Microbial Pathogenesis 102 (2017) 8–11

Contents lists available at ScienceDirect

Microbial Pathogenesis

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

Overexpression of heat shock GroEL stress protein in leptospiral biofilm

K. Vinod Kumar [a], Chandan Lall [a], R. Vimal Raj [a], K. Vedhagiri [b], C. Kartick [a], P. Surya [a], K. Natarajaseenivasan [c], P. Vijayachari [a, *]

[a] Regional Medical Research Centre (ICMR), WHO Collaborating Centre for Diagnosis, Reference, Research and Training in Leptospirosis, Port Blair 744101, Andaman and Nicobar Islands, India
[b] National Hub for Healthcare Instrumentation Development (NHHID), Centre for Biotechnology, Anna University, Chennai 600 025, India
[c] Bharathidasan University, Department of Microbiology, School of Life Sciences, Tiruchirappalli 620 024, India

ARTICLE INFO

Article history:
Received 9 August 2016
Received in revised form
24 September 2016
Accepted 14 November 2016
Available online 16 November 2016

Keywords:
Biofilm
Leptospira
Hsp
Planktonic
GroEL

ABSTRACT

Leptospira is the causative agent of leptospirosis, which is an emerging zoonotic disease. Recent studies on *Leptospira* have demonstrated biofilm formation on abiotic surfaces. The protein expressed in the biofilm was investigated by using SDS-PAGE and immunoblotting in combination with MALDI-TOF mass spectrometry. The proteins expressed in *Leptospira* biofilm and planktonic cells was analyzed and compared. Among these proteins, one (60 kDa) was found to overexpress in biofilm as compared to the planktonic cells. MALDI-TOF analysis identified this protein as stress and heat shock chaperone GroEL. Our findings demonstrate that GroEL is associated with *Leptospira* biofilm. GroEL is conserved, highly immunogenic and a prominent stress response protein in pathogenic *Leptospira* spp., which may have clinical relevance.

1. Introduction

Leptospirosis is an emerging zoonosis caused by pathogenic spirochetes belonging to the genus *Leptospira*. The disease is transmitted to humans through environmental surface waters contaminated by the urine of reservoir host and domestic animals, which are chronically colonized with *Leptospira*. It is also reported that leptospires can survive in nutrient deficient conditions in soil and water, for long periods [1]. However, not much is known about the mechanisms by which pathogenic leptospires persist in aqueous environments, chronically infected mammals and carrier hosts. It is widely accepted that bacteria can exist in two different modes of growth, the first being as single planktonic cells and the second as structured, multicellular communities known as biofilms [2]. Biofilm formation in *Leptospira* is a recently studied phenomenon and is demonstrated in environment and *in vitro* [3,4]. *Leptospira* biofilm may play a significant role, not only in environmental survival but also for successful infection and pathogenesis [5]. Similar to other bacterial biofilms *Leptospira* biofilm is a complex structural arrangement of bacteria that are enclosed in an extracellular polymeric substance (EPS) [3]. However the EPS of the leptospiral biofilm are yet to be studied.

Polysaccharides, proteins, nucleic acids, metal ions and other humic materials can be associated with the EPS matrix of the biofilms. Embedded cells within this EPS matrix can protect the bacteria from environmental stress and may also offer protection from the host immune responses [6]. A detailed understanding of the biofilm matrix composition especially proteins are critical for the rational understanding of numerous clinical and environmental implication associated with biofilms [7].

The architecture of leptospiral biofilms are rather well studied. However, the molecular mechanism of biofilm formation remains to be explored. Proteomic comparison of other bacterial biofilms has revealed the presence of several differentially expressed proteins. Heat shock proteins (HSPs) are the major and best-studied proteins known for their involvement in bacterial biofilm [8]. These HSPs are considered to be important in the pathology of various bacterial and parasitic infections. They are reported to protect pathogens against the hostile environment of host phagocytic cell [8]. In this study, an attempt was made to analyse the

* Corresponding author. Regional Medical Research Centre (ICMR), Post Bag No.13, Dollygunj, Port Blair 744101, Andaman and Nicobar Islands, India.
E-mail address: pblicmr@sancharnet.in (P. Vijayachari).

http://dx.doi.org/10.1016/j.micpath.2016.11.010
0882-4010/

comparitive proteomics of the proteins in planktonic and *Leptospira* biofilm.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Leptospira interrogans strain, Salinem, was originally isolated from human, while *Leptospira fainei* is of animal origin. All strains were obtained from national reference centre for leptospirosis at the Regional Medical Research Centre, Port Blair, India. These strains were sub-cultured at 30 °C in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium with fatty acid supplements (Bovine Serum Albumin + Tween 80) with 1% BSA and were maintained with periodic sub culturing every 7 days.

2.2. Biofilm formation

The biofilm formation assay was performed for the two strains, *L. Interrogans* Salinem and L. *fainei* But-6, in EMJH liquid medium, as per the methodology described earlier [9]. Briefly, the biofilms were allowed to form in U-bottom 96-well (polyvinyl plate) tissue culture by incubating at 30 °C for 10 days, without shaking. Every 24 h the growth medium was discarded and freshly added. Fresh EMJH medium was added to the wells as a control. Each well was washed thrice with phosphate-buffered saline (PBS) under aseptic conditions to eliminate unbound bacteria. The experiment was repeated at least thrice for the reproducibility. The ability of these strains to form biofilm was determined as per the crystal violet staining method, as described by O'Toole [10].

2.3. Microscopic examination

Leptospira strains were grown in the EMJH medium to approximately 2×10^8 cells mL^{-1} (without shaking). Sterile glass slides (76 × 26 mm, Hi-Media) were submerged (20 mm) in 50 ml of leptospiral cell suspension in EMJH growth medium (initial concentration of 2×10^5 cells mL^{-1}) and incubated at 30 °C for 7–12 days. After incubation, slides were carefully removed and rinsed two twice with PBS (pH- 7.4). One side of the slide was wiped with 70% ethanol and the attached cells were observed on the other side by Dark Field Microscope (DFM, Zeiss AXIO SCOPE A.1, Germany) [9].

2.4. SDS-PAGE and immunoblotting of biofilm and planktonic cells

L. interrogans Salinem biofilm was grown in 5 ml EMJH liquid medium in a wide mouthed glass tubes with starting inoculum size of ~10^5 cells ml^{-1} (1:10, vol/vol), incubated at 30 °C for 4–7 days without shaking. Biofilm formation was achieved at air liquid interphase on the walls of the tube, the unbounded cells were aspirated and attached cells were gently rinsed with PBS (pH- 7.4). Biofilm was collected by scraping, using sterile plastic loop. Detachment of the leptospires from the biofilm was carried out as described earlier [9]. Further, ethanol insoluble EPS was precipitated along with leptospiral cells overnight at 4 °C with 3 vol of ice-cold ethanol. The content was centrifuged at 15000 rpm for 30 min and the pellet was subjected to protein estimation and further solubilised in 2X SDS-PAGE sample buffer with 1% 2-mercaptoethanol. Similarly, in planktonic (unbound cells) leptospiral cells were centrifuged at 12,000 g for 10 min. The cell pellet was washed twice in 5 mM $MgCl_2$-phosphate buffered saline (PBS) and protein was estimated using BCA method. The protein concentration was optimised to proteins from biofilm state and solubilised in 2 X SDS-PAGE sample buffers. After boiling, the content was loaded on a 10% polyacrylamide gel with pre stained protein marker as a standard (Bio-rad, USA) and electrophoresed [11]. The resulting lysates of the two phenotypes were utilised as antigens. Pooled sera of acute phase leptospiraemic patient's (Microscopic Agglutination Test positive and IgM ELISA positive sera) were used as primary antibody for IgM recognition in immunoblotting as per the methodology described elsewhere [12,13].

2.5. Identification of over expressed proteins by MALDI – TOF

The target protein band was excised from SDS-PAGE and mass spectrometry analysis was carried out. The protein bands were digested following the standard protocol [14]. Peptide mass fingerprint was measured on an Opti-TOF 384 well insert (Applied Biosystems/MDS Sciex, Foster City, CA) with 0.3 μl of 5 mg ml^{-1} alpha-cyano-4-hydroxycinnamic acid (Aldrich, St. Louis MO) in 50% CH_3CN and 0.1% trifluoroacetic acid. Crystallized samples were washed with cold 0.1% trifluoroacetic acid and were analyzed by an Applied Biosystems 4800 MALDI TOF/TOF Proteomics Analyzer. An initial MALDI MS spectrum was acquired for each spot (400 laser shots per spectrum) and a maximum of 15 peaks, with a signal-to-noise ratio of greater than 20 were automatically selected for MS/MS analysis (1000 shots per spectrum) by post-source decay or by collisionally-induced dissociation using air at a pressure of $5e^{-7}$ Torr. Peak lists from the MS/MS spectra were submitted for database similarity searching using Protein Pilot (Applied Biosystems) Ver. 2.0, Rev. 50861. The involvement of the identified proteins in the molecular function and biological process were assigned according to the gene ontology database (http://www.geneontology.org) and the Swiss prot/uniprot database (http://beta.uniport.org).

2.6. RNA extraction and cDNA synthesis

Total RNA from 1 ml of leptospiral biofilm and planktonic cells from *L. interrogans* strain Salinem was extracted using Trizol reagent (Invitrogen, USA) in accordance with manufacturer's instructions. The air dried RNA pellets were re-suspended in diethyl pyrocarbonate water. RNAase free DNAase (Fermentas) was used to remove the genomic DNA content and the concentration was estimated at optical density at 260/280 nm. 200 ηg of total RNA was reverse transcribed using EuroScript RT kit (Euorgentec, Belgium). After RT, 0.25 U of RNAase (Fermentas) was added and the mixture was incubated at 37 °C for 20 min to remove any residual RNA from the reaction mixture. The cDNA was stored in −20 °C until use.

2.7. Real-time quantitative (qRT- PCR)

The real time quantitative RT- PCR for the cDNA targeting *GroEl* gene was performed, using 16sRNA gene as calibrator. Primers (Table 1) were designed using primer3 (http://frodo.wi.mit.edu/primer3/). The qRT- PCR was performed for the final volume of 25 μl which contained 100 nm each of forward and reverse primer, 1X reaction buffer (2.5 mM dNTPs including dUTP, 0.25U Meteor Taq DNA polymerase, 4 mM $MgCl_2$ and SYBR green I). Samples in duplicate were kept in 96 well plates and amplified in an automated real time PCR machine (7500, Applied bio-systems). The PCR conditions were followed, viz, 95 °C for 5 min, 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. The melt curve was performed at a holding step of 50 °C. Quantification was carried out using the comparative cycle threshold (CT) and demonstrated relative transcription or the ($2^{-\Delta\Delta Ct}$) n-fold difference relative to calibrator gene.

3. Results and discussion

The biofilms of *L. Interrogans* Salinem and *L. fainei* But-6 were