Identification and characterization of a peptidoglycan hydrolase from *Rhodobacter johrii*

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Abstract

The bacterial whole genome sequences are available in the database therefore explored for the varieties of known and unknown proteins. Bacteria harbor various peptidoglycan hydrolases that cleave peptidoglycan and play an important role in the cell division, growth, spore differentiation and development. In the present study, we report a peptidoglycan hydrolase in an endospore producing phototrophic proteobacterium *Rhodobacter johrii*. The Peptidoglycan Hydrolase of *Rba. johrii* (PgHR) can actively hydrolyze the intact spore cortex peptidoglycan (sacculi). The protein contains a pre-peptide precursor which has a Hydrolase-2 (PF07486) family conserved domain. PgHR protein has SleB like properties which are spore cortex-lytic enzymes involved in the depolymerization of cortex peptidoglycan present and characterized in *Bacillus* spp. The expression pattern of PgHR through qRT-PCR suggests its role in stationary phase of *Rba. johrii*. This is a new type of peptidoglycan hydrolase reported from a proteobacterium.

Introduction

In bacteria there are diverse groups of peptidoglycan hydrolase enzymes which cleaves the bonds of the peptidoglycan polymers and its fragments (Shockman et al. 1996). These enzymes play an important role in cell motility, cell division and competence, cell-wall turnover, peptidoglycan remodeling, cell enlargement, differentiation into spores and germination, digestion of asymmetric septum for prespore engulfment, maturation of cortex of spore, lysis of mother cell and spore cortex lysis (Vollmer et al. 2008; Holtje 1995). These hydrolase cleaves the bond between the cell walls creating pores essential for incorporation of more PG material and expansion of cell wall during cell growth and development of the bacteria (Lee and Huang 2013).

Germination specific lytic enzymes (GSLE) actively cleave and depolymerizes the peptidoglycan of spore cortex (Ishikawa et al. 1998), these types of enzyme are unable to cleave the intact cell wall of a new germinating bacterial cell which is present just beneath the cortex of spore (Popham et al. 1996). GSLE are two types (i) Spore cortex lytic enzymes (SCLEs) which initially hydrolyze the PG of the spore cortex (ii) Cortical fragment lytic enzymes (CFLEs) further degrade partially hydrolyzed cortex. The *B. subtilis* has three GSLE– CwlJ, SleB and YkvT of these SleB and CwlJ are well characterized. In *B. cereus* cortex lytic enzyme is SleL which shows glucosaminidase activity (Chen et al. 2000). There are still peptidoglycan hydrolase that harbor in the genome of bacteria which is yet to be explored, also the function of hydrolase depends on the type of hydrolase.

Rhodobacter johrii is a photosynthetic proteobacterium that was isolated from a pasteurized soil and produces refractile structures which are similar to spores since these structures were stained with malachite green (Girija et al. 2010). In present study, we identified a SleB like protein from *Rba. johrii*, and its comparative characterization involving bioinformatic analysis, enzyme assay and differential expression study was undertaken to understand the property of the new hydrolase.

Material And Methods

Bacterial strains, growth conditions and plasmids

Rhodobacter johrii strain JA192^T (= DMS 18678^T = JCM 14543^T = MTCC 8172^T) was grown in fully filled screw-capped tubes (10 × 100 mm) phototrophically (30°C, 2400 lx) in a mineral medium described previously (Lakshmi et al. 2011) with ammonium chloride (0.1%, w/v) and pyruvate (0.1%, w/v) as nitrogen and carbon sources/e⁻ donor, respectively. *Bacillus subtilis* strain FW2 grown in nutrient broth (Himedia M002) at 37°C overnight in shaking incubator was used for sporulation. The purity of cultures was checked at each step by streaking on nutrient agar (Himedia M001). *Escherichia coli* strains DH5α and Rosetta (DE3) were grown in LB medium (Himedia M1245) at 37°C and the antibiotic used was kanamycin (34 µg.ml⁻¹). The plasmid used for this study was pET SUMO vector. All cultures were maintained in aseptic conditions with sterility precautions to ensure the absence of contamination.

Bioinformatic analysis

The whole genome sequence of *Rba. johrii* with Accession: PRJNA323784 was used for analysis from NCBI database. For full-length protein alignment the sequences used were *B. cereus* strains, ATCC 14579 (NCBI Accession No. WP001249053), M13 (ASK14870) and MLY1 (WP100910207); *B. anthracis* strains, ATCC 14578 (WP001249045), Sterne (YP028821) and Ames (AAP26584) using Clustal Omega (Madeira et al., 2019) and rendered sequence similarity by ESPript 3.0 (Robert and Gouet, 2014). The SWISS-MODEL server (Waterhouse et al. 2018) was used to generate a model of the PgHR protein. The model obtained from SWISS-MODEL was verified using PROCHECK (Laskowski et al. 1993). Protein domains were searched by a Simple Modular Architecture Research Tool (SMART) (Letunic and Bork 2018). Protein models were superimposed based on Ca position using PyMol (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC). The genetic features including promoter were predicted through the SoftBerry server (Salamov and Solovyev and 2011). The theoretical Mw (molecular weight) and pI were computed through ExPASy online tool (Gasteiger et al. 2005).

Cloning, protein overexpression, and purification

The *pghr* gene ORF lacking starting 23 codons, including codons 24-240 was amplified from *Rba. johrii* genomic DNA by using forward primer (BSCLFSUMOE: 5'-ATATGGATCC GATGTGACGGTGAGCCAGTC-3') and reverse primer (HSCLRSUMOE: 5'-TATAAAGCT TTCAGGAGCCGCCGGGCTG-3'). DNA was extracted using the necessary precautions to prevent contamination. PCR amplified product of 674 bp as well as pET SUMO expression vector was digested with BamHI and HindIII restriction enzymes, purified and ligated using kits (QIAquick PCR purification kit; Qiagen, Inc., USA). The *pghr* fused with pET SUMO plasmid produced the plasmid pET-SUMO-PGHR, which encodes an N-terminal His₆ - SUMO tagged with PGHR truncated fusion protein. This plasmid was transformed into *E. coli* DH5α cells and was confirmed by DNA sequencing.

The his₆-SUMO- PGHR₂₄₋₂₄₀ fusion protein was overexpressed into *E. coli* Rosetta (DE3) which was grown in 1 Liter LB medium (Himedia M1245) with 34 µg.ml⁻¹ kanamycin at 37 °C till the OD reached 0.6. At this point, 0.2 mM isopropyl-β-D- thiogalactopyranoside was added and culture was incubated at 37 °C for an additional 6 hours. Cells were harvested by centrifugation (6,500 rpm for 10 mins at 4°C) and resuspended into 25 ml ice-chilled buffer A (50 mM Tris base pH 8.0, 300 mM NaCl, 10 mM Imidazole, 0.5 mM phenylmethylsulfonyl fluoride [PMSF]). Cell disruption was done by sonication (35% amplitude, 10 min processing time, 20 s intervals). The cell lysate was pelleted by centrifuging (7,000 rpm for 30 min at 4°C) and the supernatant containing the soluble homogenized fusion protein with His-tag was purified by metal affinity chromatography with the Ni-NTA resins (Takara Bio.). Proteins were eluted at 100 to 350 mM imidazole gradient in buffer A. Fractions with eluted pure proteins with no other protein contaminants were pooled together, run on SDS-PAGE gels and the appropriate band was excised and sent for MALDI-TOF MS-MS sequencing, outsourced to Sandor Life Sciences, Hyderabad, India. The pooled fractions were dialyzed in buffer with SUMO protease without imidazole and passed again through Ni-NTA resins to yield pure PGHR₂₄₋₂₄₀ protein. This was concentrated by using an Amicon Ultra 15 centrifugal filter device (Merck Millipore).

Isolation of peptidoglycan substrates

Vegetative cell wall (peptidoglycan) from *B. subtilis* (Sigma Aldrich, Inc., USA) was procured for enzyme assay. Spore sacculi (peptidoglycan) from *B. subtilis* FW2 was isolated from the purified spore isolated as previously described (Bosak et al. 2008). Spore sacculi was isolated from the prepared spores as previously described (Dowd et al. 2008) with few modifications. Briefly, spore pellet was harvested from 300 ml of *B. subtilis* FW2 was treated with 35 ml solution containing 50 mM Tris HCl (pH 7.5), 1% SDS, 50mM Dithiothreitol (DTT). This was boiled for 20 min and washed with warm autoclaved Milli-Q water till SDS was removed and centrifuged for 10,000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in 1 ml of a solution containing 100 mM tris HCl (pH 7.4), 20 mM MgSO₄, 10 µg DNase I, 50 µg RNase A and incubated at 37°C for 2 hr. To this 100 µg trypsin (TRT PCK, Worthington) in 10 mM, CaCl₂ was added and incubated at 37°C overnight. The solution was boiled with 1% SDS and washed in autoclaved Milli-Q water till SDS was removed and dissolved in autoclaved Milli-Q water for analysis (or stored at 4°C).

Enzyme activity assay

Spore sacculi was dissolved in 1 ml reaction mixture containing 30 mM NaPO₄ (pH 7), 1mM EDTA, 1mM DTT in a cuvette. The enzyme assay was done by observing the % OD₆₀₀ loss per minute (1 cm path length cuvette) of 0.2 OD₆₀₀ unit.ml⁻¹ spore sacculi in which protein PgHR₂₄₋₂₄₀ from *Rba. johrii* was added at 20 nM concentrations and incubated at 25°C. The absorbance at 600 nm was recorded at each 5 min time interval after agitation for 40 min. Similarly, the cell wall was used as a substrate in the same buffer and the same conditions used above in which protein PgHR₂₄₋₂₄₀ was added at 20 nM

concentrations and incubated at 25°C. The enzyme assay was done in triplicate and repeated at least three times.

RNA extraction, cDNA synthesis, and Quantitative RT-PCR for real-time expression

Total RNA was extracted from 10 ml of culture samples at different time points using the miRNeasy mini kit (Qiagen Inc., USA) according to the manufacturer's instructions and treated with RNase-free DNase I (Qiagen Inc., USA) to remove DNA contamination in RNA before reverse transcription. 1 µg of RNA was used to synthesize cDNA in a 10 µl reaction mixture by using Easy Script™ cDNA Synthesis Kit (Applied biological materials, Inc., Canada). The obtained first-strand cDNA was amplified using SYBR green FAST qPCR Master Mix (Kappa Biosystems, Inc., USA) in a 10 µl reaction mixture with target RNA specific primers on StepOne Real-time PCR detection system (Applied Biosystems). Primer design and specificity were done through Geneious Primer 3 for qRT-PCR (Table S1). Cycling conditions were as follows: an initial denaturation at 95°C for 10 min, followed by 42 cycles of 95°C for 15s, 62°C for the 30s, and 72°C for 30s. qRT-PCR reaction product specificity was tested by melting curve examination and each sample was tested in triplicates. The expression levels of the putative *pghr* gene were calculated using a relative standard curve method, Ct values of *pghr* gene were normalized to the Ct values obtained from the amplification of the housekeeping gene 16S rRNA gene and relative quantification was done by comparative $\Delta\Delta C_t$ method ($2^{(-\Delta\Delta C_t)}$).

Results

Identification and sequence analysis

From the genome of *Rba. johrii* a hydrolase protein sequence (Accession. no. WP069331589) was retrieved. Protein has 28% amino acid identity, 67% coverage with *B. subtilis*, and 26% identity, 65% coverage with *B. cereus* spore cortex lytic enzyme (SleB). Hydrolase protein of *Rba. johrii* was further characterized and compared with *Bacillus* spore cortex lytic enzyme (SleB). The functional domain and characteristics of the protein were analyzed with the SMART search tool (Table S2), which predicted a signal peptide from 1 to 23 a.a and a conserved hydrolase 2 superfamily (pfam07486) domain from 131 to 234 a.a (Fig. 1A). The genetic features were identified, its promoter is having -10 box (CGCAAAAAT) from 121 bp to 113 bp and -35 box (TGGACG) from 141 bp to 136 bp upstream of the start codon (ATG) of coding sequence having total length of 723 bp of this hydrolase gene (Fig. 1B). The gene encodes a polypeptide of 240 amino acid residues with an expected molecular mass of ~25,557 Da, which is highly basic having a theoretical pI of 9.82.

We named this protein as Peptidoglycan Hydrolase from *Rba. johrii* abbreviated as PgHR. Multiple sequence alignment of full-length PgHR protein with the other SleB of *Bacillus* spp. was highly conserved at the catalytic domain with divergence at other parts (Fig. 2). Further, the sequence motif EXRGE having alternate glutamate (E) residues has been considered as a signature sequence of SleB proteins (Jing et

al. 2012) which is strictly conserved in PgHR protein at position Glu128 and Glu132, simultaneously the catalytic residue glutamate (Glu128) was also conserved in PGHR of *Rba. johrii* (Fig. 2).

Secondary structure prediction and model superimposition

PgHR protein of *Rba. johrii* secondary structure was model through homology modeling using the SWISS-MODEL server, from 109-234 a.a using a template of germination-specific lytic transglycosylase SleB crystal structure (PDB ID - 4f55.1) of *Bacillus cereus* ATCC 14579 (Li et al. 2012). The structure has 7 α -helices, 3 β -sheets, 11 loops and turns (Fig. 3A). PROCHECK tool was used to validate the model obtained from SWISS-MODEL with Ramachandran plot indicating >99% residues in the allowed region, only one residue amounting to ~1% in the disallowed region (Fig. 3B). PGHR model of *Rba. johrii* and template model was superimposed based on Ca position using PyMol. The root means square deviation (RMSD) for the superimposition of the model and the template was found to be 1.96 Å (Fig. S1).

The predicted model for PgHR of *Rba. johrii* and SleB of *B. cereus* were superimposed which indicated that catalytic residue Glu128 (glutamate) is conserved and present exactly in the same location of the groove between the α and β domains as previously reported in *B. cereus* (Li et al. 2012). The PgHR groove is lined with aromatic residues Phe167, Phe209, Trp218, His231, Phe233 which are conserved in all SleB type proteins (Fig. 4A) and these groove amino acids are responsible for binding and cleavage of spore cortex peptidoglycan (Li et al. 2012). In the superimposition of PgHR model of *Rba. johrii* with SleB of *B. anthracis* ATCC 14578 (Fig. 4B) it was found that the residues Tyr153, Gly164, are replaced by the same functional group amino acid and Trp218 is conserved, shown in red color. These residues are hypothesized for their role in the binding of muramic- δ -lactam (Jing et al. 2012). Whereas the catalytic, signature, aromatic residues Glu132, Arg147, Val148, Pro154, Phe167 were conserved in the exact location around the catalytic groove (Fig. 4B).

Substrate specificity and in vitro enzyme assay

The first twenty-three amino acids at the N-terminal of PgHR₂₄₋₂₄₀ protein are predicted as signal sequence and were deleted. The C-terminal hydrolase domain containing 24 to 240 amino acids was overexpressed in the pET-SUMO- PgHR₂₄₋₂₄₀ vector along with histidine and Small Ubiquitin-like modifier tag (His₆-SUMO) at N-terminal as a fusion protein in *E. coli* Rosetta (DE3). Overexpressed protein PGHR₂₄₋₂₄₀ with N-terminal His₆-SUMO tag remained in a soluble form which was purified with Ni-NTA affinity followed by cation-exchange chromatography was confirmed by western blotting using antibodies against the His₆ tag. The sequence from MALDI-TOF MS-MS completely identical (Fig. S2) with the sequence of PgHR protein (Accession. no. WP069331589) of *Rba. johrii* and also checked on SDS gel (Fig. 5A). The tag was removed by DeSUMOylases cysteine protease treatment and the SDS-PAGE shows the expected molecular mass of PGHR (~25 KDa) after digestion of *Rba. johrii* (Fig. 5B). The digested protein was further purified with cation-exchange chromatography resulting in a pure protein free from His₆-SUMO tag. Purified PgHR₂₄₋₂₄₀ protein activity of cleaving the spore cortex was analyzed

by enzyme assay. When *B. subtilis* FW2 spore sacculi were incubated with recombinant PgHR₂₄₋₂₄₀ protein for 40 min, a loss of 50% of the substrate was observed at OD₆₀₀ (Fig. 6A). This confirms that purified PGHR shows activity on *B. subtilis* spore cortex peptidoglycan. When vegetative cell wall (peptidoglycan) from *B. subtilis* was used as a substrate for PgHR₂₄₋₂₄₀ protein, OD loss was not observed showing no enzyme activity (Fig. 6B).

Differential expression pattern analysis of PGHR in growth phases

The expression pattern of *pghr* was analysed at different growth phases (lag, mid-log, stationary, and decline) of *Rba. johrii*. The results showed no significant difference in the expression pattern until the stationary phase as compared to lag phase (Fig. 7). However, during the late stationary phase, the expression levels of *pghr* were up-regulated (~4.5-fold) and eventually down-regulated in the late decline phase. We do not find any differentiation in the cells from lag to stationary phase and also the increased expression of *pghr* at late stationary (Fig. 7). At the late decline phase, the *pghr* expression was decreased.

Discussion

This study provides evidence (Accession. no. WP069331589) of a Peptidoglycan Hydrolase in *Rba. johrii* that specifically cleaves the spore cortex peptidoglycan (sacculi) but unable to cleave the cell wall peptidoglycan. This protein has a spore cortex lytic enzyme (SleB) specific sequence motif EXRGE having alternating glutamate (E) residues (at positions 128 and 132) and catalytic residue (E128) present in PgHR of *Rba. johrii*. Further, PgHR has conserved hydrolase-2 superfamily (pfam07486) catalytic domain which is present in SleB type proteins (Jing et al. 2012).

In silico predicted secondary structure of PGHR was similar to the SleB of *B. cereus* with the conserve catalytic groove. Structural comparison of PGHR of *Rba. johrii* with SleB of *Bacillus* spp. showed the conserve catalytic glutamate residue and aromatic amino acids present within the catalytic groove responsible for binding and cleavage of spore cortex peptidoglycan (Fig. 4A) as reported in SleB proteins (Li et al. 2012; Jing et al. 2012). SleB enzymes cleave the spore cortex by specifically identifying the muramic- δ -lactam (MAL) residue present in the cortex peptidoglycan of spore and it is unable to cleave the intact cell wall (Popham et al. 1996). Three amino acids which play an important role in identification and binding to muramic- δ -lactam (MAL) was found to be conserved in the Sle protein of *Rba. johrii* (Fig. 4B). Overall bioinformatics analysis suggested that PgHR protein has spore cortex lytic enzyme like properties. SleB protein has peptidoglycan (PG)-binding domain in the N-terminal region, the role of this domain is insignificant, as the truncated protein without this domain did not affect the function of the protein (Heffron et al. 2011). Similarly, PgHR protein does not have peptidoglycan (PG)-binding domain but has the spore cortex lytic enzyme like properties.

The enzyme assay was also performed with the cell wall peptidoglycan as a substrate where SleR protein did not cleave this substrate (Fig. 6B). This assay implicates the association of the enzyme PgHR in cleaving the spore cortex and not the cell wall *in vitro*. Real-time expression profiling of *pghr* in *Rba. johrii* at different growth phases demonstrated that *pghr* expression was not significant between lag to stationary phase, while its levels spiked during the late stationary phase (Fig. 7). The expression pattern of SleR protein clearly indicates that this protein is not a prerequisite during early phases of development like in the peptidoglycan remodeling for cell division and growth (seen during the log phase). Further, it also indicates the role of PgHR during stationary phase which corroborates with sporogenesis. However, there is a need for further in-depth study to elucidate the exact function of PgHR protein in *Rba. johrii*.

Likewise, comparably in a gram-negative δ -proteobacteria *Myxococcus xanthus* forming spore-like fruiting bodies, a CbgA protein was identified as a homolog based on sequence similarity (35% identity and 57% similarity) to SpoVR protein of *B. subtilis* (Beall and Moran 1994). When CbgA protein was characterized in *M. xanthus* it was found to be a similar property protein (Tengra et al. 2006). Similarly, in this study we identified a new peptidoglycan hydrolase protein from *Rba. johrii* and further confirmed through *in silico*, *in vitro*, and expression profile-based characterization.

Conclusion

The available whole genome sequences are exploited for the discovery of new uncharacterized proteins. In this study, the bioinformatics analysis with current available tools, enzyme assay and expression study provides insights into a new peptidoglycan hydrolase from *Rba. johrii* that needs in depth functional study.

Declarations

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Conflicts of interest

The authors declare that there is no conflict of interest.

Ethical statement: Not applicable

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Figures

Figure 1

Protein domain and genetic features (A) Topology of PGHR protein showing signal peptide (Red region), hydrolase catalytic domain (grey box). (B) Promoter and gene properties of *pghr* from *Rba. johrii*. Promoter region (grey region) consist of -10 box and -35 box (red region) transcription start site transcribes the 5' untranslated (5'UTR) region (Blue region at 5') from start codon (ATG) protein is coded till the stop codon (TGA) (Pink region) after which is the 3' untranslated region (3'UTR) (Blue region at 3').

Figure 2

Multiple sequence alignment of full length PGHR protein from *Rba. johrii* with SleB protein of *B. cereus* ATCC14579 (SLEB_{BC_ATCC14579}), *B. cereus* M13 (SLEB_{BC_M13}), *B. cereus* MLY1 (SLEB_{BC_MLY1}), *B. anthracis* ATCC14578 (SLEB_{BA_14578}), *B. anthracis* Sterne (SLEB_{BA_sterne}), *B. anthracis* Ames (SLEB_{BA_ames}). Conserved amino acids are shown in red boxes, green asterisk mark represents the similar residues. The catalytic residue Glu is highlighted with red star mark and another Glu with blue star mark these alternate sequences are part of SleB specific EXRGE motif shown in an orange box. Blue line donates the catalytic domain of the proteins.

Figure 3

Structure prediction and conformation.(B) Ribbon structure of PGHR model based on structure of SleB from *B. cereus* (PDB code - 4f55.1). The N- and C- termini are labeled, α helices are in red color, β - sheets are in yellow color and other colors represent loops and turns. (C) Ramachandran plot analysis of PGHR of *Rba. johrii*.

Figure 4

Superimposition of PGHR structure. (A) Superimposition of PGHR (orange) of *Rba. johrii* and SleB (cyan) of *B. cereus* backbones showing the side chains of structurally conserved aromatic acid residues (Phe167, Phe209, Trp218, His231, Phe233) present in the catalytic groove along with the catalytic residue (Glu128) and conserved amino acids are written with the same colours (B) Superimposition of PGHR (cyan) of *Rba. johrii* and SleB (green) of *B. anthracis* backbones showing the side chains of structurally conserved residues (Tyr153, Gly164, replaced by same functional group amino acid and Trp218, shown in red colour, these amino acid are responsible for binding to muramic- δ -lactam (MAL)) and residues (Glu132, Arg147, Val148, Pro154, Phe167) are present in exact same location in the groove in both proteins.

Figure 5

Purification of recombinant PGHR protein of *Rba. johrii* expressed in *E. coli*. (A) Soluble overexpressed protein His6-SUMO- PGHR24-240 marked with black arrow, M- protein marker; kDa, S- supernatant, W1, W2 – washed eluates, 1-4 Lanes - Ni affinity eluates from 100 mM, 200 mM, 300 mM, 400mM imidazole. (B) M- protein marker; kDa, 1-DeSUMOylases cysteine protease digestion, 2 - Ni affinity eluate.

Figure 6

Enzyme activity of PGHR protein. (A) Spore sacculi as substrate using PGHR24-240 protein in enzyme reaction represented with the line with squares show consistent reduction in the OD while line with circles indicating control reaction in which substrate was not present and no OD loss is observed. (B) cell wall (peptidoglycan) as substrate when treated with the PGHR24-240 protein in enzyme reaction is represented with the line having triangles do not show any significant reduction in OD as compare to the control represented by line with circle here substrate was not present showing no OD loss. Data shown are averages for three independent reactions. Error bars represent 1 standard deviation of the mean.

Figure 7

Expression pattern of PGHR at different growth phases of *Rba. johrii* through qRT-PCR analysis shown in the graph. Bars represent mean standard deviation of three biological replicates.

Supplementary Files

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