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Quorum quenching activity in the cell-free lysate of *Enterobacter ludwigii* isolated from beef and its effect on quorum sensing regulation in *Yersinia enterocolitica*

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Abstract

A wide range of Gram negative bacteria regulates their phenotypic characteristics through the auto-inducing signaling mechanism called Quorum Sensing (QS). AHL-mediated quorum sensing mechanism plays a crucial role in biofilm formation and other virulence factors. Hence, disrupting the QS signaling mechanism might be a striking approach. In this study, bacterial isolates from beef were screened for its quenching activity. The cell free lysate of test bacterial isolates were confirmed for its quenching activity using the reporter strain C. *violaceum* CV026 and quantified by means of HP-TLC. *In-silico* techniques like molecular docking and dynamics simulation studies were applied to the predicted tertiary structure of lactonase. The cell free lysate of *Enterobacter ludwigii* PUFSTb09 (KR476387) exhibited pronounced quorum quenching activity by reducing 78.20% of violacein production by the reporter strain. Furthermore, the cell free lysate of test bacteria exhibited significant ($p<0.05$) reduction in biofilm formation and EPS production of *Y. enterocolitica* (KT266804) by 66.15% and 70.18% respectively. The biofilm inhibitory activity of the test isolate was further confirmed by means of confocal laser microscopy and scanning electron microscopy which revealed the suppression of total biomass. Molecular analysis revealed the presence of aiiA gene in *Enterobacter ludwigii* PUFSTb09 and in docking analysis it was predicted that the affinity towards OHL-lactonase complex was lesser than that of LasR-OHL complex which might be due to the degrading nature of lactonase enzyme towards the natural ligand. The study shows the potential of AHLs degradation by AHL lactonase present in cell free lysate of *E. ludwigii* and regulation of QS-dependent phenotypes in food borne *Y. enterocolitica.*

Keywords

Quorum quenching, AHL lactonase, Molecular docking, aiiA gene, Biofilm

1. Introduction

Quorum sensing (QS) is a population dependent regulation mechanism used by bacteria to control their gene expression through the release of small diffusible signaling compounds.[1] Acyl homoserine lactones (AHLs) are the major group of signaling molecules used by many Gram negative bacteria.[2] As the AHLs concentration reaches the threshold level, it binds with LuxR receptor protein to form a complex. These signaling molecules play a vital role in regulating QS dependent phenotypes such as virulence determination, biofilm formation, motility and exopolysaccharide (EPS) production.[3]

Quorum sensing inhibitors from natural and synthetic sources are reported to be the most effective in disrupting QS signaling pathway.[4] Quorum quenching (QQ) activity leads to inactivation/degradation of QS molecules,[5] as it prevents sufficient accumulation in the bacterial cell thereby disrupting the quorum sensing.[3] So far, numerous strategies are known for disrupting QS signaling pathway and one of that is the enzymatic degradation or modification of AHL signaling molecules.[6] AHL lactonases are the degrading enzymes which favors the action of hydrolyzing lactones to open the AHL ring, on the other hand AHL-acylase are group of AHL degrading enzyme know to hydrolyze the amide bond.[7]

AHL lactonase encoded by aiiA gene which mediates the cleavage of lactone ring attenuates the cell-to-cell communication leading to blocking of virulence and pathogenic phenotypes. Expression of this lactonase gene is known to attenuate the virulence factors in many bacteria which include *Aeromonas hydrophila,* and *Erwinia carotovora.*[8] Many species of bacteria like *Bacillus thuringiensis*,[7] *Agrobacterium tumefaciens*,[9] and *Rhodococcus erythropolis*[10] were shown to degrade AHLs by lactonase production encoded by aiiA gene. Most of the AHL-lactonase are belongs to the metallo-β-lactamase super family as it shows

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.[11] 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.[12]

Presence of AHL-producing and degrading bacteria coexist in various ecosystems such as plant surfaces, fish intestine and shrimp gut.[13-15] The isolation of both quorum sensing and quenching bacteria from beef meat has not been reported so far. Therefore bacterial isolates from beef were screened for both AHLs production and degradation using biosensor strain. The AHL-degradation activities were further tested on the QS regulated phenotypes of positive isolate. Also, aiiA homologous gene was identified by amplification of conserved regions; elucidated with putative tertiary structure of AHL-lactonase.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Thirty five bacterial isolates cultured from beef (Departmental Culture Collection, Food science and Technology, Pondicherry University) was used as a test strains for quorum quenching assay. In addition *Chromobacterium violaceum* CECT 5999, a mutant strain which cannot produce its AHL but do respond to short and medium chain AHLs was used as a reporter strain. *Yersinia enterocolitica* (KT266804) a beef isolate which was already reported for its quorum production in our earlier report[16] was used as a test strain and *Bacillus cereus* (MTCC 1272) was used as a positive control in AHL degradation assay. All the cultures except CECT

5999 was cultured in nutrient broth and stored as 60% glycerol stocks at -80 °C until further use. *C. violaceum* CV026 was routinely cultured in LB broth supplemented with Kanamycin (20 µg/ml) in rotary shaker incubator (Scigenics Biotech, India) at 30 °C prior to experiments. Ten micro molar of N-octanoyl-DL-homoserine lactone (Sigma-Aldrich, India) was added to induce the violacein production in CV026, when required.

2.2. Cell-free lysate

Test bacterial strains seeded into M9 minimal media was incubated at 30 °C for 48 h in a shaker incubator. Bacterial cell pellets were harvested by centrifugation at 5,000 g for 10 min and resuspended in 10 ml of Tris-Hcl buffer (10 mM, pH 7.0). Dispensed cells were then placed in a sample container immersed in an ice bath. Cells were then disrupted by sonicating the suspension for 60-90 sec. After sonication, the cell lysate was centrifuged at 8,000 g for 10 min at 4 °C. Supernatant was carefully collected and filtered in 0.22 µm syringe filter which was then stored at -20 °C until further use. All the assays were performed with Tris-Hcl buffer as assay control.

2.3. Agar overlay assay

Test isolates producing AHL lactonase were screened as described by Kawaguchi et al.[17] Briefly, micro-centrifuge tubes containing 10 µl of cell-free lysate were added with 100 µM OHL and LB broth to attain the final volume of 2 ml. Reaction mixtures were incubated at 30 °C for 2 h and heated at 95 °C for 10 min. After incubation the reaction mixture was heated at 95 °C for 10 min. LB agar plates (1.5%) overlaid with reporter strain (0.8%) were loaded with 20 µl of the reaction mixture in the bored wells. Inoculated plates were then incubated overnight at 30 °C. To confirm the enzymatic activity, the reaction mixture was again heated at 95 °C for 10 min. Also, the reaction mixture was incubated with proteinase K at a protease-to-protein (wt/wt) ratio

of 1:10 at 30 ºC for 30 min[18]. The residual activities were detected under standard conditions with Synthetic OHL, heat killed and no protease-treated enzyme as the control. Absence of violacein pigmentation around the wells was scored as a positive result for AHL degradation.

2.4. Flask incubation assay

In flask incubation assay overnight culture of CV026 was inoculated into 50 ml of LB broth seeded with cell free lysate of test bacterial isolates along with OHL. Inoculated flasks were then incubated at 30 °C for 24 h in a shaker incubator. AHL degradation was quantified by extracting the violacein produced. Briefly, 1 ml of culture from each flask was centrifuged at 8,000 g for 5 min to precipitate violacein. Pellet dissolved in 1 ml of DMSO was vortexed robustly to solubilize the violacein completely. Above mixture was centrifuged again to remove the cells and quantified at 585 nm using microplate reader (Biotek, USA). Harvested bacterial cells were resuspended in sterile PBS for bacterial cell measurement at 600 nm.

2.5. Bacterial identification

The 16S rRNA of positive isolate was extracted using HiPURA bacterial genomic DNA purification kit (Himedia, India) and was amplified by set of forward and reverse universal primer U1F-5'-AGAGTTTGATCCTGGCTCAG-3' and U1R-5'-GGTTACCTTGTTACGACTT -3') respectively. Thermocycling conditions were maintained as initial denaturation of 92 ºC for 2 min and 10 s followed by 35 cycles of 92 ºC for 1 min, 48 ºC for 30 s and 72 ºC for 2 min and 10 s and a final extension step of 72 ºC for 6 min and 10 s using mastercycler (Eppendrof, Germany). Amplified products were resolved using 1.5% agarose, ethidium bromide gel. DNA sequencing was performed and subjected to BLAST analysis for determining nucleotide sequence similarities in NCBI. Similar sequences were subjected to multiple sequence alignment using CLUSTAL W. The phylogenetic tree was constructed using MEGA 4 using neighbor

joining method. The identified isolates were deposited in NCBI (National Center for Biotechnology Information databases) and MTCC (Microbial Type Culture Collection) was assigned with accession number.

2.6. Quantification of AHL degradation by HP-TLC

To extract the AHL produced by the quorum positive culture, test isolate was inoculated into 100 ml of M9 minimal media and incubated at 30 °C. Spent culture supernatant was extracted with ethyl acetate and the pooled extract was evaporated to dryness. Residues were resuspended in 100 µl of HPLC grade acetonitrile and stored at -40 ºC until further use.[19] For the quantification of AHL degradation by quorum quenching bacteria, cell free lysate suspended in 10 mM Tris-Hcl buffer (pH 7.0) was added with AHL extract and incubated at 30 °C for 12 h. The reaction was stopped by adding equal volume of dichloromethane and the solvent was evaporated to dryness using nitrogen gas. The residue was resuspended in 100 µl of HPLC grade acetonitrile and stored at -40 ºC. OHL was used as a positive control in all the assays, when required.

High-performance thin layer chromatographic method was optimized for the quantitative evaluation of AHLs profile and quorum quenching activity. Two microliter of extracts was applied onto C18 RP - Silica gel 60 F254 (10 × 10 Merck, Germany) using automatic TLC sampler and the chromotagrams were developed using methanol and water (60:40 v/v). Developed plate was air dried and overlaid with a thin film of soft agar seeded with reporter strain *C. violaceum* CV02, incubated for 24 h at 32 °C. AHLs detected by blue pigmentation were scanned at 585 nm by TLC scanner (CAMAG, Switzerland). AHL produced and the percentage of degradation was determined by comparing with known concentration of OHL and control.

160 2.7. Reduction in EPS production

161 Overnight culture of test isolate (adjusted to 0.1 OD@600 nm) was inoculated into 9.5 ml
162 LB broth along with 0.5 ml of cell free lysate and incubated at 30 ºC for 24 h. Late-log phase
163 cells adhered to the walls of the test tubes were harvested by centrifugation at 8,500 rpm for 30
164 minutes at 2 ºC. Filtered supernatant was added with three volumes of chilled ethanol and
165 incubated overnight at 2 ºC to precipitate the dislodged EPS. Precipitated EPS was collected by
166 centrifugation at 5,000 g for 30 min which was then dissolved in 1 ml of de-ionized water.
167 Culture broths without enzyme added with PBS served as control. Bacterial cells were removed,
168 resuspended in sterile PBS and read at 600 nm. Collected EPS was quantified by means of
169 phenol-sulfuric acid method.[20]

170 2.8. Effect of CFL on biofilm formation

171 The effect of cell free lysate on biofilm formation was screened as described by Junker et
172 al.[21] with minor modifications. Briefly, overnight culture of test isolate (adjusted to 0.1 OD@600
173 nm) was inoculated into 950 µl LB broth in a 12-well microtiter plate along with 50 µl of cell
174 free lysate and incubated at 30 ºC for 24 h with agitation. After incubation, plates were carefully
175 rinsed with double-distilled water to remove loosely attached cells. Adhered cell on the walls
176 were stained with 100 µl of crystal violet solution (HiMedia, India) for 10 min. Excess stain was
177 removed by rinsing with distilled water and washed with 100 µl decolorizer. Intensity was
178 measured at OD585 nm by using microplate reader (Biotek, USA), for quantification of biofilm
179 biomass. Culture broths without enzyme added with PBS served as control. Bacterial cells were
180 removed, resuspended in sterile PBS and read at 600 nm.

181 2.9. In-situ visualization

Overnight grown bacterial culture was resuspended in PBS to attain 0.1 OD@600nm and inoculated into LB broth. Bacterial biofilms were allowed to develop on 1x1cm glass slides on 90mm petridish with and without cell free lysate for fluorescence microscopy. After 24hrs of incubation, glass slides were rinsed with sterile distilled water, air dried and stained with acridine orange (0.1%) for 5 min. biofilm formed on the glass slides were visualized under fluorescence microscope at 10× (Gippon, Japan).

For scanning electron microscopy (SEM) sample preparation was done as described by Lembke et al.[22] Briefly, biofilms formed on the glass slides were fixed with 2.5% glutaraldehyde for 1 hour. Fixed glass slides were washed using 0.1M sodium acetate buffer (pH 7.3). Slides were then dehydrated with ethanol; air dried, carbon sputtered and analyzed using a scanning electron microscope (Hitachi S-3000H, Japan).

2.10. PCR amplification and alignment of aiiA homologue gene

Genomic DNA of AHL degrading bacteria was amplified with forward and reverse primer aiiAF2 (5'-CGGAATTCATGACAGTAAAGAAGCTTTA-3') and aiiAR2 (5'-CGCTCGAGTATATATTCAGGGAACACTT-3').[23] Thermocycling conditions were maintained as initial denaturation at 94 °C for 5 min, 5 cycles of 94 °C (45 s), 44ºC (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 using mastercycler (Eppendroff, Germany).[24] Amplified product was resolved using 2× agarose, ethidium bromide gel.

2.11. Homology sequence analysis

The partial gene sequence of *Enterobacter ludwigii* strain PUFSTb09 (GI: 849976757; accession number: KR476387) was obtained from NCBI database for homology analysis. Nucleotide blast was performed with non redundant database for comparing its homology with

partial coding sequences of *Enterobacter ludwigii* strain VT70 and VT66 AHL-lactonase (aiiA) gene (KF768741 and KF768742). Homology of the genes coding for the lactonase enzyme was screened through sequence alignment.

2.12. Tertiary structure prediction of lactonase enzyme

Putative tertiary structure for AHL lactonase was predicted with Modeller 9.14.[25] The gene sequence of *Enterobacter ludwigii* was analyzed for its ORF and its transcription into full length protein sequence. Followed by transcription of protein sequence, protein blast was done to predict the suitable template for three-dimensional structure prediction.

2.13. Docking

Predicted three-dimensional structure of AHL lactonase enzyme was docked with N-acyl homoserine-lactone using Schrodinger suite 2010. All the residues were selected for blind docking with the prepared grid. The N-acyl homoserine-lactone was prepared through LigPrep and docked with the lactonase grid through Glide module.

2.14. Molecular dynamics simulation

Molecular dynamics simulation of lactonase-AHL complex was performed to study the effect of lactonase on AHL. This simulation was performed with the help of Gromacs 4.5.3[26] by using GROMOS force field. The protein-ligand complex was solvated using SPCE[27] water model at 1.5 nm cubic box. System was neutralized by substituting solvent molecule from CL- ion. Periodic boundary condition was applied in all direction. Subsequently, a maximum of 50,000 energy minimization steps were carried out for the predicted models using a conjugate gradient algorithm[28] with a tolerance of 1000 kJ mol^{-1} nm^{-1} by following steepest descent minimization[29]. The system was equilibrated for 2 ns by keeping pressure and temperature constant in subsequent steps. This pre-equilibrated systems were subsequently used in the 10000

ps (10 ns) production MDS with a time-step of 2 fs. Structural coordinates were saved every 2 ps and analyzed using the analytical tools in the GROMACS package. The lowest potential energy conformations were fetched from 10 ns MDS trajectory for further analysis.

3. Results and discussion

3.1. Screening of AHL degrading bacteria

Quorum quenching activity of cell free lysate of beef isolates was screened with N-octanoyl-DL-homoserine lactone molecules by agar overlay and flask incubation assay. Inhibition of violacein production was considered as positive for degradation of AHL molecules using *C. violaceum* CV026 biosensor. Out of 35 bacterial isolates, PUFSTb09 showed the degradation of AHLs in agar overlay assay (Fig. 1). Further, to confirm the enzymatic activity in cell-free lysate, it was heat killed at 95 °C for 10 min and subjected to Proteinase K degradation which 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 beef isolate. Further, in flask incubation assay for the quantification of AHLs degradation by beef isolates it was observed that the cell free lysate of PUFSTb09 inhibited 78.20% ($p<0.05$) of violacein pigmentation in the reporter strain without inhibiting cell biomass (Fig. S1). Inactivation of AHLs degradation on heat treatment and proteolytic degradation indicates the enzymatic activities in cell free lysate of beef isolate. Strains belonging to the genera *Bacillus, Rhodococcus, Agrobacterium and Pseudomonas* have been reported for their AHL degrading activity. The quorum quenching enzyme of these isolates have been identified which includes lactonase, acylase, and oxidoreductase.[7, 9, 30, 31]

3.2. Bacterial identification

The isolate degraded AHL was identified by amplifying the genomic DNA followed by 16S rRNA gene sequencing. The isolate was tentatively identified by BLAST analysis of obtained 16S rRNA sequence. Sequence analysis 16S rRNA of beef isolate was identified as *E. ludwigii* PUFSTb09 which shared 99% similarity with *E. ludwigii* group in the local alignment search (Fig. S2). The isolate *E. ludwigii* identified for its AHL degradation in this study has been previously isolated from the plant *V. madraspatana* and from strawberry fruit as endophyte.[32,11]. The identified bacteria was deposited at NCBI and assigned with accession number.

3.3. Quantification of AHL degradation

Culture broth of *Y. enterocolitica* extracted for AHL was quantified using high performance thin layer chromatography and compared with the known concentration of N-octanoyl homoserine lactone as standard. N-octanoyl homoserine lactone resolved with an active peak with the retention factor 0.86. AHLs extracted from the culture broth of *Y. enterocolitica* also, induced a sharp active peak at the same retention time of standard compound. AHL present in the culture broth of *Y. enterocolitica* was estimated to be 545.8 nM/L. Degradation of AHL by the cell free lysate of *E. ludwigii* PUFSTb09 was also analyzed by HPTLC. It was revealed that *E. ludwigii* PUFSTb09 exhibited significant hydrolysis of AHL extracted from the culture broth of *Y. enterocolitica* PUFSTb04. Under test condition it was estimated that *E. ludwigii* PUFSTb09 hydrolyzed 52.78% of QS molecule (Fig. S3). Cell free lysate of positive culture exhibited 46.61% of AHL hydrolysis which confirms the presence of quorum quenching enzyme. The above result corroborates well with the findings of Cirou et al.[33] who reported the degradation of AHL by *B. megaterium.* Many genera of Gram negative bacterium including *Burkholderia*, *Acinetobacter* and, *Alteromonas* have been proved for their quorum quenching activity.[34-36]

3.4. Reduction in biofilm formation and EPS production

The cell free lysate of *E. ludwigii* PUFSTb09 was screened for its effect on the biofilm formation of *Y. enterocolitica* PUFSTb04 by microtiter plate method. In quantitative assay for screening anti-biofilm activity of cell free lysate, results exhibited that the biofilm formation by the test isolate was inhibited by 66.15% ($p<0.05$) when compared to control. It was noteworthy that the planktonic cells were not influenced by the treatment (Fig. S4a). The cell free lysate of positive control *B. cereus* inhibited the biofilm formation by 68.89% when compared with control. It was reported that the cell free lysate of entophytic bacteria inhibited 60% of biofilm formation of *P. aeruginosa*.[32]

The effect of cell free lysate on the EPS production of test bacteria was screened as it is positively correlated with biofilm forming potential. Quantitative analysis of EPS extracted from treated and untreated culture broths of test bacteria revealed that EPS production decreased by 70.18% (Fig. S4b). In addition on screening the effect of *B. cereus* CFL on EPS production, 71.37% decrease in the EPS production was observed ($p<0.05$).

3.5. In-situ visualization

In-situ visualization of the biofilm developed with and without cell free extracts of *E. ludwigii* and *B. cereus* was analyzed using fluorescence microscopy and scanning electron microscopy. Results exhibited the suppression of total biomass in treated slides when compared with the control (Fig. S5). Both SEM and fluorescence microscopic images revealed a thick biofilm formation on the control slide but the same was not observed on the slides treated with cell free lysate of *E. ludwigii* and *B. cereus.* Results obtained are in accordance with that of Rajesh and Ravishankar,[32] who demonstrated that the biofilm inhibitory activity of *B. firmus* PT18 and *E. asburiae* PT39 cell free lysate.

3.6. PCR amplification and alignment of aiiA homologue gene

Basic screening by agar overlay assay and the inactivation of degrading activity on heat treatment suggest the presence of quorum quenching enzyme in the cell free lysate of *E. ludwigii.* Further HPTLC analysis confirms the presence of degrading enzyme and its coding genes. Using the extracted genomic DNA, PCR amplicons of about 700 bp was visualized on 1.5 × gels on electrophoresis (Fig. 2). The sequence was submitted to NCBI database and assigned with the accession number (KU53012). The amplification of aiiA homologous gene indicated the presence of AHL lactonase as QS degrading enzyme in the cell free lysate of beef isolate. Dong et al.[6] and Sakr et al.[37] have reported the presence of aiiA gene in many bacteria and in *B. weihenstephanensis* respectively. The amino acid sequence alignment of our enzyme with AHL lactonases from other species was done using NCBI database and Clustal W. The homology analysis of the genes coding for aiiA by means of sequence manipulation suite (http://www.bioinformatics.org/Sms2/ident_sim.html) revealed that *E. ludwigii* (KU53012) shared around 34% homology with amino acid sequences of *E. ludwigii* (AHE80976.1) and *E. aerogenes* (AHE80975.1). The intensity of the similarity bars shows the similarity between the sequences (Fig. S6). It was reported that AHL-acylases of *Ralstonia* sp. are found to be diverse which shares 39% identity at their amino acid level. It has also showed 36% identity with amino acid sequences of acylases reported from organisms such as *P. aeruginosa*.[38]

3.7. Tertiary structure prediction of lactonase enzyme

The putative tertiary structure of lactonase was modeled through Modeller 9.14 by using 2BR6 as template. PDBSum database[39] was used for the analysis of predicted model. The predicted three-dimensional model of lactonase was shown in the Fig. 3 Metallo-beta-lactamase super family of the lactonase was revealed from NCBI CD search which includes thiolesterases, members of the glyoxalase II family. These enzymes catalyze the hydrolysis of S-D-lactoyl-

glutathione to form glutathione and D-lactic acid and a competence protein that is essential for natural transformation in *Neisseria gonorrhoeae* and could be a transporter involved in DNA uptake. Except for the competence protein these proteins bind two zinc ions per molecule as cofactor. PDBSum analysis of this protein reveals all the secondary structural components which comprise 2 β sheets, 3 βαβ units, 3 β hairpin, 2 β, 8 β strands, 11 helices, 3 helix-helix interaction, 18 β turns, and 1 gamma turn.

3.8. Docking and molecular dynamics simulation

The molecular docking analysis was performed to screen the hot spot residues of the protein. These residues are interacting with lactonase enzyme and the natural ligand (N-octanoyl homoserine lactone) to change its conformation for functioning activity. On blind docking, AHL-lactonase complex exhibited docking score of -2.42 Kcal/mol. The complex was submitted to PDBSum databases for analysis. The LigPlot module of this database helps to visualize the H-bond interaction between the residues which revealed the three hydrogen bonds formed in the complex (Table 1). The above complex with no hydrophobic bonds shows weak binding affinity towards each other (Fig. 4). This is in contrast with the LasR-AHL complex which shows strong binding affinity towards each other with the dock score of -4.42 Kcal/mol.

Molecular dynamics simulation was performed to analyze the conformation changes for activation and deactivation of LasR receptor protein in the presence of signaling molecule and lactonase respectively. The simulations were performed with two complexes, LasR-OHL and Lactonase-OHL for 10ns. The RMSD profile was generated for both the complexes as shown in the Fig. S7. It was observed in the profile that the deviation recorded in the OHL-lactonase complex was higher than the LasR-OHL complex which might be because of the degrading activity of lactonase.

4. Conclusion

In brief, the current study evidenced the AHLs degrading activity of *Enterobacter* species isolated from beef. The cell free lysate of quorum quenching bacteria efficiently regulated QS dependent phenotypes like EPS production, and biofilm formation of quorum sensing bacteria isolated from the same source. Further, *In-silico* studies evidenced the alignment of aiiA gene to other known sources proposed the relevant enzymatic activity and the tertiary structure of AHL-lactonase. The study appends additional note on the potential application of AHL-lactonase extracted from quorum quenching bacteria to manage QS regulated phenotypes in *Y. enterocolitica*.

Conflict of interest

Authors declare that they have no conflict of interest.

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Figure legends

Fig. 1 Plates showing degradation of AHLs by agar overlay assay using biosensor strain CV026. Suppression of violacein production in the reporter strain by cell free lysate, heat killed and proteinase K treated controls of *E. ludwigii* (PUFSTb09) and *B. cereus* MTCC 1272 (A and B).

Fig. 2 PCR amplification of aiiA gene from *E. ludwigii* (PUFSTb09) and *B. cereus* (MTCC 1272) using the specific primers. Lane 1: 100bp marker; Lane 2: aiiA gene of *E. ludwigii* (PUFSTb09), and Lane 3: aiiA gene of *B. cereus* (MTCC 1272).

Fig. 3 Showing the putative three dimensional, tertiary structure of AHL lactonase gene from *E. ludwigii* (PUFSTb09)

Fig. 4 Docked conformation of AHL lactonase gene with natural ligand. Indexed figure represent the residues of AHL lactonase interacting with acylated homoserine lactone. H-bonds formed in the complex were displayed in black color dashed lines.

Table1 Residues of N-octanoyl-DL-homoserine lactone interacting with AHL lactonase and LasR receptor protein through various interactions.

Molecules (Drug Bank ID)	Hydrogen Bonding interactions			Dock score (Kcal/mol)	Glide-Emodel score	Hydrophobic interactions
	Donor	Acceptor	Length (Å)			
N-Octanoyl-DL-homoserine lactone (OHL) 3474204	Lig:: O2 Thr 75:OG1 Asp 73: OD1	Trp 60:NH1 Lig:: N1 Lig:: N1	3.01 3.30 3.00	-4.28	-42.2	Leu 110, Phe 101, Tyr 93, Ala 105, Trp 88, Tyr 56, Ser 129, Tyr 64, Leu 36, Ala 127, Tyr 47, Ala 50, Val 57, Ile52
AHL-Lactonase 3474204	M 1(H1) G 60(H) Lig (16)	Lig (O3) Lig (O1) H 57(O)	2.2 2.0 2.1	-2.42	-25.4	-

Fig. 1

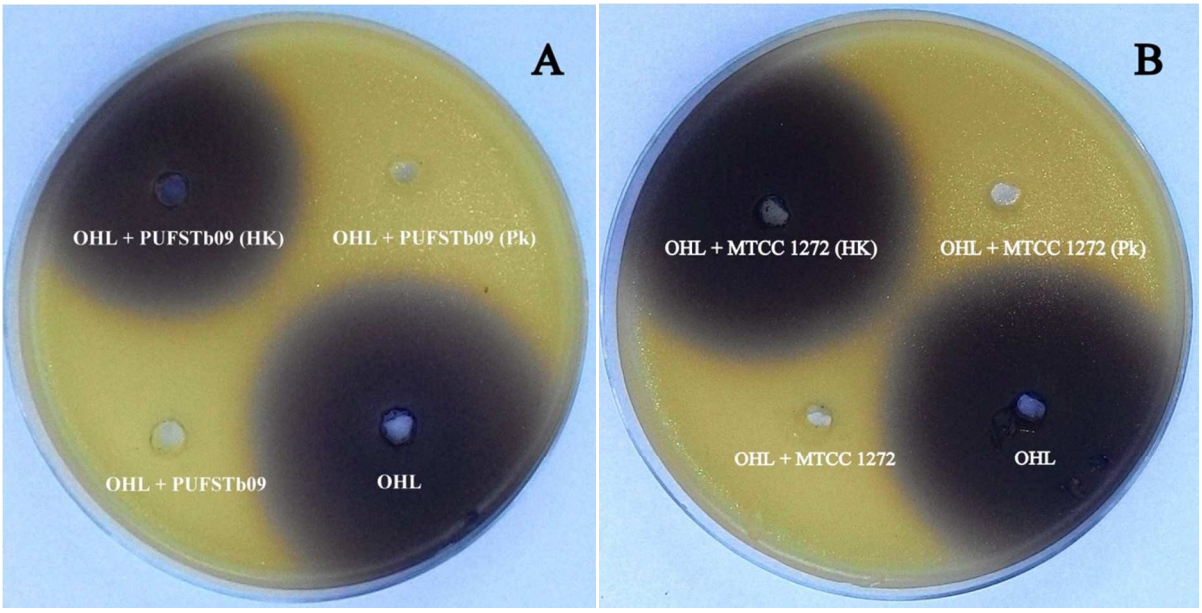

Fig. 2

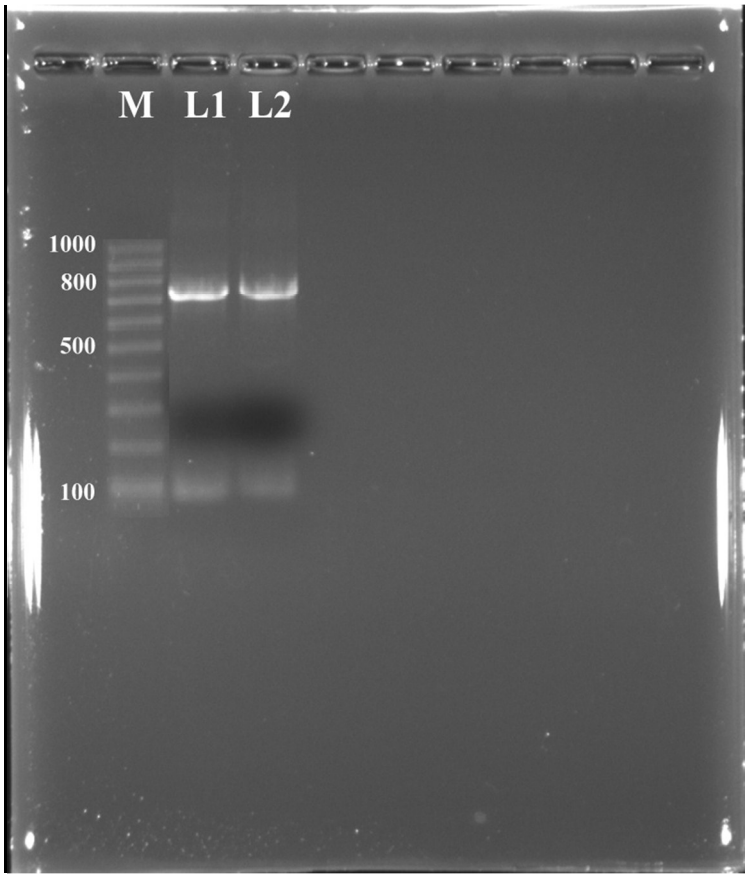

Fig. 3

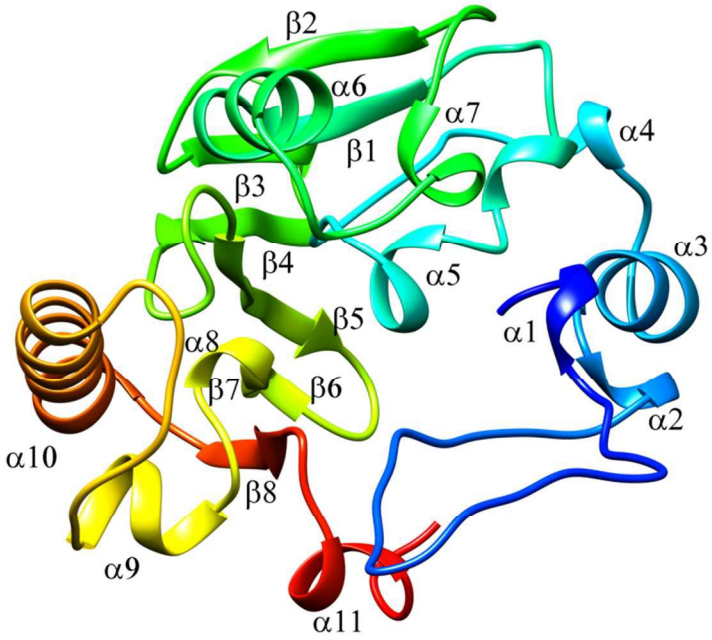

Fig. 4