. *P. aeruginosa* PAO1-JP2 (*lasI* and *rhlI* mutant) were used for the biofilm study with and without the supplementation of substrates (C_4-HSL and 3-oxo-C_{12}-HSL). *Aeromonas hydrophila* subsp. *hydrophila* (MCC-2052) was procured from Microbial Culture Collection, (Pune, India) and maintained in nutrient broth at 30 °C.

2.2. Isolation of endophytes and preparation of cell-free lysate

The plant sample of *Coscinium fenestratum* Gaertn was collected from forest of Western Ghat in Karnataka, India (13.08 °N, 75.45 °E). The plant was identified by consulting taxonomists and the herbarium of the plant was preserved in the Department of Studies in Microbiology (MGMB/001/2013–14), University of Mysore (India). The sequence of surface sterilization and isolation of endophytic bacteria were performed as per the previously reported method (Rajesh and Rai, 2014a,b).

The endophytic bacterial isolates were grown in the minimal medium at 30 °C in shaker incubator for 48 h and then cells were harvested by centrifugation at 20000 Xg for 10 min. Cell-free lysate was prepared by extracting with potassium phosphate buffer (100 mM; pH 7.0) and centrifuged; supernatant was filtered through 0.45 µm filter, the filtrate was stored at −20 °C until use.

2.3. Detection of quorum quenching phenotypes: AHL hydrolysis assay

Quorum quenching was determined using C_4-HSL as substrate for *C. violaceum* CV026 biosensor as reported previously (Zhu et al., 2011). Briefly, substrate, *C. violaceum* CV026 and cell-free lysate of endophytic bacterial isolates were incubated in Erlenmeyer flasks containing LB broth in shaker incubator (120 rpm) at 30 °C for 24 h. The control was maintained without cell-free lysate. Then, violacein was extracted with equal volume of Dimethyl Sulphoxide and centrifuged at 7000 Xg for 10 min to get clear supernatant. Two hundred microlitres of the violacein were added to 96 well F bottomed microtiter plates (Tarsons, F plates) and absorbance was measured at 585 nm. The growth was estimated by reading OD at 600 nm to determine the effect of cell-free lysate on growth of biosensor. The assay was repeated using C_6-HSL and C_8-HSL as substrates for *C. violaceum* CV026 biosensor.

2.4. Identification of endophytic bacteria

The16S rRNA genes were amplified from genomic DNA using universal 16S rRNA primers (Rajesh and Rai, 2014a,b) and amplicons were sequenced by Sanger's method. Brifely, 25 µl of reaction mixture contained 1 µl of each primer (27F-5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R-5′ACGGCTACCTTGTTACGCTT-3′, Bangalore Genie, India), 1 µl of template DNA and 22 µl of one fold diluted master mix (Bangalore Genie, India). 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 72 °C for 8 min. The final PCR product purified using the GenElute gel elution kit (Sigma–Aldrich, USA) and sequenced. Endophytic bacterial isolates were identified by homology analysis of the sequences 16S rRNA by the NCBI BLAST tool. The sequence was deposited in GenBank and accession numbers were obtained.

2.5. Amplification of aiiA homologous gene

Endophytic bacteria with the potential of AHL inactivation were confirmed for the presence of AHL-lactonase encoding *aiiA* homologous gene (Rajesh and Rai, 2014a,b). The isolates CS9, CS16 and CS28 were amplified using forward and reverse primers as shown in Table 1. Amplification 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); 25 cycles of 94 °C (45 s), 53 °C (45 s), 72 °C (1 min) for *aiiAF1* and *aiiAR1* primers; 30 cycles for *aiiAF2* and *aiiAR2* followed by primer extension at 72 °C for 8 min. The amplicons were resolved by 2% agarose gel electrophoresis.

Table 1
List of primers used for amplification of lactonase gene from endophytic isolates.

Strains (Endophytic isolates)	List of primers used
CS9	*aiiAF1* (5′-ATCGGATCCATGACAGTAAAGAAGCTTTAT-3′)
CS16	*aiiAR1* (5′-GTCGAATTCCTCAACAAGATACTCCTAATG-3′)
CS28	*aiiAF2* (5′-CGGAATTCATGACAGTAAAGAAGCTTTA-3′) aiiAR2 (5′-CGCTCGAGTATATATTCAGGGAACACTT-3′).

Table 2
Quorum quenching activity of endophytic bacteria using *C. violaceum* CV026 as biosensor.

Endophytic isolate	Identified bacteria	*aiiA* gene GenBank accession number	Type of AHL-lactonase	AHL degradation (nmol/h/ml)		
				C_4-HSL	C_6-HSL	C_8-HSL
CS9	*Enterobacter* sp. CS9 (KP096501)	KR011986	AiiA-CS9	173.7 ± 2.9	202.9 ± 3.1	164.1 ± 3.0
CS16	*Enterobacter ludwigii* CS16 (KP096502)	KR049073	AiiA-CS16	167.3 ± 1.8	187.7 ± 3.9	151.5 ± 2.4
CS28	*Enterobacter aerogenes* CS28 (KP096504)	KR049074	AiiA-CS28	152.8 ± 1.9	180.7 ± 2.6	142.3 ± 3.9

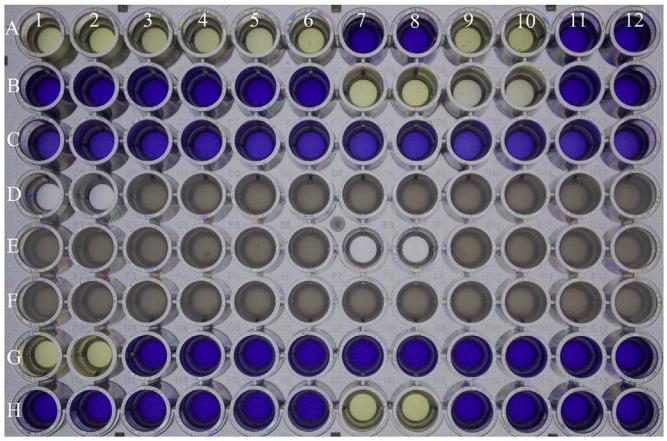

Fig. 1. Quorum quenching assay by microtiter plate method. Quorum quenching was analyzed by measuring the intensity of violacein production extracted from *C. violaceum*. CV026 (row number A1-A6 sterile control, A9 and A10 treated with *Enterobacter* sp. CS9, B7 and B8 treated with *E. ludwigii* CS16 and B9 and B10 treated with *E. aerogenes* CS28. Column number D–F indicates the growth measurement at 600 nm and column number G–H indicates the absence of QSI and QSA.

2.6. Multiple sequence alignment of aiiA homologous gene

The amplicons of *aiiA* homologous gene were purified and sequenced to identify the conserved regions. The sequence was assembled using CAP3 Sequence Assembly Program (Huang and Madan, 1999). Open reading frame (ORF) was detected using Geneious 8.1 and sequence was deposited in GenBank database.

2.7. In-silico analysis of putative tertiary structure of quorum quenching enzyme

The putative tertiary structure of AHL-lactonase encoded by *aiiA* homologous gene was predicted using Swiss-Model Workspace (Arnold et al., 2006). The predicted structure was compared with Phyre server (Kelley and Sternberg, 2009) for further confirmation of model building/confidence measure.

2.8. Enzyme-substrate binding analysis

The binding modes of substrates (C_4-HSL, C_6-HSL, 3-oxo-C_6-HSL, C_8-HSL, C_{10}-HSL, C_{12}-HSL, 3-oxo-C_{12}-HSL) with putative AHL-lactonase were performed using docking procedures. Template was selected based on best homologous AHL-lactonase model from Swiss-Model Workspace. The substrate structures were generated using PRODRG2 server (Schüttelkopf and van Aalten, 2004), and same substrates were used for molecular interaction study. Predicted AHL-lactonase was docked with server generated substrates using SwissDock server (Grosdidier et al., 2011). All the parameters of docking were kept default with server for each docking study. Best poses were selected based on pairing with zinc ions and direction of acyl chains (Liu et al., 2008). The best pose of enzyme-substrate complex was observed using UCSF Chimera software (Pettersen et al., 2004) to determine substrate binding energy.

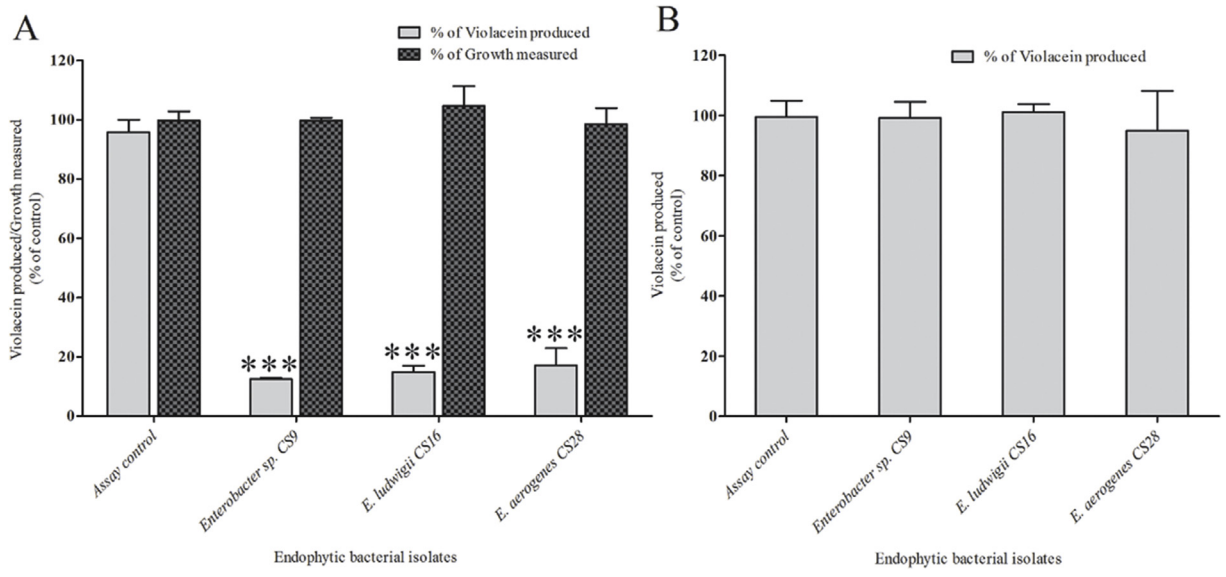

Fig. 2. Quantification of violacein production (quorum quenching) and measurement of growth by microtiter plate method. (A) Quantification of violacein produced in percentage with respect to control (without treatment), more than 70% inhibition of violacein production (*** indicates $p < 0.001$), was observed by *Enterobacter* sp. CS9, *E. ludwigii* CS16 and *E. aerogenes* CS28 and absence of growth inhibition. (B) Absence of QSI and QSA, measured by amount of violacein production with respect to the control.

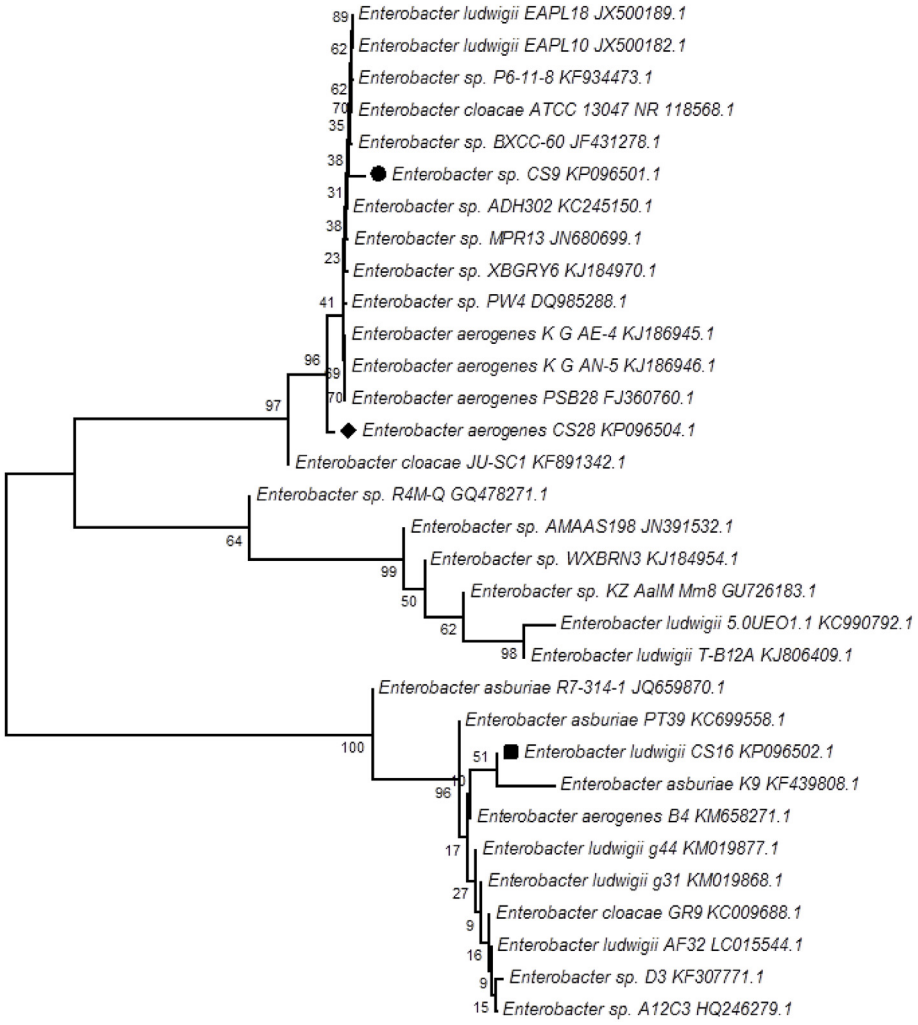

Fig. 3. Unrooted phylogenetic tree deriving from neighbor-joining showing the evolutionary relationship of endophytic *Enterobacter* sp. CS9, *E. ludwigii* CS16 and *E. aerogenes* CS28 with its closest BLAST hits. The neighbor-joining tree was maintained by 1000 boot straps using MEGA-6 software.

2.9. Purification of AHL-lactonase

The cell-free lysate from endophytic isolate of CS9 was extracted and filtered through 0.45 µm membrane. Then, cell-free lysate was saturated with 60% ammonium sulphate (Sigma-Aldrich) at 4 °C and the precipitate was collected by centrifugation at 10000 Xg for 30 min. The precipitate was dissolved in 1 mL of 10 mM potassium phosphate buffer (pH 7.0), dialyzed (dialysis tubing, MW cut-off 12 kDa; Himedia, India) overnight against 10 mM potassium phosphate buffer. The enzyme solution was concentrated, applied to gel filtration column Sephadex G-75 (30 × 1.5 cm) equilibrated with 50 mM, Tris-HCl buffer (pH 7.5). Then, 2 mL of each enzyme fraction was eluted with gradient of 0.0–1 M NaCl and active fractions were pooled and concentrated. The concentrated fraction was applied onto an anion exchange column DEAE-cellulose (30 × 1.5 cm) equilibrated with 50 mM, Tris-HCl buffer (pH 7.5) and eluted with gradient of 0.4–0.8 M NaCl, 2 mL each fractions were collected. The active enzyme fraction was pooled and concentrated, used as pure AHL-lactonase and stored at −20 °C.

2.10. Control of biofilm formation in Aeromonas hydrophila by AHL-lactonase

Inhibition of biofilm was tested by static microtiter plate assay as reported previously (Cady et al., 2012). To demonstrate effect of quorum sensing inhibition on biofilm formation, the purified enzyme was treated to *P. aeruginosa* PAO1 as a model organism. Briefly, overnight culture of *P. aeruginosa* PAO1 was loaded into 190 µL of LB broth in microtitre plate and 10 µL of filter (0.45 µm) sterilized purified AHL-lactonase. Then, the plates were incubated at 37 °C for 24 h without agitation. To confirm the effect of AHL-lactonase on planktonic cells, this was quantified spectrophotometrically at 600 nm. The biofilms were stained with crystal violet [0.1% (w/v) in water] and incubated for 15 min at room temperature (26–28 °C) and then washed with sterile distilled water. Crystal violet bound to biofilm was extracted with ethanol (95%) and quantified spectrophotometrically at 590 nm.

Further, to confirm the effect of quorum quenching activity by AHL-lactonase on biofilm formation, the strain *Pseudomonas aeruginosa* PAO1-JP2 was grown in 10 µM of AHL (C_4-HSL and 3-oxo-C_{12}-HSL) supplemented LB broth, with AHL lactonase treatment. The controls were maintained without the treatment of AHL lactonase and the biofilm was quantified as previously reported method (Rajesh and Rai, 2014a,b).

Effect of AHL-lactonase treatment on formation of biofilm in *A. hydrophila* was studied using sterile polystyrene, 96-well microtiter plate as explained earlier with 100 µg/ml of purified AHL-lactonase.

2.11. Microscopy

Visualization of biofilm formation and effect of AHL-lactonase were performed as reported earlier (Rajesh and Rai, 2015). Briefly, *P.*

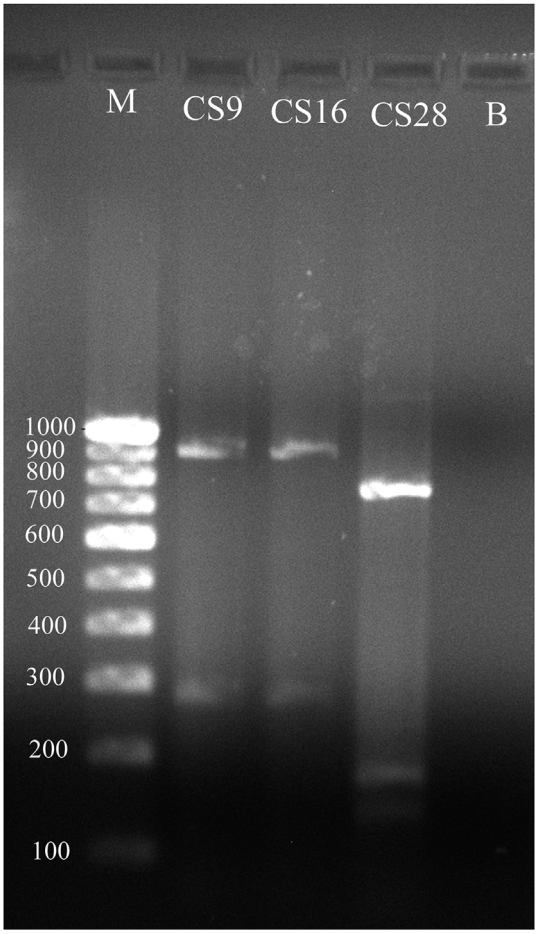

Fig. 4. Amplification of *aiiA* homologous gene from reported endophytic isolates. Lane M; 100 bp molecular marker, Lane CS9; *Enterobacter* sp. CS9, Lane CS16; *E. ludwigii* CS16 and Lane CS28; *E. aerogenes* CS28, Lane B; PCR blank (without DNA template).

aeruginosa PAO1 and *A. hydrophila* was grown in 50 mm glass slide taken in 90 mm petridish along with and without (control) AHL-lactonase treatment. The slides were rinsed with sterile distilled water, air dried and stained with 0.5 ml of a 10 μg/ml prefiltered solution of DAPI (Himedia, India) and incubated for 5 min at 28 °C. The biofilm cell attachment was observed under fluorescence microscope and inhibition of biofilm formation was compared visually with treated and control samples.

2.12. Statistical analysis

Statistical significance of variance for quantification of quorum quenching by microtiter plate assay was analyzed by one way ANOVA followed by Tukey's test. Graphpad Prism 5.03 software was used for statistical determinations.

3. Results

3.1. Endophytic bacteria and their QQ activity

Based on morphological differences, a total of 87 isolates were selected from *C. fenestratum* Gaertn., as bacterial endophytes. Cell-free lysates from all the isolates were screened for presence of QQ activity using C_4-HSL, C_6-HSL and C_8-HSL as substrates for *C. violaceum* CV026. CS9, CS16 and CS28 isolates exhibited degradation of AHL molecules (Table 2). The extent of decrease in intensity of violacein produced expressed as percent violacein with respect to control is given in Fig. 1. Among the tested strains, the isolate CS9 exhibited significantly ($p < 0.001$) higher degradation activity against C_6-HSL molecule (Fig. 2).

3.2. C. fenestratum associated endophytic QQ bacteria belong to the genus Enterobacter

Isolates showing degradation of synthetic AHLs were identified by 16S rRNA gene sequencing. Using BLAST search tool the isolated strains CS9, CS16 and CS28 were identified as *Enterobacter* sp., *Enterobacter ludwigii* and *Enterobacter aerogenes* respectively. The phylogenetic tree showed a unique subclade suggesting that novel strains were isolated as endophytic bacteria (Fig. 3).

3.3. Amplification of aiiA homologous gene

The identified isolates were further tested for the presence of genes that encode for the quorum quenching enzymes. Using specific primers about 900 bp amplicon from CS9 and CS16, and about 800 bp from CS28 were observed (Fig. 4). The obtained amplicons were sequenced. The homology analysis of the sequence and phylogeny analysis showed more than 95% similarity to previously reported AHL-lactonase (Fig. 5) (Dong et al., 2000).

3.4. Multiple alignments of sequence and prediction of putative tertiary structure of AHL-lactonase

Amplicons of *aiiA* gene were sequenced and closest hits in BLAST analysis showed presence of AHL-lactonase. The *aiiA* homologous gene belongs to Metallo-β-lactamase superfamily, which are zinc dependent hydrolases. The multiple sequence alignment analysis using NCBI database and ClustralW for these sequences with AHL-lactonase from other species revealed the presence of motif "HXHXDH" as well as tyrosine (Y) residue at position 194 (Fig. 6). Furthermore, the putative tertiary structure was predicted using Swiss-Model and using N-acyl homoserine lactone hydrolase of *B. thuringiensis* as a template accession No. 3dh.1 for *Enterobacter* sp. CS9, 2a7m.1 for *E. ludwigii* CS16 and 4j5f.1 for *E. aerogenes* CS28 along with each showing two Zinc binding sites (Fig. 7) and Phyre server confirmed the predicted structure with Swiss-Model workspace. The model showed QMEAN4 score of 0.866 for *Enterobacter* sp. CS9, 0.851 for *E. ludwigii* CS16 and 0.822 for *E. aerogenes* CS28 (Benkert et al., 2011).

3.5. Enzyme-substrate binding analysis

We assembled the predicted putative tertiary structure of reported endophytes containing the active sites responsible for active inactivation of AHLs based on best pose of substrate-enzyme complex. Many substrates from short chain to long chain AHLs were selected for docking study and the result showed multiple substrate affinity along with ring opening mechanism. All selected substrates positioned as lactone ring facing cavity and nitrogen atom facing outside (Fig. 8). The estimated binding energy and Gibbs free energy favour the substrates with long chains (Table 3) but variations in AHL degradation were not significant in catalysis of AHLs as estimated by *in vitro* analysis using *C. violaceum* CV026 strain in terms of relative AHL-lactonase activity.

3.6. Purification of AHL-lactonase

Column purified AHL-lactonase active fractions after final step of purification, were purified to 61.5 fold with the yield of 62.7% (Table 4). Furthermore, SDS-PAGE analysis of purified AHL-lactonase showed a band having size of approximately 30 kDa (Fig. 9).

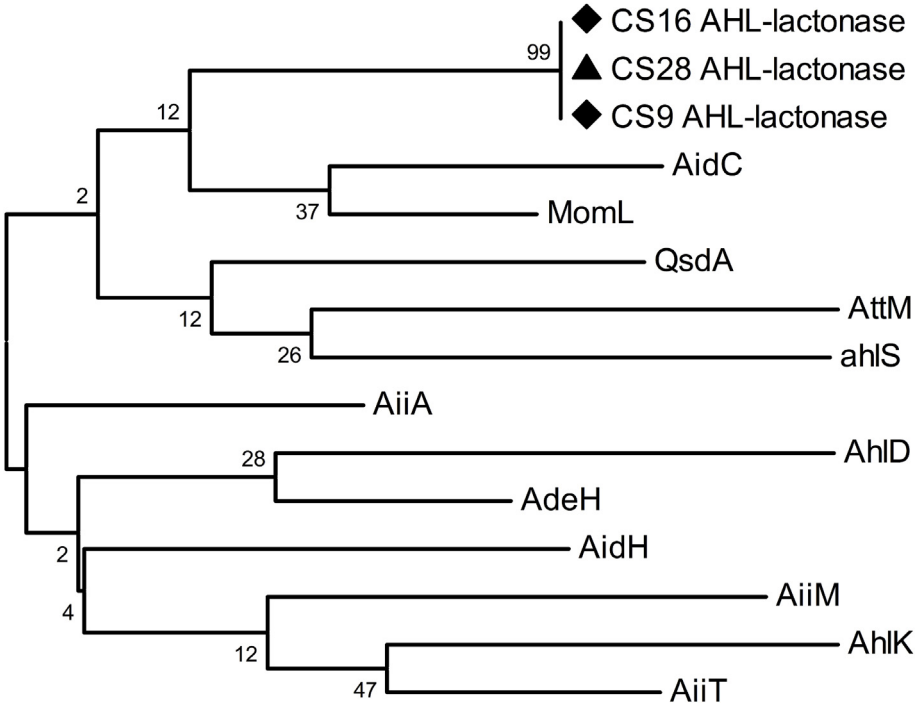

Fig. 5. Phylogenetic tree of *aiiA* homologous gene deriving from neighbor-joining shows the evolutionary relationship between selected AHL-lactonase gene and *aiiA* homologous gene from endophytic *Enterobacter* species. The phylogeny tree was constructed with 1000 boot straps using MEGA-6 software.

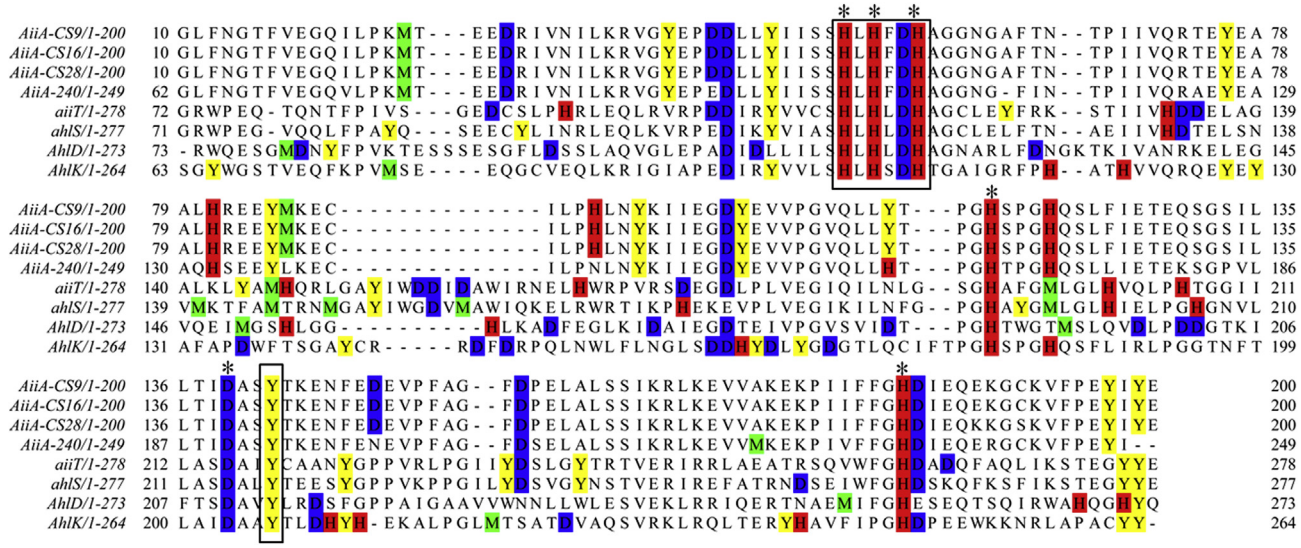

Fig. 6. Multiple sequence alignment of amino acid of AHL-lactonase from *Enterobacter* sp. CS9 (AiiA-CS9), *E. ludwigii* CS16 (AiiA-CS16) and *E. aerogenes* CS28 (AiiA-CS28) aligned with *Bacillus* sp. 240B1 (AiiA-240, accession number: AF196486.1), *Thermaerobacter composti* (aiiT, accession number: AB935248.1), *Solibacillus silvestris* (ahlS, accession number: AB648919.2), *Arthrobacter* sp. IBN110 (AhlD, accession number: AF525800.1) and *Klebsiella pneumoniae* (AhlK, accession number: AY222324.1). ClustralW2 was used for alignment of sequences and Jalview was used to shade identical residue with blue for aspartic acid, red for histidine and yellow for tyrosine conserved residues. A short conserved sequence for metal ligands of "HXHXDH" motif and Tyrosine (194) were boxed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.7. Antibiofilm activity of AHL-lactonase

The effect of AHL-lactonase on biofilm formation of *A. hydrophila* was determined by microtiter plate method as well as fluorescence microscopy. *P. aeruginosa* PAO1 and PAO1-JP2 were used as model organisms to determine the activity of AHL-lactonase on quorum sensing regulated biofilm formation. AHL-lactonase treated PAO1 biofilm significantly reduced the total biomass ($p < 0.001$) and with the PAO1-JP2 strain it was confirmed that, formation of biofilm is regulated by presence of AHL molecules. Furthermore, PAO1-JP2 supplemented quorum sensing molecules significantly reduced the formation of biofilm on treatment with AHL-lactonase, suggesting that AHL-lactonase could be an antibiofilm agent against *P. aeruginosa* infection (Fig. 10A–C).

Similarly, AHL-lactonase also exhibited significant antibiofilm activity ($p < 0.001$) against *A. hydrophila*. The AHL-lactonase treatment did not show inhibitory activity on planktonic cells and formation of biofilm was inhibited by more than 80% of total biomass (Fig. 10D). Furthermore, the antibiofilm activity was visually confirmed by fluorescence microscopy using DAPI. The control slides were fluorescing

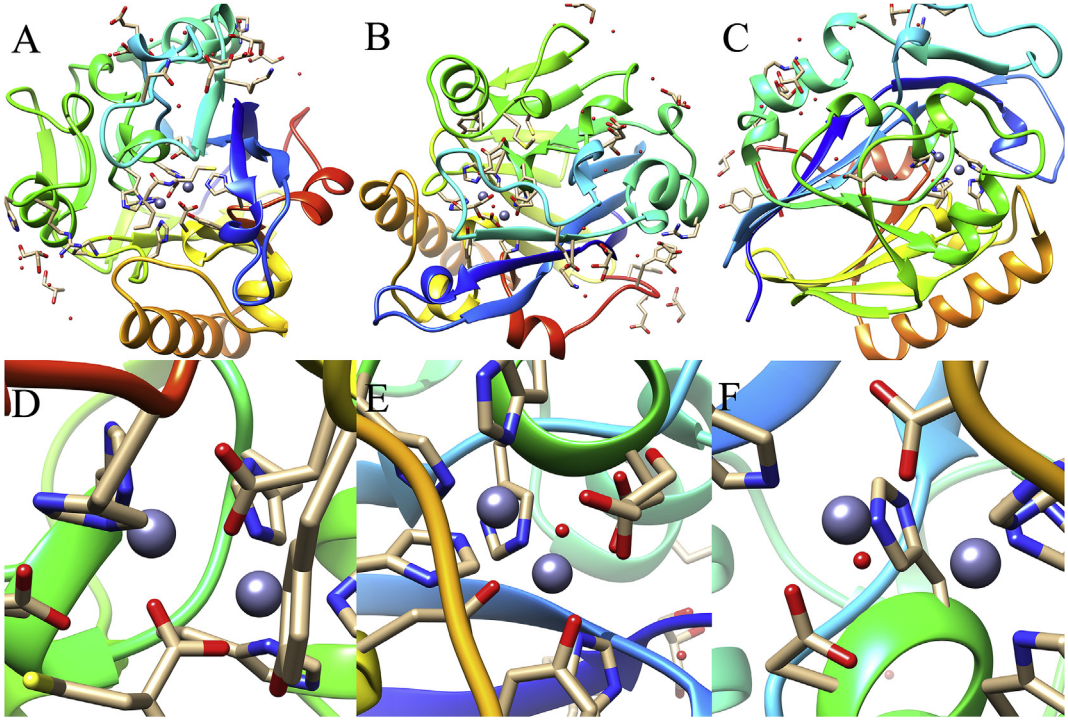

Fig. 7. Predicted putative tertiary structure of AHL-lactonase from *Enterobacter* sp. CS9 (A), *E. ludwigii* CS16 (B) and *E. aerogenes* CS28 (C) and metal ligands for dinuclear zinc binding (D, E and F respectively).

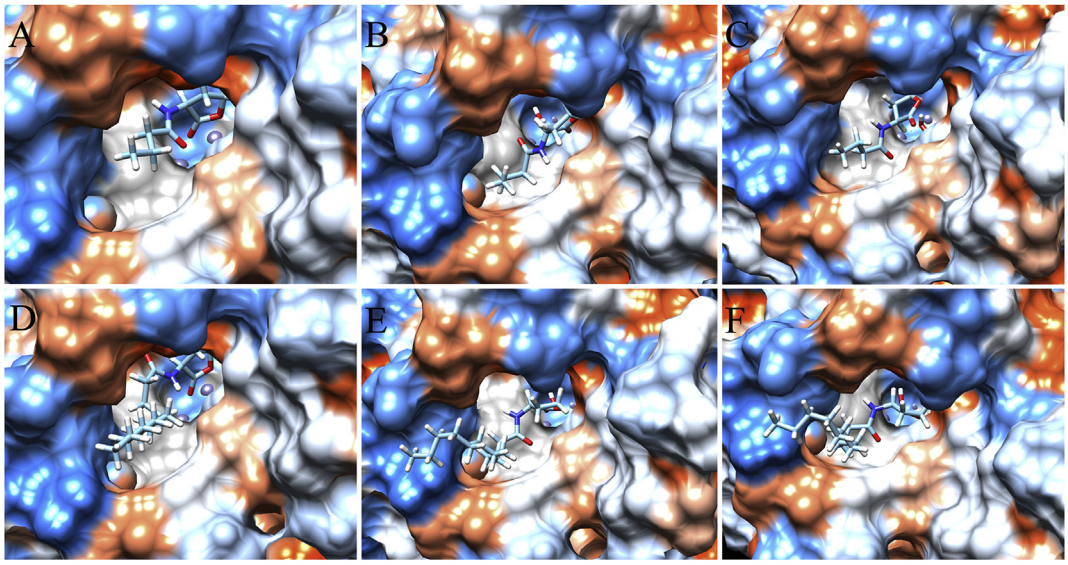

Fig. 8. Product-bound predicted putative AHL-lactonase. The predicted AHL-lactonase from *Enterobacter* sp. CS9 (A), *E. ludwigii* CS16 (B) and *E. aerogenes* CS28 (C) bound with substrate C_4-HSL. Similarly AHL-lactonase bound with C_{12}-HSL (D, E and F respectively). The broad substrate binding cavity surfaces were shown with each substrate in relation to the product's hydrophobic acyl chains. Figure was prepared using coordinates given from SwissDock server and observed using USCF Chimera.

showing biofilm formation by *A. hydrophila* and *P. aeruginosa* PAO1 (Fig. 11). The AHL-lactonase treated slides showed significantly reduced biofilm intensity.

4. Discussion

The quorum sensing mechanism is widely distributed in microorganisms to regulate the threshold population and control of QS regulated genes. Quorum quenching enzymes have diverse applications as they are capable of degradation of quorum sensing signal molecules, thereby control severity of pathogenesis. AHL-lactonase is one such quorum quenching enzyme that belongs to the superfamily of metallo-β-lactamase and shows sequence similarity with glyoxalase II and arylsulfatase (Dong et al., 2000).

In our study, AHL-lactonase from three different endophytic bacteria was isolated and confirmed by the presence of *aiiA* homologous gene. Phylogenetically *Enterobacter* sp. CS9, *E. ludwigii* CS16 and *E. aerogenes* CS28 belong to separate subclade suggesting that the isolated bacteria were novel endophytes. Previous reports show that, many isolated endophytic bacteria from peanut, maize and strawberry fruit also impart plant growth promotion (Rajesh and Rai, 2014a,b; Wang et al., 2013). Therefore, the presence of AHL-lactonase in endophytes

Table 3
Estimated substrate binding energy and Gibbs free energy (ΔG) of AHL-lactonase from endophytic bacteria.

AHL-Lactonase	Substrate	Estimated Substrate Binding Energy	Estimated ΔG (kcal/mol)
AiiA-CS9	C_4-HSL	−37.4792	−9.92
	C_6-HSL	−42.7335	−10.51
	3-oxo-C_6-HSL	−48.1111	−10.98
	C_8-HSL	−54.8449	−10.11
	C_{10}-HSL	−47.5457	−11.06
	C_{12}-HSL	−60.5811	−11.53
	3-oxo-C_{12}-HSL	−55.0878	−11.57
AiiA-CS16	C_4-HSL	−22.4323	−6.56
	C_6-HSL	−23.9506	−6.78
	3-oxo-C_6-HSL	−25.3062	−7.84
	C_8-HSL	−34.7218	−6.75
	C_{10}-HSL	−39.6959	−7.69
	C_{12}-HSL	−37.6431	−7.25
	3-oxo-C_{12}-HSL	−31.9013	−7.88
AiiA-CS28	C_4-HSL	−23.2565	−6.79
	C_6-HSL	−29.5868	−7.28
	3-oxo-C_6-HSL	−30.0367	−7.35
	C_8-HSL	−35.2611	−7.25
	C_{10}-HSL	−30.4424	−8.11
	C_{12}-HSL	−40.6991	−8.08
	3-oxo-C_{12}-HSL	−37.6118	−8.78

support the plant by enhancing the resistance power against invading pathogens and also used as biological control tool against human, animal or plant pathogens (Chankhamhaengdecha et al., 2013; Dong et al., 2000).

Prediction of putative tertiary structure of reported quorum quenching enzymes shows that they belong to AHL-lactonase family and zinc dependent active enzyme for degradation of AHL. The presence of a conserved motif "HXHXDH" as well as tyrosine (Y) residue at the position 194 suggest that the similarity in enzymatic degradation pattern with reported *aiiA* encoding enzyme AHL-lactonase (Dong et al., 2002, 2000). The presence of dinuclear zinc explains the catalytic mechanism of metallo-β-lactamase superfamily enzymes (Liu et al., 2008).

Molecular docking and molecular interaction studies on enzyme-substrate binding patterns exhibited the required substrate orientations and specificity of AHL-lactonase catalytic activity. The substrate orientation affects the enzymatic activity and results in variations in hydrolysis of AHLs due to hydrophobic interactions between AHL-lactonase and AHLs as substrate (Momb et al., 2008). Molecular docking suggests that AHL substrate binds to dinuclear zinc facing the lactone ring and hydrophobic acyl side chain facing outside the AHL-lactonase (Liu et al., 2008; Momb et al., 2008). Similar results of docking for endophytic AHL-lactonase from *Enterobacter* sp. CS9, *E. ludwigii* CS16 and *E. aerogenes* CS28 with AHLs as substrates (C_4-HSL, C_6-HSL, 3-oxo-C_6-HSL, C_8-HSL, C_{10}-HSL, C_{12}-HSL, 3-oxo-C_{12}-HSL) were observed in this study.

As reported earlier, the dinuclear zinc present in AHL-lactonase helps to stabilize the charge of the tetrahedral intermediate and ring opening mechanism. Initially, nucleophilic attack on AHL carbonyl carbon by the bridging hydroxide results in ring opening by direct ester C-O bond cleavage. Then, the Zn1 is required to stabilize the charge of the tetrahedral intermediate, which facilitates the nucleophilic attack, similarly Zn2 stabilizes the charge of the alkoxide which leads to ring opening of lactone moiety (Liao et al., 2009).

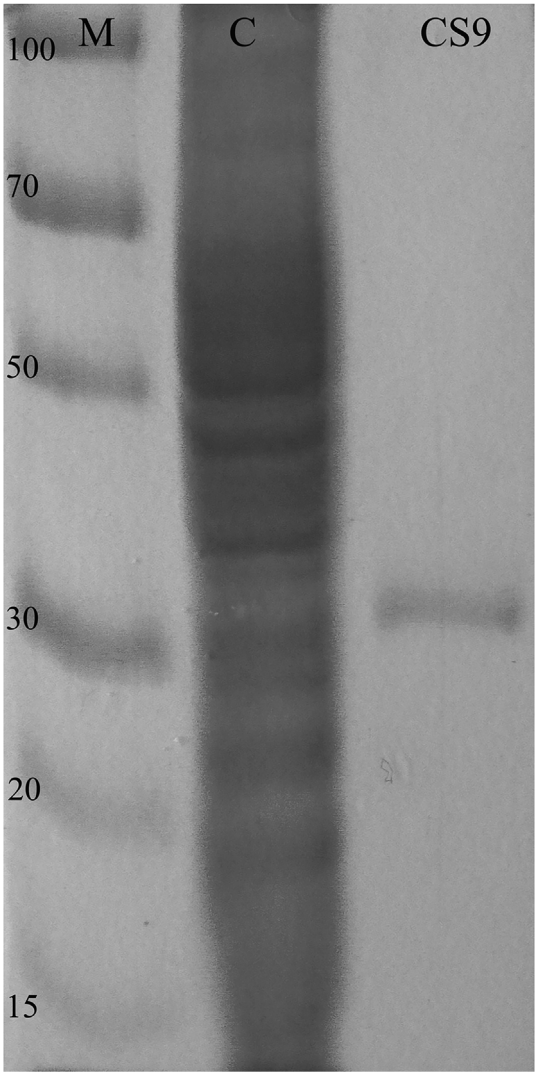

Fig. 9. SDS-PAGE analysis of AHL-lactonase from *Enterobacter* sp., strain CS9. Lane M: Protein marker, Lane C: Crude cell free lysate, Lane CS9: Purified AHL-lactonase from *Enterobacter* sp., strain CS9.

A. hydrophila widely exist in aquatic environment as an opportunistic pathogen using quorum sensing system for formation of biofilm and expression of virulence factors. Further, it is demonstrated that addition of 0.05% glucose significantly impairs biofilm formation only at the early stages (Jahid et al., 2013). According to previous reports of the 24 genes that are associated with biofilm formation, 14 genes enhance and 10 genes reduce formation of biofilm. Biofilm formation is also regulated by 11 genes encodes for histidine kinase, LuxR, fimbrial

Table 4
Purification of the AHL-lactonase enzyme from *Enterobacter* sp. CS9.

Purification step	Total protein (mg)	Relative AHL-lactonase activity (nmole/h/ml)	Specific activity (nmole/h/ml)	Yield (%)	No. of fold purification
Cell-free lysate	10.45	202.9	19.4	100	1
Ammonium sulphate	6.85	190.4	27.8	98.8	1.43
Sephadex® G-75	1.078	160.8	149.2	83.4	7.67
DEAE-cellulose	0.101	120.9	1197.0	62.7	61.5

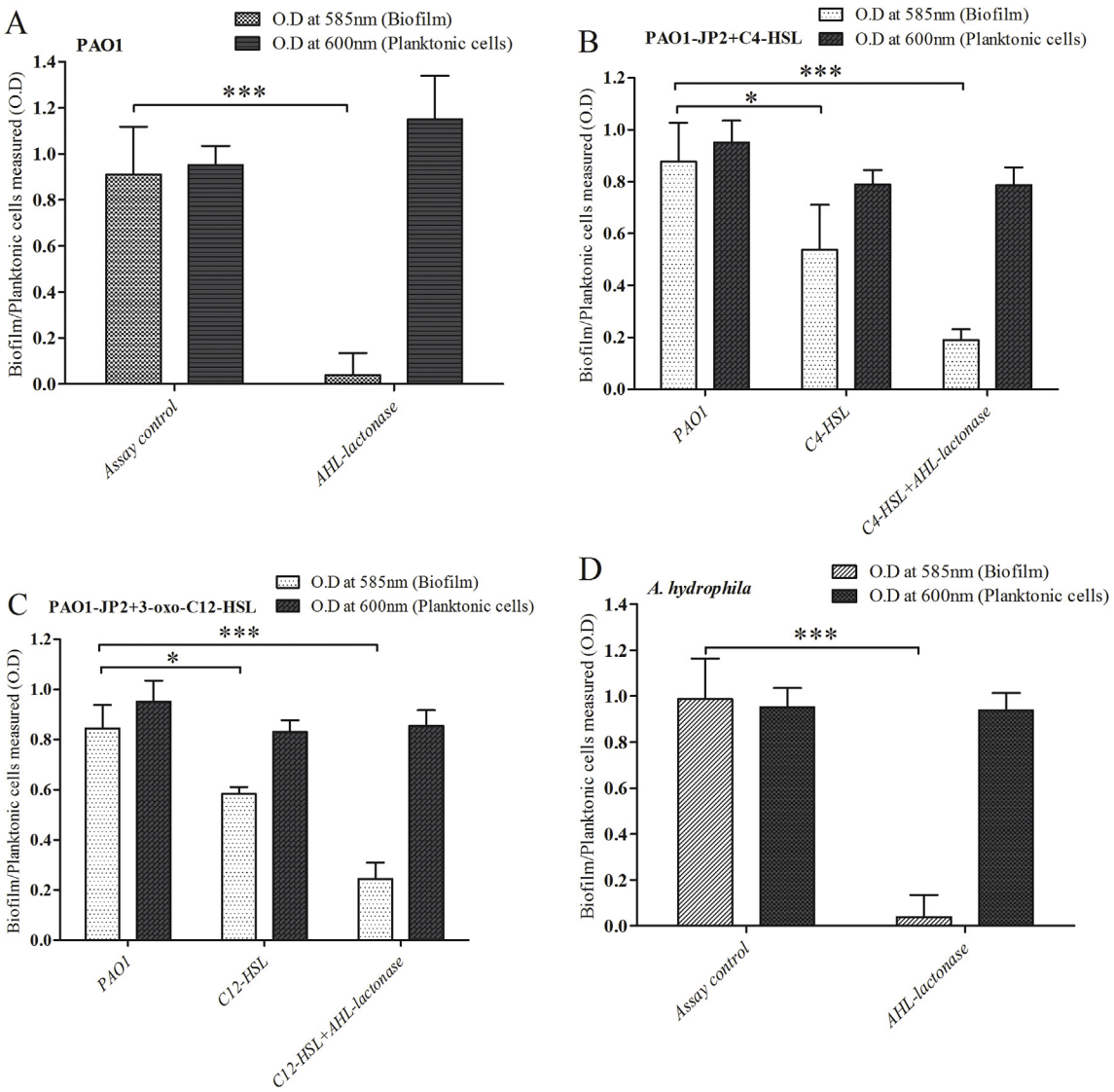

Fig. 10. Effect of AHL-lactonase from *Enterobacter* sp., strain CS9 on quorum sensing regulated biofilm formation in *P. aeruginosa* PAO1 and PAO1-JP2 as model organisms and *A. hydrophila*. (A) Measured optical density of *P. aeruginosa* PAO1 biofilm inhibition by AHL-lactonase. Significant biofilm inhibition was observed ($p < 0.001$) in treated cells. (B) Biofilm measured in PAO1, PAO1-JP2 with 10 μM C4-HSL and in presence of AHL-lactonase. (C) Biofilm measured in PAO1, PAO1-JP2 with 10 μM 3-oxo-C12 HSL and in presence of AHL-lactonase. (D) Effect AHL-lactonase treatment on *A. hydrophila* biofilm formation ($p < 0.001$). The error bar represents standard deviations reproduced from results of three repeated experiments.

protein, membrane protein, helicase, trehalose-6-phosphate hydrolase, transcriptional regulator of the ArsR family and phosphodiesterase, which plays a major role in LuxI/LuxR QS system (Du et al., 2016).

Since, AHL-lactonase showed hydrolysis activity on AHLs molecules, we allowed enzyme to act on biofilm formation by *A. hydrophila.* Treatment with AHL-lactonase on *P. aeruginosa* PAO1 biofilm significantly reduced biofilm formation in presence of provided quorum sensing molecules suggesting that biofilm formation is regulated by quorum sensing system in this bacterium. Similarly, treatment with AHL-lactonase on *A. hydrophila* reduced the formation of biofilm without inhibiting the planktonic cells as confirmed by microtiter plate assay and fluorescence microscopy. Similar results are observed with AHL-Lactonase from *Bacillus licheniformis* DAHB1 and *Pseudomonas aeruginosa* PsDAHP1 against biofilm forming strains of *Vibrio parahaemolyticus* and *Proteus* species respectively (Vinoj et al., 2015a, 2015b, 2014). Treatment with antibiotics would create survival pressure harsher for cells and promoting antibiotic resistance. However, control of quorum sensing generally do not create harsher environment for bacteria and reduce the succession of resistance against treated antibiotics.

5. Conclusion

Novel AHL-lactonase encoding *aiiA* homologous genes were identified from three different endophytic *Enterobacter* species. The study demonstrated that, the AHL-lactonase having significant similarity with the predicted tertiary structure of the Metallo-protein AHL-lactonase. Molecular interaction of AHL-lactonase with AHLs as substrate would result in lactone ring opening reaction using dinuclear zinc metal ions. These AHL lactonases can be effectively used against *A. hydrophila* biofilm formation and can be extended to other bacteria against AHL mediated quorum sensing inhibition.

Acknowledgment

The authors are thankful to Department of Science and Technology, New Delhi for providing fellowship for the first author (Grant order number DST/INSPIRE Fellowship/2011 (265) dated 17 July 2011) and

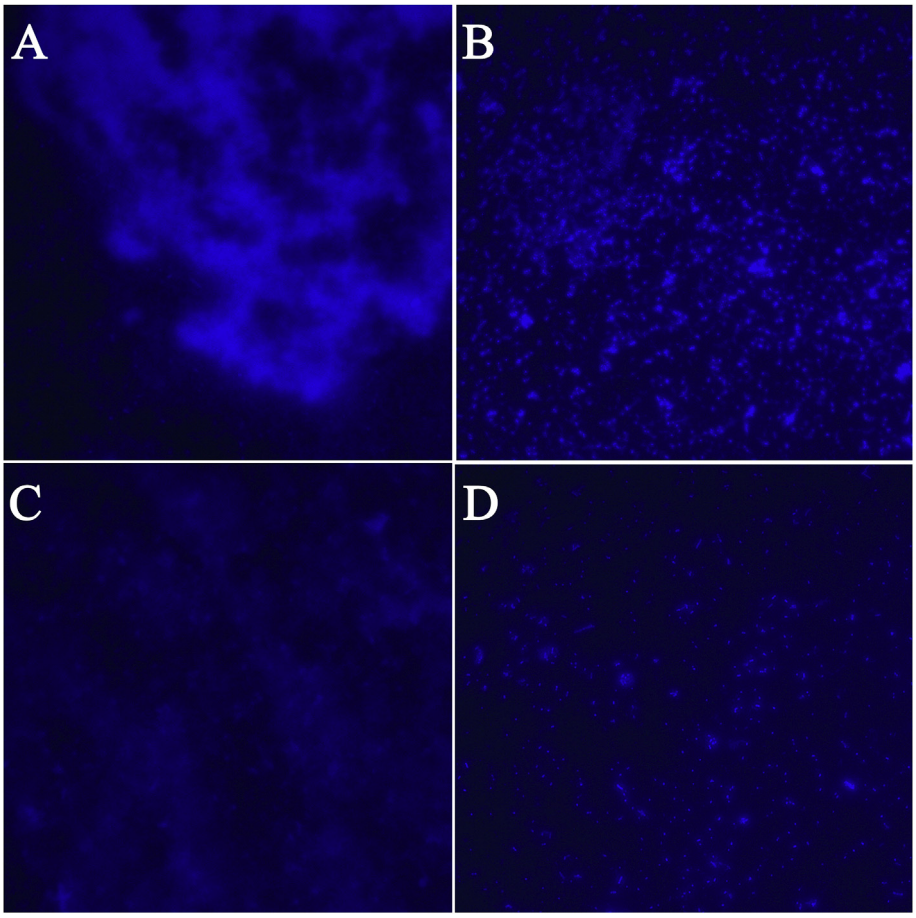

Fig. 11. Fluorescence microscopy for the visualization of biofilm formation by *P. aeruginosa* PAO1 and *A. hydrophila* stained with DAPI. Figure shows observed biofilm without the treatment of AHL-lactonase (A and C) and with treatment of AHL-lactonase (B and D) respectively.

Prof. K. Gopalakrishna Bhat for identifying the medicinal plant. We thank Robert McLean, Professor, Department of Biology, Texas State University, for providing *C. violaceum* CV026 and to Dr. Sang Sun Yoon, Assistant Professor, Yonsei University college of Medicine, Seoul, Korea for the gift of C_4-HSL.

References

Arnold, K., Bordoli, L., Kopp, J., Schwede, T., 2006. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinforma. Oxf. Engl. 22, 195–201. https://doi.org/10.1093/bioinformatics/bti770.

Benkert, P., Biasini, M., Schwede, T., 2011. Toward the estimation of the absolute quality of individual protein structure models. Bioinformatics 27, 343–350. https://doi.org/10.1093/bioinformatics/btq662.

Cady, N.C., McKean, K.A., Behnke, J., Kubec, R., Mosier, A.P., Kasper, S.H., Burz, D.S., Musah, R.A., 2012. Inhibition of biofilm formation, quorum sensing and infection in *Pseudomonas aeruginosa* by natural products-inspired organosulfur compounds. PLoS One 7 e38492. https://doi.org/10.1371/journal.pone.0038492.

Cao, Y., 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, 1899–1908. https://doi.org/10.1128/AEM.06139-11.

Chankhamhaengdecha, S., Hongvijit, S., Srichaisupakit, A., Charnchai, P., Panbangred, W., 2013. Endophytic actinomycetes: a novel source of potential acyl homoserine lactone degrading enzymes. BioMed Res. Int 2013. https://doi.org/10.1155/2013/782847.

Chen, F., Gao, Y., Chen, X., Yu, Z., Li, X., 2013. Quorum quenching enzymes and their application in degrading signal molecules to block quorum sensing-dependent infection. Int. J. Mol. Sci. 14, 17477–17500. https://doi.org/10.3390/ijms140917477.

Dong, Y.-H., Gusti, A.R., Zhang, Q., Xu, J.-L., Zhang, L.-H., 2002. Identification of quorum-quenching N-acyl homoserine lactonases from *Bacillus* species. Appl. Environ. Microbiol. 68, 1754–1759. https://doi.org/10.1128/AEM.68.4.1754-1759.2002.

Dong, Y.-H., Xu, J.-L., Li, X.-Z., Zhang, L.-H., 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. Proc. Natl. Acad. Sci. Unit. States Am. 97, 3526–3531. https://doi.org/10.1073/pnas.97.7.3526.

Du, H., Pang, M., Dong, Y., Wu, Y., Wang, N., Liu, J., Awan, F., Lu, C., Liu, Y., 2016. Identification and characterization of an *Aeromonas hydrophila* oligopeptidase gene pepF negatively related to biofilm formation. Front. Microbiol. 7. https://doi.org/10.3389/fmicb.2016.01497.

Garde, C., Bjarnsholt, T., Givskov, M., Jakobsen, T.H., Hentzer, M., Claussen, A., Sneppen, K., Ferkinghoff-Borg, J., Sams, T., 2010. Quorum sensing regulation in *Aeromonas hydrophila*. J. Mol. Biol. 396, 849–857. https://doi.org/10.1016/j.jmb.2010.01.002.

Grosdidier, A., Zoete, V., Michielin, O., 2011. SwissDock, a protein-small molecule docking web service based on EADock DSS. Nucleic Acids Res. 39, W270–W277. https://doi.org/10.1093/nar/gkr366.

Huang, W., Lin, Y., Yi, S., Liu, P., Shen, J., Shao, Z., Liu, Z., 2012. QsdH, a novel AHL lactonase in the RND-type inner membrane of marine *Pseudoalteromonas byunsanensis* strain 1A01261. PLoS One 7 e46587. https://doi.org/10.1371/journal.pone.0046587.

Huang, X., Madan, A., 1999. CAP3: a DNA sequence assembly program. Genome Res. 9, 868–877.

Jahid, I.K., Lee, N.-Y., Kim, A., Ha, S.-D., 2013. Influence of glucose concentrations on biofilm formation, motility, exoprotease production, and quorum sensing in *Aeromonas hydrophila*. J. Food Protect. 76, 239–247. https://doi.org/10.4315/0362-028X.JFP-12-321.

Kelley, L.A., Sternberg, M.J.E., 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nat. Protoc. 4, 363–371. https://doi.org/10.1038/nprot.2009.2.

Koch, G., Nadal-Jimenez, P., Cool, R.H., Quax, W.J., 2014. *Deinococcus radiodurans* can interfere with quorum sensing by producing an AHL-acylase and an AHL-lactonase. FEMS Microbiol. Lett. 356, 62–70. https://doi.org/10.1111/1574-6968.12479.

Liao, R.-Z., Yu, J.-G., Himo, F., 2009. Reaction mechanism of the dinuclear zinc enzyme N-Acyl-l-homoserine lactone hydrolase: a quantum chemical study. Inorg. Chem. 48, 1442–1448. https://doi.org/10.1021/ic801531n.

Liu, D., Momb, J., Thomas, P.W., Moulin, A., Petsko, G.A., Fast, W., Ringe, D., 2008. Mechanism of the quorum-quenching lactonase (AiiA) from *Bacillus thuringiensis*. 1. Product-bound structures. Biochemistry (Mosc.) 47, 7706–7714. https://doi.org/10.1021/bi800368y.

Miao, C., Liu, F., Zhao, Q., Jia, Z., Song, S., 2012. A proteomic analysis of *Arabidopsis thaliana* seedling responses to 3-oxo-octanoyl-homoserine lactone, a bacterial quorum-sensing signal. Biochem. Biophys. Res. Commun. 427, 293–298. https://doi.org/10.1016/j.bbrc.2012.09.044.

Momb, J., Wang, C., Liu, D., Thomas, P.W., Petsko, G.A., Guo, H., Ringe, D., Fast, W., 2008. Mechanism of the quorum-quenching lactonase (AiiA) from *Bacillus thuringiensis*. 2. Substrate modeling and active site mutations. Biochemistry (Mosc.) 47, 7715–7725. https://doi.org/10.1021/bi8003704.

Morohoshi, T., Tominaga, Y., Someya, N., Ikeda, T., 2014. Characterization of a novel thermostable N-acylhomoserine lactonase from the thermophilic bacterium *Thermaerobacter marianensis*. J. Biosci. Bioeng. https://doi.org/10.1016/j.jbiosc.2014.11.014.

Park, S.-Y., Lee, S.J., Oh, T.-K., Oh, J.-W., Koo, B.-T., Yum, D.-Y., Lee, J.-K., 2003. AhlD, an N-acylhomoserine lactonase in *Arthrobacter* sp., and predicted homologues in other bacteria. Microbiol. Read. Engl. 149, 1541–1550.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., Ferrin, T.E., 2004. UCSF Chimera–a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612. https://doi.org/10.1002/jcc.20084.

Ponce-Rossi, A., dos, R., Pinto, U.M., Ribon, A. de O.B., Bazzolli, D.M.S., Vanetti, M.C.D., 2016. Quorum sensing regulated phenotypes in *Aeromonas hydrophila* ATCC 7966 deficient in AHL production. Ann. Microbiol. 66, 1117–1126. https://doi.org/10.1007/s13213-016-1196-4.

Rai, R.V., Rajesh, P.S., Kim, H.-M., 2012. Medicinal use of *Coscinium fenestratum* (Gaertn.) Colebr.: an short review. Orient. Pharm. Exp. Med. 13, 1–9. https://doi.org/10.1007/s13596-012-0094-y.

Rajesh, P.S., Rai, V.R., 2014a. Molecular identification of *aiiA* homologous gene from endophytic *Enterobacter species* and in silico analysis of putative tertiary structure of AHL-lactonase. Biochem. Biophys. Res. Commun. 443, 290–295. https://doi.org/10.1016/j.bbrc.2013.11.101.

Rajesh, P.S., Rai, V.R., 2014b. Quorum quenching activity in cell-free lysate of endophytic bacteria isolated from *Pterocarpus santalinus* Linn., and its effect on quorum sensing regulated biofilm in *Pseudomonas aeruginosa* PAO1. Microbiol. Res. 169, 561–569. https://doi.org/10.1016/j.micres.2013.10.005.

Rajesh, P.S., Rai, V.R., 2015. Purification and antibiofilm activity of AHL-lactonase from endophytic *Enterobacter aerogenes* VT66. Int. J. Biol. Macromol. 81, 1046–1052. https://doi.org/10.1016/j.ijbiomac.2015.09.048.

Schüttelkopf, A.W., van Aalten, D.M.F., 2004. PRODRG: a tool for high-throughput crystallography of protein-ligand complexes. Acta Crystallogr. D Biol. Crystallogr. 60, 1355–1363. https://doi.org/10.1107/S0907444904011679.

Sio, C.F., Otten, L.G., Cool, R.H., Diggle, S.P., Braun, P.G., Bos, R., Daykin, M., Cámara, M., Williams, P., Quax, W.J., 2006. Quorum quenching by an N-Acyl-Homoserine lactone acylase from *Pseudomonas aeruginosa* PAO1. Infect. Immun. 74, 1673–1682. https://doi.org/10.1128/IAI.74.3.1673-1682.2006.

Vinoj, G., Jayakumar, R., Chen, J.C., Withyachumnarnkul, B., Shanthi, S., Vaseeharan, B., 2015a. N-hexanoyl-L-homoserine lactone-degrading *Pseudomonas aeruginosa* PsDAHP1 protects zebrafish against Vibrio parahaemolyticus infection. Fish Shellfish Immunol. 42, 204–212. https://doi.org/10.1016/j.fsi.2014.10.033.

Vinoj, G., Pati, R., Sonawane, A., Vaseeharan, B., 2015b. In vitro cytotoxic effects of gold nanoparticles coated with functional acyl homoserine lactone lactonase protein from *Bacillus licheniformis* and their antibiofilm activity against *Proteus* species. Antimicrob. Agents Chemother. 59, 763–771. https://doi.org/10.1128/AAC.03047-14.

Vinoj, G., Vaseeharan, B., Thomas, S., Spiers, A.J., Shanthi, S., 2014. Quorum-quenching activity of the AHL-lactonase from *Bacillus licheniformis* DAHB1 inhibits Vibrio biofilm formation in vitro and reduces shrimp intestinal colonisation and mortality. Mar. Biotechnol. 16, 707–715. https://doi.org/10.1007/s10126-014-9585-9.

Wang, K., Yan, P., Ding, Q., Wu, Q., Wang, Z., Peng, J., 2013. Diversity of culturable root-associated/endophytic bacteria and their chitinolytic and aflatoxin inhibition activity of peanut plant in China. World J. Microbiol. Biotechnol. 29, 1–10. https://doi.org/10.1007/s11274-012-1135-x.

Zheng, Z., Fuqua, C., Chen, L., 2012. The quorum sensing transcriptional regulator TraR has separate binding sites for DNA and the anti-activator. Biochem. Biophys. Res. Commun. 418, 396–401. https://doi.org/10.1016/j.bbrc.2012.01.035.

Zhu, H., He, C.-C., Chu, Q.-H., 2011. Inhibition of quorum sensing in Chromobacterium violaceum by pigments extracted from *Auricularia auricular*. Lett. Appl. Microbiol. 52, 269–274. https://doi.org/10.1111/j.1472-765X.2010.02993.x.