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Full Length Research Paper

Expression of chitinase and chitin binding proteins (CBP's) by *Listeria monocytogenes* J0161 in biofilm and Co-culture broths

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Listeria monocytogenes **is a dreaded pathogen colonizes to form biofilm in the natural environments and in food processing facility. Biofilm state makes the bacterium resistant to antimicrobial agents. *L. monocytogenes*, an intracellular parasite also colonizes the mammalian epithelial cells in the gut. Chitinase and other chitinolytic proteins expressed by *L. monocytogenes* contribute to the colonization of the bacterium in the intestine. It has been reported that chitinase and chitin binding proteins are essential for attachment of the bacterium to the epithelial cells. In this study, we have pursued to analyze, if chitinase and chitin binding proteins play a role in biofilm formation and the expression of these proteins otherwise in a co-culture state. We report the expression pattern of these proteins, which supports our claim that chitinase and chitin binding proteins have a role to play in biofilm formation.**

Key words: *Listeria monocytogenes*, chitinase, microarray, biofilm, co-culture, gene expression.

INTRODUCTION

Listeria monocytogenes is a Gram-positive saprophyte that is found in soil, water, and decaying vegetation (Chaturongakul et al., 2008; Czuprynski, 2005; Freitag et al., 2009). If consumed by a susceptible host, *L. monocytogenes* can adopt a pathogenic lifestyle and lead to fatal infections (Allerberger and Wagner, 2010; Barbuddhe and Chakraborty 2009). While significant attention has focused on the gene products of *L. monocytogenes* that specifically contribute to sustenance within host cells, relatively less is known on the genes that contribute to bacterial life outside the infected host.

The role of chitinases in the growth of *L. monocytogenes* in environmental conditions is well documented (Leisner et al., 2008). In fact, chitin binding proteins (CBP's) and chitinases are hypothesized to contribute to adherence and sustenance of some bacterial species in the environment (Kirn et al., 2005).

Chitinases are glycosyl hydrolases that catalyze the hydrolysis of the 1,4-beta-linkages in chitin. Chitinases are found in a wide range of species from all kingdom of life, which includes bacteria, viruses, higher plants as well as mammals. Chitinases have diverse physiological and biological roles depending on the organisms. They also play an important part in maintaining the chitin balance in the natural environment. In addition, to the functions of regulating cellular growth and proliferation, chitinases also play an important biological role in defending the host cells or by increasing interaction with pathogens due to their ability to hydrolyze and/or interact with chitin present in pathogens or host cells. In *Vibrio cholerae,* chitinase have been reported to contribute to virulence and pathogenicity. The role of bacterial CBP's in the processes of pathogenesis (for example, adhesion, colonization) to the host cells has been reported (Kirn et

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Abbreviations: CF, Cystic fibrosis; **TSB,** tryptone soy broth; **PBS,** phosphate buffer solution; **DNA,** deoxyribonucleic acid.

al., 2005). DebRoy et al. (2006) reported that deletion of the *chiA* gene expression in *L. pneumophila*, reduced the bacterial persistence in the lungs of infected mice. The study strongly suggests that chitinase enhances bacterial survival in mammalian host cells. Furthermore, deoxyribonucleic acid (DNA) microarray analysis by Salunkhe et al. (2005) revealed that the expression of CBP in *Pseudomonas aeruginosa* was significantly up-regulated in the cystic fibrosis (CF) associated strains as compared to the laboratory strain, suggesting the potentially pathogenic role of CBP as a virulent factor of the *P. aeruginosa* infection in CF patients (Salunkhe et al., 2005).

The chitinolytic activity is through the expression of putative 'CBP's, chitinase-A and chitinase-B. Recently it was observed that *L. monocytogenes* encodes two chitinases, *chi-A* and *chi-B*, that can hydrolyze Chitin (Leisner et al., 2008). *Prf A*, a virulence regulator and *lhr A*, a global regulator, have been reported to regulate the expression of chitinolytic gene products in *L. monocytogenes* (Nielsen et al., 2011). Recent reports state that *chi-A* and *chi-B* both contribute to the chitinase activity and in turn to the growth of *L. monocytogenes* in liver and spleen of mice (Chaudhuri et al., 2010) with the background of significant reports on the role of chitinase and CBP's in the survival and sustenance and the pathogenesis of some pathogens including *L. monocytogenes*, our objective was to understand the expression pattern of chitinolytic gene products and their regulators under different growth conditions. We report the expression pattern of chitinase, CBP's and *prf A* (positive regulatory factor A) as their regulator in biofilm and co-culture broths of *L. monocytogenes*, in comparison to *L. monocytogenes* pure culture in broth.

MATERIALS AND METHODS

Bacterial Strains and culture conditions

L. monocytogenes j0161 was chosen for our study, since its complete annotated transcriptome is available at the *L. monocytogenes* database of the Broad Institute. The other criteria that helped us choose this strain is that the functional annotation of this strain's genome was of very good standards with the highest percentage (78.5%) of the genes annotated (2335 of 2973) with definitive functions. The *L. monocytogenes* strain was obtained from the Agriculture Research Services (ARS), United States Department of Agriculture (USDA). *Bacillus subtilis* ATCC 11774 was used in the co-culture experiments.

Microarray studies

A pure culture of *L. monocytogenes* J 0161 was grown in two sets, in broth as well as biofilm using tryptone soy broth (TSB) as the medium for growth. In the first set, *L. monocytogenes* was grown as monoculture for 24 h at 37°C, while in the second set; the strain was grown in the presence of *B. subtilis* for 4, 12 and 24 h in different tubes individually. Similarly, pure culture biofilm were also grown on a set of three different slides, for studying the gene expression at 4, 12 to 24 h of incubation, respectively.

Deoxyribonucleic acid (DNA) extraction and evaluation

Cells were pelleted after (i) 4, 12 and 24 h of incubation as mixed cultures in broth, (ii) 24 h of pure culture in broth and (iii) 24 h of mixed biofilm. Pelleted cells were washed thrice with phosphate buffer solution (PBS). The cells were then further processed for RNA extraction using Ribo Pure-Bacteria Kit (catalog no. 1925; Ambion) according to the manufacturer's instructions. The concentration of the RNA extracted was evaluated using Bioanalyzer (Agilent; 2100); while the purity of the RNA extracted was determined using the standard procedure for the same by measuring A_{260} and A_{280} on a Nanodrop Spectrophotometer (Thermo Scientific; 1000).

Probe and microarray slide design

Unique probes for hybridization were designed for the 2973 transcripts identified, annotated and available in the database of the Broad's Institute for *L. monocytogenes j0161*. Considering the co-culture condition, BLAST was performed against *L. monocytogenes J0161* and *B. subtilis* to eliminate the possible cross-hybridizing probes with the transcripts within *L. monocytogenes* and *B. subtilis.* Further, Agilent Custom Microarray Gene Expression *Listeria monocytogenes* 8×15 k (AMADID: 030831) consisting of 15,000 probes of 60 mer length, designed by Genotypic Technology Pvt. Ltd, was used for the Microarray experiment.

Deoxyribonucleic acid (DNA) labelling, amplification and hybridization

Poly (A)-tails were added to the 3'-end of RNA by using A-plus Poly (A) polymerase tailing kit (Epicentre Biotechnologies). The samples were then labeled using Agilent Quick Amp Kit PLUS (Part number: 5190-0442). 500 ng of polyadenylated RNA was reverse transcribed using oligodT primer tagged to T7 promoter sequence. Complementary deoxyribonucleic acid (DNA) thus obtained was converted to double stranded cDNA in the same reaction. Further the cDNA was converted to cRNA in the in-vitro transcription step using T7 RNA polymerase enzyme and Cy3 dye was added into the reaction mix. During cRNA synthesis Cy3 dye was incorporated into the newly synthesized strands. cRNA obtained was cleaned up using Qiagen Rneasy columns. Concentration and amount of dye incorporated was determined using Nanodrop. Samples that pass the QC for specific activity were taken for hybridization. 600 ng of labeled RNA were hybridized on the array.

Hybridization, scanning and data analysis

Post amplification, the cRNA was subject to hybridization using the Gene Expression Hybridization kit (Part Number 5188-5242; Agilent) in Sure hyb Chambers (Agilent) at 65°C for 16 h. Hybridized slides were washed using Agilent Gene Expression wash buffers (Part No: 5188-5327). The hybridized, washed microarray slides were then scanned on a G2505C scanner (Agilent Technologies) and Images were quantified using Feature Extraction Software (Version-10.5.1.1, Agilent). The extracted raw data was analysed and normalized using GX Version 11.0 software from Agilent. Normalization of the data was done in Gene Spring GX using the 75^{th} percentile shift (Percentile shift normalization is a global normalization, where the locations of all the spot intensities in an array are adjusted. This normalization takes each column in an experiment independently, and computes the n^{th} percentile of the expression values for this array, across all spots (where n has a range from 0 to 100 and n=75 is the median). It subtracts this value from the expression value of each entity) and normalized to specific

Table 1. Reference to the list of genes transcripts identified for the study and Log_2 fold expression values at different time intervals in co-culture broth and biofilm state.

S/N	Gene ID (*L. monocytogenes* J0161)	Gene description
1	LMOG_01358T0	Chitinase
2	LMOG_01507T0	Chitinase B
3	LMOG_02397T0	Chitin binding protein
4	LMOG_03055T0	Virulence regulatory factor *prfa*
5	LMOG_00418T0	Host factor *hfq* family protein

control samples. Significant genes up and down regulated showing one fold and above within the samples with respect to control sample were identified. Differentially regulated genes were clustered using hierarchical clustering based on Pearson coefficient correlation algorithm to identify significant gene expression patterns [Clustering algorithm measures the similarity (difference) between genes or conditions] Gene Spring GX 11 Software. The fold values shown are log base 2 normalized values.

Pathway annotations

All the pathway and gene ontology function data for available strains of *L. monocytogenes* and the protein sequences for available pathway data were collected from Uniprot. Transcript sequences for *L. monocytogenes* J0161 were BLASTed against the protein database. All the significant genes showing hits greater than 90% identity were selected for pathway annotation.

Microarray data accession number

The Microarray data have been deposited and made available at the Gene Expression Omnibus database under the accession number GSE27936 (www.ncbi.nlm.nih.gov/geo).

RESULTS

Microarray studies on the gene expression of *L. monocytogenes* J0161 in different growth states were carried out. The transcriptome of the strain consisted of 2974 transcripts. In this study we discuss the expression of chitinase related genes only. Some of the regulatory genes that have been reported to have a regulatory role in the expression of chitinase and other chitinolytic genes have also been considered (Table 1). However, only few of the known regulatory transcripts were annotated in the strain J0161 and the same have been considered. *prf A* and *hfq* family protein are the regulatory factors that have been included in the study.

Chitinase and chitin binding proteins (CBP) expression in co-cultured broth

The strain J0161 of *L. monocytogenes* as described earlier was co-cultured along with *B. subtilis* in broth. After fixed intervals of 4, 12 and 24 h of growth, the expression pattern was observed (Figure 1). Chitinase (LMOG_01358T0) showed an ascending pattern of up-regulation by time. From 0.87 Log_2 fold variation at 4 h of growth to 3.12 at 24 h of growth. Similarly, the *prf A,* a regulatory factor (LMOG_03055T0), known to regulate chitinase expression, showed an ascending form of expression. The CBP (LMOG_02397T0) was up-regulated only at the 4^{th} hour of growth. It showed a descending pattern of expression over time.

Chitinase-B, the other chitinolytic protein was continuously down-regulated, while the transcript of *Hfq* family protein (LMOG_00418T0) continued to show up-regulation from 4 to 24 h of growth.

Chitinase and chitin binding proteins (CBP) expression in co-cultured biofilm

The strain J0161 was grown as biofilm as described earlier. The expression pattern of *L. monocytogenes* strain at fixed time intervals 4, 12 and 24hrs were studied (Figure 2).

Chitinase (LMOG_01358T0) was up-regulated only at the 4^{th} hour of growth as biofilm, after which the expression was down-regulated. The expression of CBP (LMOG_02397T0) was up-regulated at all time intervals studied. The *prfA,* (LMOG_03055T0), also showed up-regulated expression only at the 4^{th} hour of biofilm growth, similar to that of chitinase (LMOG_01358T0). Chitinase-B (LMOG_01507T0) was continuously down-regulated and the transcript of *Hfq* family protein (LMOG_00418T0) was down-regulated over time.

DISCUSSION

Attachment and adherence are preliminary prerequisite stages in biofilm formation. The mechanism of biofilm formation with respect to individual bacterial species has been an unsolved riddle for a long period. Biofilm formation by pathogens is perceived as a major risk. Understanding the molecules and their mechanism for biofilm formation is essential. Therefore, we carried out a gene expression study of *L. monocytogenes* (Strain J0161) in order to decipher the contributions of specific

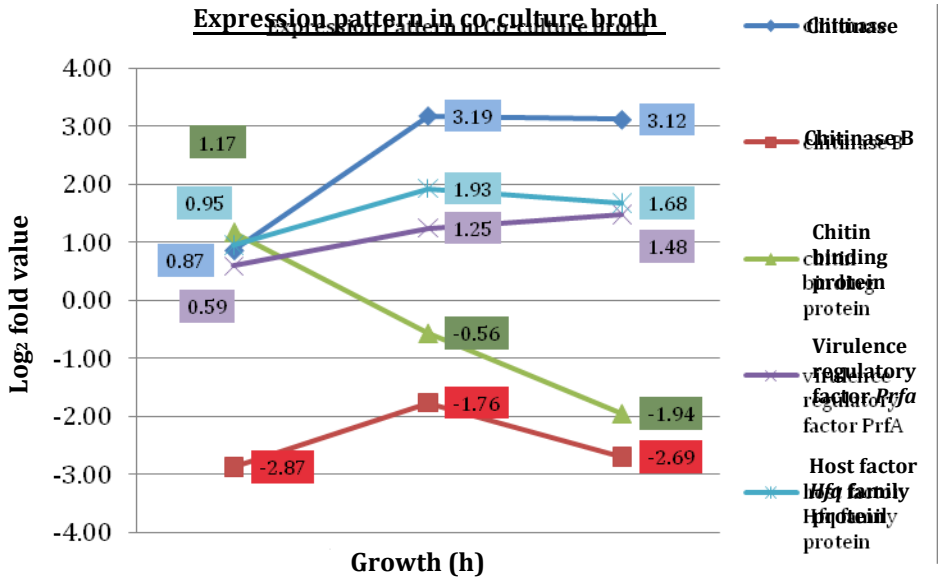

Figure 1. Expression pattern of chitinase and associated genes in co-cultured broth.

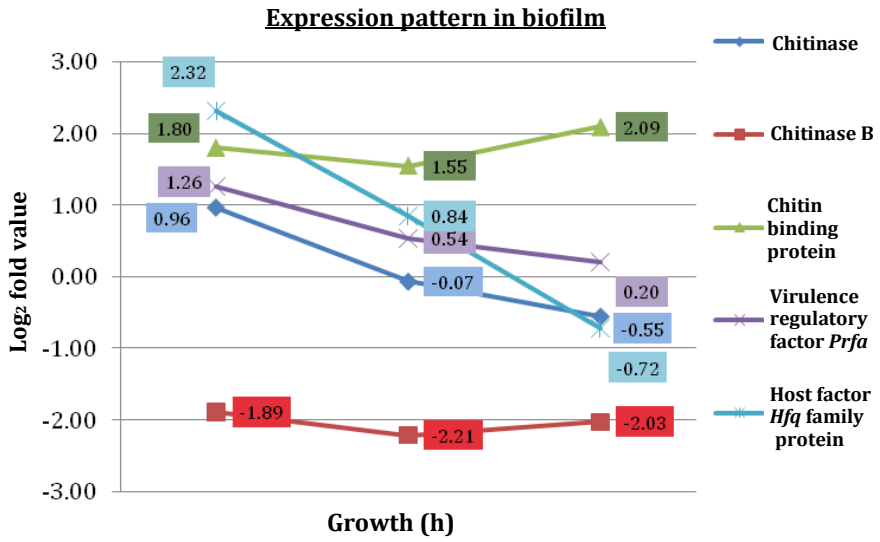

Figure 2. Expression pattern of chitinase and associated genes in biofilm.

molecules involved in biofilm formation. Leisner et al. (2008) in discussing the chitin hydrolysis by Listeria sp., concluded that it will be of considerable interest to understand chitinolytic activity in support to growth and survival of *L. monocytogenes* in the environment. Further, Kirn et al. (2005) had discussed the role of GbpA (a GlcNAc, sensitive chitin-binding protein) as a colonization factor. They have discussed the importance of GbpA for not only intestinal colonization but also for colonization in the environment. In their report they state that, by mutating the wild type strain of *V. cholerae*, at the *gbpA* promoter region, the strain was deficient in colonizing the intestinal epithelial cells. They also stated that, GbPA affects the binding of the bacterium in environmental conditions. With the background of earlier discussions on the role of chitinase, in this study, we report and discuss the expression pattern of Chitinase and its related proteins to suggest the probable role of chitinase and CBP's in the biofilm formation.

We report that, concurrent to the reports by Kirn et al. (2005), the expression of chitinase and CBP's in *L. monocytogenes* was typically at the initial stage of biofilm formation. The three annotated gene transcripts of chitinase and chitin binding proteins were up-regulated at the 4th hour of the biofilm formation as compared to the broth culture of *L. monocytogenes.* However, chitinase-B

remained down-regulated. Though we could not ascertain the cause for the down-regulation of chitinase-B, we observed that the *Prf A*, a virulence regulator gene that has been reported to regulate the expression of chitinases in *L. monocytogenes* (Nielsen et al., 2011), is also over expressed specifically at the 4th hour of biofilm formation. Further the expression pattern of chitinase and CBP, reduced concurrently with the expression of *PrfA* at the 12th and 24th hour of biofilm formation. These observations significantly show that chitinase and CBP might play a definite role in the initial stage of the biofilm formation. They probably contribute to the attachment and adherence of the bacterial cells to an abiotic surface. Furthermore, it has been noted that the abiotic surface used in our study for biofilm growth were plain glass slides immersed in tryptone soy broth (TSB – HiMedia-M011). CBP (GbpA), as reported earlier bind to chitinous surfaces. Our observation signifies that, if CBP or chitinase were to play a role in attachment, then they could also bind to non-chitinous surfaces. Although it is difficult to say if chitinase and CBP contribute to attachment for biofilm formation by *L. monocytogenes* without performing a mutation (knock-out) studies, previous reports and this study put together can be stated as an evidence to suggest the significance of chitinase, CBP and *PrfA* in the initial stage of biofilm formation.

It is not a veiled fact that, unlike the lab grown cultures, microbes in their natural environment seldom exist in pure culture. Therefore, we also carried out a gene expression study of *L. monocytogenes* (strain-J0161) in co-culture state with *B. subtilis*, to understand the difference in the expression pattern. Here, we report a significant variation in the expression of chitinase and CBP by *L. monocytogenes* in co-culture state. The CBP was down regulated after the 4th hour of growth in this state and the chitinase and the *PrfA* were concurrently upregulated. The down regulation of CBP in the broth culture state and the up-regulation in biofilm signifies the role of CBP in biofilm formation specifically.

In addition, we also report that contrary to the previous reports on the repression of chitinase expression in the presence of glucose (Leisner et al., 2008), in this study, though the medium used (TSB) for biofilm formation contained glucose as dextrose, the expression of chitinase and CBP was not repressed.

The up-regulated expression of chitinase in broth culture, and gradual regulation in biofilm could be attributed to regulation by *PrfA* (a positive regulator factor) that has already been reported to regulate expression of chitinase in *L. monocytogenes* (Nielsen et al., 2011). Furthermore, it has been reported that quorum sensing in *V. harveyi* negatively regulates chitinase expression (Defoirdt et al., 2010), therefore it could be hypothesized that quorum sensing that is known to be the basis of complex biofilm formation, as in *V. harveyi* could be down regulating expression of chitinase by time.

It is to be noted that the up-regulation of chitinase was observed in 'co-cultured' broth. It has been reported that, *L. monocytogenes* can digest the cell walls of the microflora of bacteria and obtain carbon and energy (Premaratne et al., 1991). Considering this report, we could assume that the presence of *B. subtilis* in the medium stimulates a competitive mode of life in *L. monocytogenes*, in which chitinase was up-regulated in order to digest the cell wall of the competing population of *B. subtilis.*

Chitinase-B, another chitinolytic protein remained down-regulated in both biofilm and co-culture broth. This gives us reason to hypothesize that regulation of chitinases encoded in *L.monocytogenes* are independent of each other. The hypothesis on the role of Chitinase in biofilm formation needs to be further understood by induced mutation or knockout studies.

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