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EXPRESSION OF VIRULENCE GENES BY *LISTERIA MONOCYTOGENES* J0161 IN NATURAL ENVIRONMENT

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ABSTRACT

Majority of studies concerning the gene expression of *Listeria monocytogenes* have been done on pure culture states. Our objective was to study *L.monocytogenes* in a co-cultured state and to understand if microbes in their natural state of existence are different in their expression than that of the purely cultured lab grown forms. For a long period discussions have been on the expression of *prfA*, (which is a virulence gene regulator) in a mammalian host and its role in causing the switch from a saprophytic to pathogenic form of *L.monocytogenes*. We, in this paper for the first time report the expression of *prfA* and other virulence genes by *L.monocytogenes* under different extracellular conditions, and also as a pure culture biofilms, that is different from the previous reports. We also report that the expression of *prfA* seems to vary considerably when co-cultured with *Bacillus subtilis.*

Key words: *Listeria monocytogenes,* prfA, Virulence, Microarray, Biofilms, Co-culture, Gene Expression.

INTRODUCTION

Gram positive *Listeria monocytogenes* is an oppurtunistic pathogen that causes severe food-borne illness, commonly known as Listeriosis (8), and ranks as one of the major causative factors for the high fatality rate amongst all food-borne bacterial infections. Occurence of Listeriosis has been sporadic in Asian countries. Of late, there has been an increase in the occurrence of human Listeriosis, including food contamination in India (1,2,16,17,22).

Given the high fatality rate caused by Listeriosis, considerable research has been devoted towards understanding the pathogenicity of *L.monocytogenes* However, our understanding of the bacterium in its natural environmental niche as flagellum propelled free-living forms, or as part of a biofilm community (13), is poor. One of the major areas of concern with respect to L. monocytogenes growth dynamics is the transformational phenomena of *L.monocytogenes* from saprophytic to pathogenic and virulent form. Several groups (8, 13, 21, 25.)have reported and explained the role of one particular gene namely *prfA* coding for the *P*ositive *R*egulatory *F*actor *A* (*PrfA*)in this transformation of *Listeria sp*. Further, Lemon, Freitag & Kolter (13) have clearly demonstrated that the expression of *prfA* is dependent on the stage(s) of *Listera*

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life cycle and its environment.

prfA regulates the expression of virulence genes, namely Internalin A & B (*inl A & B*), Listeriolysin O (*LLO*) and Phospholipases (*plcA & plcB)*(12), all of which facilitate the intracellular growth and spread of the bacterium within a mammalian host. *prfA* also induces the expression of a bile salt hydrolase (encoded by *bsh*) as well as a bile exclusion system, both of which contribute to bacterial survival in the intestine and attribute to the pertinacity of *L. monocytogenes* in the gall bladder (3). Strains that lack a functional *prfA* are considered redundant in expressing virulence (4). Mechanism of expression and regulation of *prfA* and its functional relevance to the transformation of the bacterium from saprophytic to pathogenic and virulent forms has also been studied extensively (5,10,13,17,19,20) Apart from the life style dependant expression, *prfA* expression is also temperature dependant and an RNA thermo-sensor has been linked to the control of *prfA* (11). As with most other bacterial species, *L.monocytogenes* will seldom exist as a monoculture species. The gram-positive endospore forming *Bacillus subtilis,* is very-well characterized organism, ubiquitous in its occurrence in different environments (26); it is a known probiotic (10) and potent antibiotic producer (27) proven to be effective against many pathogens (19). We present here the results of a pilot study towards understanding the expression dynamics of the virulence gene *prfA* of *L.monocytogenes* in the presence of *B.subtilis* in its natural extracellular environment in broth and in biofilms.

MATERIALS AND METHODS

Bacterial Strains and culture conditions

The strain J0161 of *Listeria monocytogenes* added to the objective of our study since its complete transcriptome was available and annotated at the *Listeria monocytogenes* database of the Broads Institute. Further, amongst the strains that were annotated and for which the transcriptome was available, strain J0161 had the highest percentage (78.5%) of annotated genes (2335 of 2973 gene transcripts).

Strain J0161 *L.monocytogenes* strain was obtained from the Agriculture Research Services (ARS), United States Department of Agriculture (USDA). As per the source (USDA) the *L.monocytogenes* J0161 is a human isolate. *Bacillus subtilis* ATCC (American Type Culture Collection)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 biofilms using Trytone Soy Broth (TSB) as the medium for growth. In the first set, *L.monocytogenes* was grown as a single culture for 24hours at 37^0 C (Celsius), while in the second set, the strain was grown in the presence of *Bacillus subtilis* for 4, 12 and 24 hours in different tubes individually. Similarly, pure culture biofilms were grown on a set of three different slides, for studying the gene expression at 4, 12 to 24 hours of incubation, respectively. Co-cultures of *L.monocytogenes* and *B.subtilis* were incubated for 24 hours in the second set of slides to allow formation of biofilms.

RNA extraction and evaluation

Cells were pelleted after (i) 4, 12 and 24 hours of incubation as co-cultures, (ii) 24 hours of mono-culture and (iii) 24 hours of co-culture biofilm. Pelleted cells were washed with phosphate buffer solution. 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 and purity of the RNA extracted were evaluated using Bioanalyzer (Agilent; 2100) and absorbance readings at 260nm and 280nm were performed using the Nanodrop Spectrophotometer (Thermo Scientific; 1000). 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

Agilent Custom Microarray Gene Expression *Listeria monocytogenes* 8x15k (AMADID: 030831) designed by Genotypic Technology Pvt. Ltd was used for the experiment. The Array consists of 15,000 probes of 60 mer length. The array contains total number of 2973 unique transcripts, which are obtained from the *L.monocytogenes* database of the Broad's Institute. 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.*

RNA Labelling, Amplification and Hybridization

Poly (A)-tails was 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). 500ng of polyadenylated RNA was reverse transcribed using oligodT primer tagged to T7 promoter sequence. cDNA (complementary DNA) thus obtained was converted to double stranded cDNA in the same reaction. Further the cDNA was converted to cRNA (Complementary RNA) 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 passed the QC for specific activity were taken for hybridization. 600ng 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 65º C for 16 hours. 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). Feature extracted raw data was analyzed using Gene Spring GX Version 11.0 software from Agilent. Normalization of the data was done in Gene Spring GX using the 75th 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 0-100 and n=75 is the median). It subtracts this value from the expression value of each entity.) and normalized to Specific 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].

Pathway Annotations

All the Pathway and Gene ontology function data for available strains of *Listeria monocytogenes* and the protein sequences for available pathway data were collected from Uniprot. Transcript sequences for *Listeria monocytogenes* J0161 were BLAST 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

Culture conditions and Biofilm formation

24 hours pure broth culture of the strain was used as reference for comparison the gene expression of the bacterium cultured in different conditions in broth and as biofilms. The strain of *L.monocytogenes* J0161 was cultured as broth co-culture with *B.subtilis* (ATCC 11774) for 24hours, mono-culture biofilm for 24hours and co-culture biofilm with *B.subtilis* (ATCC 11774) for 24hours.

As broth co-culture with *B.subtilis,* our objective was to understand how *L.monocytogenes* J0161 responds to the presence of another bacterium in its niche. Given that bacterial cells in a biofilm differ from the broth or colony grown cells and also that biofilm formation is a multistage process, we examined differential gene expression at different stages (time intervals of 4, 12 and 24 hours) of the biofilm growth. We report here the results of the same, with particular focus on (i) the virulence genes at different stages of biofilm formation, and (ii) the expression pattern in broth in the presence of *B.subtilis,* as compared to the 24hour broth culture.

Gene expression profile of *L.monocytogenes* in monoculture biofilm and in co-culture broth and biofilm with *B.subtilis*

There are 2974 reported genes from the genome of *L.monocytogenes*, of which 150 genes were up-regulated in monoculture biofilm as compared to 24hours pure culture broth. Of these 150 genes, we observed a statistically significant > 2.0 fold up-regulation of more than 20 genes.

On the other hand, when *L.monocytogenes* was co-cultured with *B. subtilis*, 469 genes out of the 2974 gene transcripts were up-regulated in broth, while 515 transcripts of *L.monocytogenes* were up-regulated in biofilm. While the up-regulation of 188 genes and 234 genes were specific to co-culture broth and co-culture biofilms respectively, 281 genes were commonly up-regulated in the presence of *B.subtilis* under both conditions (Fig. 1).

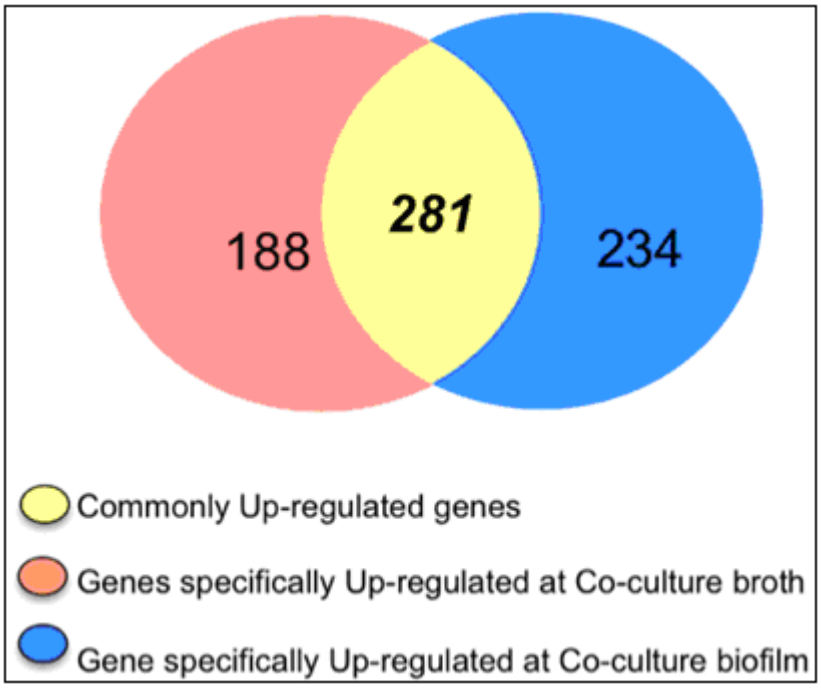

Figure 1. Depiction of genes up-regulated in the co-culture broth & biofilm.

Identification of Core Virulence genes in *L.monocytogenes* J0161

Annotated transcriptome of the Strain J0161 of *L.monocytogenes* was obtained from the database of Broad Institute web page (http://www.broadinstitute.org/annotation/genome/listeria_group). About 72 % of the gene transcripts of the strain were annotated of which, 10 are from core virulence genes could be identified, including *prfA*. (Table 1).

Table 1. List of Core Virulence Genes Identified and Annotated by Broad Institute.

S.no	Gene Description	Annotation
1	LMOG_03051T0	*Actin Assembly ActA*
2	LMOG_00341T0	*Hemolysin A*
3	LMOG_01377T0	*Hemolysin B*
4	LMOG_02646T0	*Internalin A*
5	LMOG_02645T0	*Internalin B*
6	LMOG_02173T0	*Phospholipase*
7	LMOG_03050T0	*Phospholipase C*
8	LMOG_03054T0	*PlcA*
9	LMOG_03055T0	*prfA*
10	LMOG_03053T0	*Thiol-activated Cytolysin*

Expression of *prfA* in monoculture biofilms, co-cultured broth and biofilms with *B.subtilis*

prfA (LMOG_03055T0) by *L.monocytogenes* in broth co-cultured with *B.subtilis* was up-regulated. The expression at 4hours of incubation was observed to be marginally above neutral regulation with 0.59 fold more of expression , however after 12 hours of incubation the expression increased to more than 1.0 fold which persisted even after 24hours of incubation (Fig. 2) (Table 2, 3 and 4). Though *prfA* was up-regulated in co-cultured broth, it was significantly Down-regulated to -0.39 fold expression in biofilms with *B.subtilis*.

Further, as reported earlier by Lemon *et al* (12) who worked on monoculture biofilms, *prfA* was indeed up-regulated in biofilms; however the up-regulation was observed only during the initial 4 hours of biofilms culturing.

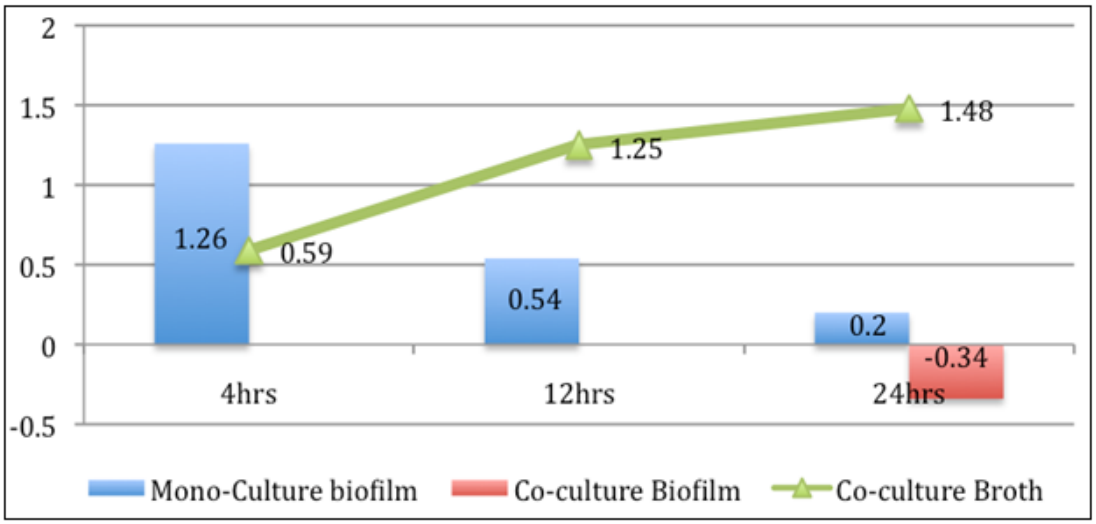

Figure 2. Expression of *prfA* in monoculture biofilms, co-cultures broth and biofilms with *B. subtilis.*

Table 2. List of Virulence Genes Up-regulated in Co-cultured Broth

S.no	Gene Description	Annotation	Fold Value Expression in Co-culture Broth at (hours)		
			4	12	24
1	LMOG_00341T0	*Hemolysin A*	0.08	0.89	2.03
2	LMOG_02645T0	*Internalin B*	0.27	2.14	1.04
3	LMOG_02173T0	*Phospholipase*	-0.21	1.83	3.00
4	LMOG_03055T0	*prfA*	0.59	1.25	1.48

Table 3. List of Virulence Genes Up-regulated in Co-cultured Biofilm

S.no	Gene Description	Annotation	Fold Value E0xpression in Co-culture Broth at 24 hours
1	LMOG_03053T0	*Thiol-activated Cytolysin*	2.78
2	LMOG_03051T0	*Actin Assembly ActA*	1.04

Table 4. List of Virulence Genes Up-regulated in Mono-culture Biofilm

S.no	Gene Description	Annotation	Fold Value Expression in Co-culture Broth at (hours)		
			4	12	24
1	LMOG_03055T0	*prfA*	1.26	0.54	0.20
2	LMOG_00341T0	*Hemolysin A*	1.09	0.55	1.12
3	LMOG_02173T0	*Phospholipase*	1.35	0.60	-0.40
4	LMOG_01377T0	*Hemolysin B*	0.98	0.56	-0.23
5	LMOG_03053T0	*Thiol-activated Cytolysin*	0.87	1.08	-0.08

Expression of other core virulence genes in Co-culture Broth

The up-regulation of *prfA* concomitantly up-regulated other core virulence genes known to be part of the regulon *prfA*. Those that were up-regulated in co-cultured broth were *Internalin-A* (LMOG_02646T0), *Hemolysin-A* (LMOG_00341T0) *Internalin-B* (LMOG_02645T0) and *Phospholipase* (LMOG_02173T0) (Fig. 3, Table 2); of these, *Internalin-A* though was initially Down-regulated was up-regulated over time (Fig. 3) while the remaining showed a similar pattern in expression

However, other core virulence genes were not up-regulated in the co-cultured broth.

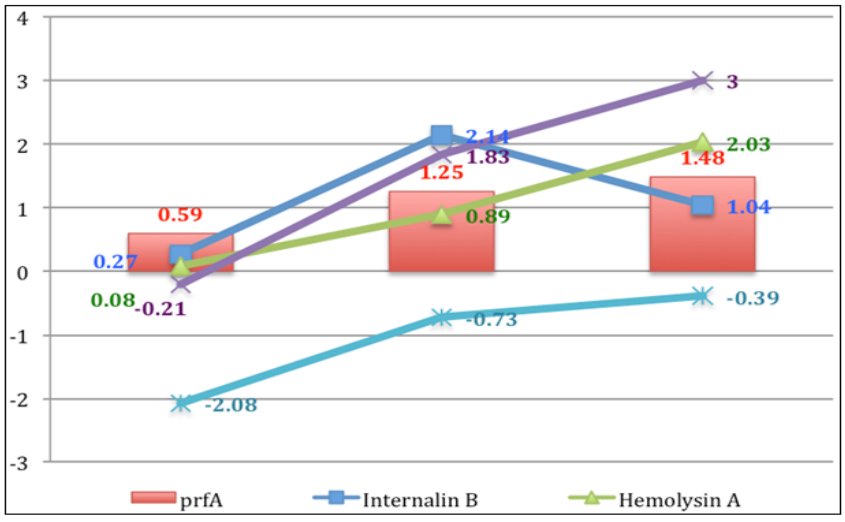

Figure 3. Expression of the core virulence genes along with *prfA* in co-culture broth.

Expression of other core virulence genes in Monoculture Biofilm

Besides *prfA,* 4 other virulene genes (out of the 10 genes) were up-regulated after 4hours of incubation as biofilms (monoculture). With the sole exception of *Hemolysin-A*, all these up-regulated genes, however showed a downward trend in expression after 12hours of incubation. These genes were namely, *Phospholipase* (LMOG_02173T0), *Hemolysin-B* (LMOG_01377T0) and *Thiol-Activated cytolysin* (LMOG_03053T0) (Fig. 4, Table 4). *Hemolysin –A* alone was up-regulated after 24hours of incubation of Biofilm.

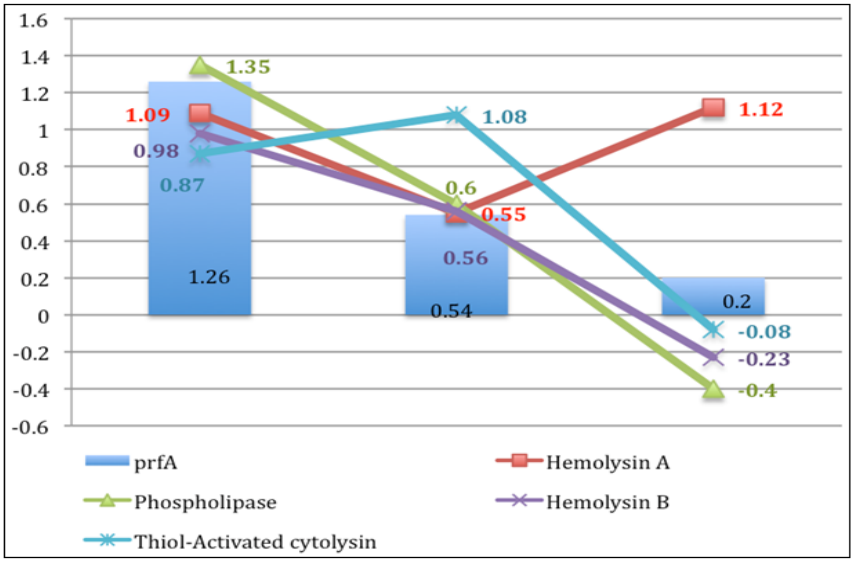

Figure 4. Expression of core virulence genes other than *prfA* in mono-culture biofilm

Expression of other core virulence genes in Co-culture Biofilm

In co-culture biofilm, 2 virulence gene transcripts *Thiol-Activated Cytolysin* (LMOG_03053T0) and *Actin* assembly (LMOG_03051T0/Act-A) were up-regulated after 24hours of incubation (Table-III). All other genes were either negatively or neutrally regulated. (Fig. 5)

Annotations	24hrs	Gene Description
prfA		LMOG_03055T0
Hemolysin A		LMOG_00341T0
Hemolysin B		LMOG_01377T0
Thiol-Activated Cytloysin		LMOG_03053T0
Internalin A		LMOG_02646T0
Internalin B		LMOG_02645T0
Plc A		LMOG_03054T0
Phospholipase		LMOG_02173T0
Actin assembly Act A		LMOG_03051T0
Up-Regulation		
Neutral Regulation		
Down-Regulation		

Figure 5. Expression of core virulence genes other than *prfA* in co-culture biofilm.

DISCUSSION

Our results also strengthen the case for understanding specifically the expression pattern of virulence genes in different environmental conditions of growth, and their implications for hypothesizing the virulence model of *L.monocytogenes* J0161.

The discussions on our results revolve around the specific issue of expression of *prfA* and other core virulence gene.

In mono-culture biofilm, *prfA* was up-regulated at the initial stages of biofilms growth in mono-culture biofilm and was gradually up-regulated in co-cultured broth from 4 to 24hours. Other virulence genes were also down regulated in tandem with the down regulation of *prfA* in mono-culture biofilm. However, Hemolysin-A was up-regulated, despite the down regulation of *prfA* in mono-culture biofilm. The core virulence genes were up-regulated in tandem from 4 to 24hours of incubation concurrent to *prfA* up-regulation in co-culture broth. Though most of the core virulence genes showed similar expression pattern as with *prfA* in all the growth conditions observed, *ActA* and Cytolysin gene transcripts were two exceptions. These two gene transcripts were up-regulated in co-culture biofilms, despite the down-regulation of *prfA*.

Biofilm formation is considered as a dynamic and complex phenomenon, the mechanism for which is still not entirely understood. Role of specific genes in the formation of biofilms by *L.monocytogenes* has not been discussed till lately (24). Role of virulence genes in biofilm formation has been speculated for a long period till Lemon *et al* (2010)(12) reported on the role of *prfA* in biofilm formation. Contrary to the reports by Lemon *et al*, about the role of *prfA* in biofilm formation, our observations that *prfA* in a monoculture biofilm of *L.monocytogenes* J0161 is up-regulated only during the initial period (4 hours) of biofilm formation, followed by down-regulation, suggests that *prfA* likely plays a role only in the initial stages of biofilm formation or alternatively the expression is not required for biofilms formation at all.

Further in the past it has been reported that the expression of *prfA* was responsible for the switching of the saprophytic form of *L.monocytogenes* to its pathogenic form and also that, under conditions of intracellular localization the bacterium switches on the expression of *prfA*. Our observation on the co-cultured broth state, indicates that expression of *prfA,* may also be switched on, under specific extracellular conditions. This observation further substantiates the hypothesis that *prfA* is either not required for biofilms formation, alternatively if it is required, then its role is refrain only at the initial stage of biofilms formation. When cells are localized intracellularly, the conditions mimic the broth conditions; *in-vivo* studies on *prfA* expression in Listeria that have already been intracellularly localized would help understand its role even better.

The expression of *prf A* in co-cultured biofilms was down-regulated after 24hours, however since, we did not study its expression at the 4h and 12 hour stages, we could not identify if the expression pattern in co-cultured biofilms showed a trend similar to what we observed with the mono-culture biofilms of *L.monocytogenes*. This could mean that the presence of *B.subtilis* makes *L.monocytogenes* less virulent in biofilms.

Another significant finding in our study was that some of the virulence genes regulated by *prfA,* were up-regulated despite the down-regulation of *prfA*. The up-regulation of '*Hemolysin-A'*, in monoculture biofilms state after 24 hours, and that of 'Thiol-Activated *cytolysin*' and 'Actin assembly *ActA*' genes in Co-cultured biofilms despite *prfA* downregulation, gives rise to a *prfA*-independent expression of these virulence genes under these conditions.

prfA is very well known for its dual role in regulating genes other than ones for virulence (14); in a similar fashion, it is likely that the few core virulence genes observed to be up-regulated at different extracellular conditions may be playing dual roles and may also be of significance in the role other than virulence.

Our results further give corroborative evidence for the 'Mr.Hyde' analogy (6,7,22) on the saprophytic

L.monocytogenes, since even in the saprophytic state if not all, some of the core virulence gene transcripts show a trend of up-ward regulation in expression. Only further studies on *in-vivo* expression of the core virulence genes as well as gene knockout experiments would help understand further the roles of the same in the virulence/pathogenicity of *L.monocytogenes*.

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