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Time-dependent gene expression pattern of *Listeria monocytogenes* J0161 in biofilms

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Background: *Listeria monocytogenes* J0161, a food-borne pathogen, forms biofilm on contact surfaces, which makes the bacterium highly resistant. Biofilm formation in vivo confers resistance to antimicrobial agents, and in vitro not only increases resistance but also increases the risk of transmission of the pathogen. Biofilm formation is a complex dynamic process. The mechanism of biofilm formation is not as yet well understood. Understanding the molecular mechanism of biofilm formation will be of significance in removal of biofilms, thereby reducing the risk of transmission.

Methods: *L. monocytogenes* cultures were grown to form biofilms on glass slides. At time intervals of 4, 12, and 24 hours, the cells were pelleted and the RNA extracted. The extracted RNA was analyzed using microarray technique and statistical tools.

Results: The microarray data showed that gene expression was specifically upregulated at each time interval. About 159, 40, and 184 genes were upregulated at 4, 12, and 24 hours, respectively. An ascending and descending pattern of gene upregulation was identified.

Conclusion: We report specific genes for biofilm growth of *L. monocytogenes* that were upregulated at particular time intervals. The role of specific genes in the formation of biofilms by *L. monocytogenes* J0161 can be studied using these data.

Keywords: *Listeria monocytogenes*, microarray, biofilms, gene expression

Introduction

Listeriosis a zoonotic bacterial disease that has emerged as a major food-borne disease during the past two decades, and has a high case fatality rate of approximately 20%–30%.[1] Listeriosis, as an important cause of severe illness accounts for 3.8% of hospitalizations for food-borne disease and 27.6% of deaths due to food-borne illness.[2] The severity of the disease includes meningitis, septicemia, and abortion. The risk of listeriosis is greatest among certain well defined high-risk groups, including pregnant women, neonates, and the elderly, but may occasionally occur in persons who have no predisposing underlying condition.[1] There have been more than 20 outbreaks of listeriosis in different parts of the globe since 1981, and involving different types of food.[3–12]

The disease is caused by *Listeria monocytogenes*. The pathogen gains entry into a food-processing facility and survives by forming microbial community niches known as "biofilms".[13–15] It has been reported that biofilm formation by *L. monocytogenes* is the major cause of transmission of the pathogen.[16] Biofilm formation increases the resistance of the pathogen to antimicrobial agents and also increases resistance to environmental stress.[17] Once established as a biofilm, removal of *L. monocytogenes* becomes a challenge.[18] Biofilm formation is a multiphase complex process, starting

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with attachment of a cell to a surface, followed by irreversible adherence, and multiplication and growth to form a three-dimensional structure. This phase-wise development of a biofilm is therefore a time-dependent process. Understanding the complexity of the process of biofilm formation has been a long-standing issue. It is believed that understanding of the mechanism behind biofilm formation will yield answers to many intriguing questions, from the very objective and need for biofilm formation by a microbe to ways and means of getting rid or making use of biofilms.[19] Therefore, it is essential to understand fundamentally the basic phenomenon, in particular the expression of genes that contribute to the formation of biofilm and are otherwise different from laboratory-grown cultures, broth, or a colony.

Here we report on the variations in gene expression of *L. monocytogenes* and patterns studied by microarray at different time intervals in biofilm formation. We chose to work on the microarray gene expression pattern of the J0161strain of *L. monocytogenes,* which is a human isolate of serotype 1/2a, and this adds to the significance of our study. We report on the genes that were upregulated specifically at 4, 12, and 24 hours.

Materials and methods

Bacterial strains and culture conditions

The J0161strain of *L. monocytogenes* was selected for study because its complete transcriptome was available and annotated in the *L. monocytogenes* database held at the Broad Institute. Further, amongst the strains that were annotated and for which the transcriptome was available, the J0161 strain had the highest percentage (78.5%) of annotated genes (2335 of 2973 gene transcripts).

The J0161 *L. monocytogenes* strain was obtained from the Agriculture Research Services, United States Department of Agriculture, and grown both in broth and as biofilm. Biofilms were grown as pure culture on three different slides for studying gene expression after set time periods of incubation, ie, at 4, 12, and 24 hours.

A pure culture of *L. monocytogenes* J0161 was grown in broth as well as biofilm using tryptone soy broth as the growth medium. *L. monocytogenes* in broth was grown for 24 hours at 37°C, and as biofilms for 4, 12, and 24 hours at 37°C on glass slides. The broth culture was used as a reference/control sample.

The culturing technique used for biofilm formation involved a static batch culture method, in which the experimental setup was incubated without nutrient replenishment at the defined time interval of the study after introducing the inoculum. Static batch cultures of this type have recently been reported to be an excellent method for genetic screening and for understanding the signals that trigger the transition of planktonic cells to form biofilms.[20]

In our earlier experiments to enumerate the cells in biofilms grown using the static batch culture method, we observed a trend of cell count that was typical of a standard growth curve (data not shown), and peaked at 12 hours. The cell count after 12 hours of incubation was fluctuating until 24 hours of incubation. There was a decline in the cell count after 24 hours. A similar pattern was observed by Chavant et al.[21] Therefore, based on our observation and inferences, we designed our experimental setup to study the gene expression pattern, taking into account the fact that the expression pattern at 4, 12 and 24 hours would depict gene expression at different stages of biofilm formation. We considered 4 hours of incubation as the stage of irreversible attachment, 12 hours as the stage of formation of microcolonies, and 24 hours as the peak stage of fruiting biofilm bodies.

In evaluating the kinetics of biofilm formation, Beresford et al[22] had reported the attachment of Listeria cells as early as 2 hours, but our attempts to extract RNA completely at incubation times earlier than 4 hours did not yield the requisite volume (less than 50 ng) and/or concentration ($<$6 ng/μL) for downprocessing by microarray. Hence the decision was made to study the expression pattern after 4 hours of incubation at the earliest.

Microarray studies

RNA extraction and evaluation

Cells were pelleted after 4, 12, and 24 hours of incubation as biofilms. Similarly, cells in pure culture broth (reference/control) were pelleted after 24 hours of incubation. The pelleted cells were washed with phosphate buffer solution. The cells were then further processed for RNA extraction using a Ribo pure bacteria kit (Ambion®, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The concentration and purity of the RNA extracted were evaluated using a bioanalyzer (Agilent 2100, Santa Clara, CA), and absorbance readings at 260 nm and 280 nm were performed using a Nanodrop spectrophotometer (ThermoScientific 1000, Hudson, NH). The concentration of the RNA extracted was evaluated using the bioanalyzer, while the purity of the RNA extracted was determined using the standard procedure of measuring A_{260} and A_{280} on the Nanodrop spectrophotometer.

Probe and microarray slide design

An Agilent *Listeria monocytogenes* 8 × 15 k (Amadid 030831) custom gene expression microarray designed by Genotypic Technology Pvt Ltd (Bangalore, India) was used for the experiment. The array consists of 15,000 probes of 60 mer length and contains 2973 unique transcripts obtained from the *L. monocytogenes* database at the Broad Institute.

RNA labeling, amplification and hybridization

Poly (A)-tails was added to the 3′ end of RNA using an A-plus Poly (A) polymerase tailing kit (Epicentre Biotechnologies, Madison, WI). The samples were then labeled using an Agilent Quick Amp Kit Plus, and 500 ng of polyadenylated RNA was reverse-transcribed using an oligodT primer tagged to the T7 promoter sequence. The cDNA 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. The cRNA obtained was purified using Qiagen RNeasy columns. The concentration and amount of dye incorporated was determined using the Nanodrop spectrophotometer. Samples that passed quality control for specific activity were taken for hybridization, and 600 ng of labeled RNA was hybridized on the array.

Hybridization, scanning, and data analysis

Following amplification, the cRNA was hybridized using the Agilent gene expression hybridization kit in Sure hyb chambers (Agilent) at 65°C for 16 hours. The hybridized slides were washed using Agilent gene expression wash buffer. The hybridized washed microarray slides were then scanned on a G2505C scanner (Agilent) and the images were quantified using feature extraction software (version 10.5.1.1, Agilent). The raw data extracted were analyzed using GeneSpring GX version 11.0 software from Agilent. Normalization of the data was done in GeneSpring 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 nth percentile of the expression values for the array across all spots (where n has a range of 0–100 and n = 75 is the median). It subtracts this value from the expression value of each entity normalized to specific control samples. Significant genes upregulated and downregulated by at least one-fold within the samples with respect to the control sample were identified. Differentially regulated genes were clustered using hierarchical clustering based on the Pearson coefficient correlation algorithm to identify significant gene expression patterns (the clustering algorithm measures the similarity or difference between genes or conditions).

Pathway annotations

All pathway and gene ontology function data for the 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 (http://www.ncbi.nlm.nih.gov/geo).

Validation by quantitative PCR

To validate our microarray data, we performed real-time polymerase chain reaction (PCR) to determine the gene expression pattern of *Prf* A (LMOG_03055T0), glycoprotein *(gp)49* (LMOG_02949T0), *chitinase* (LMOG_01358T0), and penicillin-binding protein gene (LMOG_00981T0) at different time points (4, 12, and 24 hours). Total RNA was extracted from independent biofilm cultures at 4, 12, and 24 hours, respectively, using AM-1925 (Ambion). DNase-treated RNA was used to synthesize cDNA using an Affinity Script quantitative PCR cDNA synthesis kit (Agilent). Relative quantification by quantitative PCR was done using an Invitrogen Power SYBR Green PCR Master Mix. The experiment was conducted using a Stratagene M × 3005P (Agilent) platform. The sequences and length of the primers used are as shown in Table 1. The relative gene expression levels were determined after normalizing with GAPDH as the reference gene using the Delta CT method.

Results

The results obtained from the study were analyzed and are shown with the emphasis on understanding the specific genes expressed at a higher level than the reference sample (after 24 hours of broth culture at 37°C) at different stages of biofilm formation. Further, genes showing a gradual increase in upregulation or downregulation at every stage of biofilm

Table 1 Forward and reverse primers of target genes for qPCR

Gene	Primer type	Sequence	Length
PrfA	Forward	ATTTAGAAGTCATTAGCGAGCA	22
	Reverse	CAGGATTAAAAGTTGACCGCA	21
gp49	Forward	TTAGAAGAGGCAATGAACATAG	22
	Reverse	GTTGTTCATTTTGCTGTTCGTT	22
Chitinase	Forward	GAAGGGAGACGGAGTAAATC	20
	Reverse	CGAACGCCTGCTCATCCC	18
Penicillin binding protein gene	Forward	CTATCACTACAGGACTTCGC	20
	Reverse	CAAGAGCTGTATGAATGGTTAA	22

formation have been identified. Figure 1 compares variation in the *L. monocytogenes* J0161 gene expression pattern at different stages of biofilm formation with that of the reference sample at 24 hours of broth culture. Figure 2 is a Venn diagram showing the upregulated genes for the whole transcriptome of *L. monocytogenes* J0161 at different stages of biofilm formation (4, 12, and 24 hours) and that at 24 hours of broth culture. The diagram compares the genes upregulated at specific intervals of biofilm formation and genes upregulated at all three time intervals of biofilm formation.

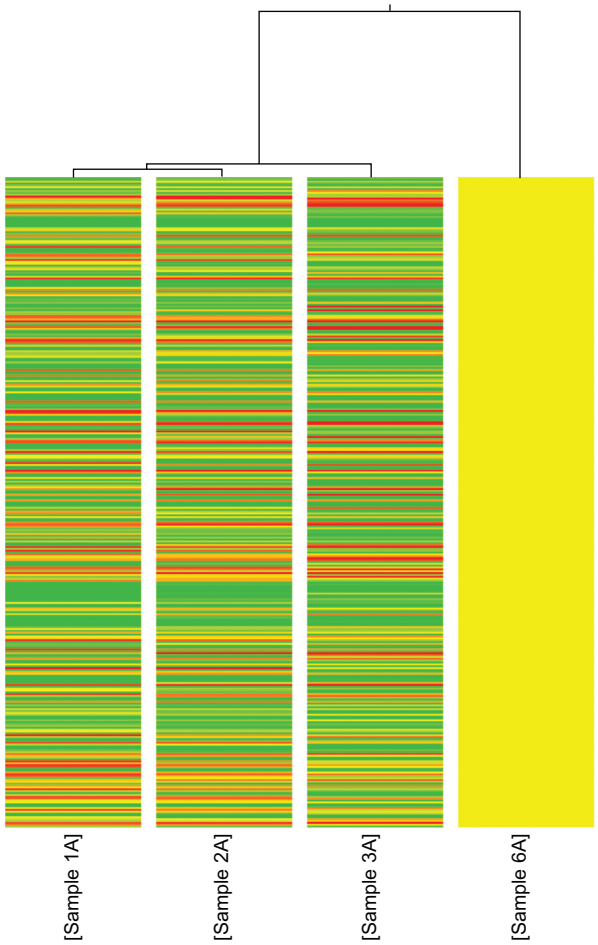
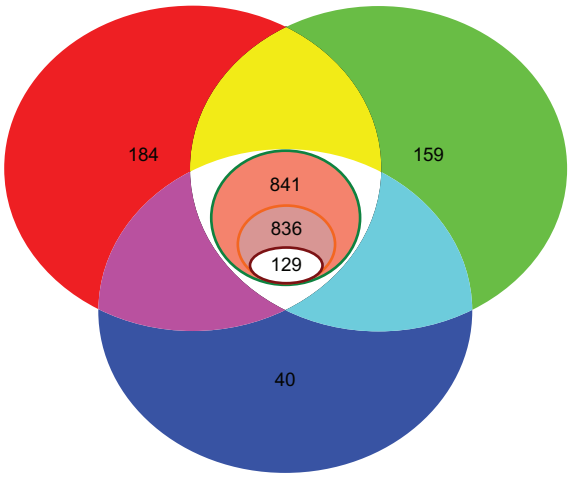


Figure 1 Microarray results in dendrogram after normalization with the reference. Columns marked as 1A, 2A and 3A, represent gene expression as bioflims (of *L. monocytogenes* J0161) at 4, 12, and 24 hours respectively. Column marked as 6A is the reference, which is gene expression at 24 hours as broth of *L. monocytogenes* J0161.
Notes: Yellow depicts neutral regulation, red depicts upregulation and green depicts downregulation.

Upregulation of genes as a function of time

More than 150 genes were upregulated after 4 hours of incubation for biofilm formation, three of which showed a more than two-fold upregulation in expression. While two of them are hypothetical protein coding genes (http://www.broadinstitute.org/annotation/genome/listeria_group/MultiHome.html), the third is a "thioredoxin" gene (LMOG_00491T0) known for its role in the response to oxidative stress.[23,24] Further analysis of the hypothetical proteins revealed that one of them (LMOG_00557T0) is likely to be involved in oxidation-reduction (http://www.uniprot.org/), as shown in Table 2.



Types of genes	No. of genes
Genes upregulated at all the three time intervals	129
Ascendingly regulated genes from 4 to 24 hours	836
Descendingly regulated genes from 4 to 24 hours	841
Genes upregulated specifically at 4 hours	159
Genes upregulated specifically at 12 hours	40
Genes upregulated specifically at 24 hours	184

Figure 2 Venn diagram showing the number of genes with a specific pattern of regulation at specified time intervals.

At 12 hours, the number of upregulated genes decreased to 40. Three of these 40 genes showed more than a two-fold increase in expression, with the "M protein transacting positive regulator" (LMOG_01126T0) showing at least a three-fold increase in expression. The other two genes were PTS system galactitol-specific enzyme IIB component (LMOG_01128T0) and a hypothetical protein (LMOG_02496T0), as shown in Table 3.

After 24 hours of incubation for biofilm formation, more than 150 genes were upregulated, of which 24 were showing more than two-fold gene expression. Among the 24 genes that showed more than two-fold gene expression, nine were glycoprotein genes, ie, *gp43*, *11, 37, 39, 15, 44, 63,* and *22* (LMOG_03098T0, LMOG_03129T0, LMOG_03097T0, LMOG_03100T0, LMOG_03133T0, LMOG_03099T0, LMOG_03115T0, and LMOG_03140T0, respectively), while five of them were hypothetical protein genes (Table 4).

Our results show incremental upregulation of genes over time. Apart from the transcripts that were specifically upregulated at a particular time interval, a total of 836 genes showed a gradual increase in expression with time (from 4 to 24 hours of incubation). Further, there were variations in the expression levels among these genes. A significant upregulation of at least six-fold in the expression of *gp49* (LMOG_03104T0) was observed at 4–24 hours, while 8, 20, and 44 genes were upregulated by five-fold, four-fold, and three-fold, respectively. Proteins of the glycoprotein family comprised the majority of the transcripts prominently exhibiting this trend (Tables 5 and 6). More than 70% of the genes downregulated at 4–24 hours of incubation were related to sugar metabolism and transport (Tables 7 and 8). Sets of 129 transcripts were upregulated at incubation time intervals of 4, 12, and 24 hours as biofilms (Table 9). It is noteworthy that these genes were upregulated only during growth as biofilms but not in broth.

Quantitative PCR was done on *Prf*A (LMOG_03055T0), *gp49* (LMOG_02949T0), *chitinase* (LMOG_01358T0), and penicillin-binding protein gene (LMOG_00981T0) genes to validate the expression pattern observed using the microarray data. In the microarray data, genes for both *Prf*A and *chitinase* showed a trend of descending regulation over time, while the genes for *gp49* and penicillin-binding protein gene showed an incremental pattern of expression. The quantitative PCR data for the target genes showed a similar pattern of expression to that observed in the microarray data (Figures 3, 4, 5, and 6).

Discussion

Biofilm formation is a multiphase dynamic process, beginning from the stage of initial attachment to its development and dispersion. Cells in the biofilm formation stage are different from culture-grown planktonic cells.[15] When a microbial culture is introduced into a medium, the bacterial cells adhere to the available contact surface, eventually forming an irreversible attachment. Thereafter, the cells grow in number to develop into a mature biofilm. Fully formed/mature biofilms, which are often multilayered structures, burst to disperse cells from within the biofilm. These dispersed cells, in turn, settle down at remote sites on a contact surface and develop further into independent biofilm. Despite the enormous amount of data available on biofilm physiology, the molecular level dynamics governing the various stages of biofilm development are not well understood. Given the phenotypic differences between biofilms and planktonic cells, the metabolic pathways and gene expression of the sessile biofilm cells are likely to be different. Using microarrays, Yi et al[25] have reported on the role of a particular regulatory protein in biofilm formation by yeasts. In this study, we have analyzed time-dependent gene expression data to delineate the probable role of genes at different stages of biofilm formation.

Several earlier studies have reported such time-dependent expression. Hautefort et al[26] have reported simultaneous time-dependent expression of three type 3 secretion systems in *Salmonella enterica*. In another study, Chan et al[27] have shown differential gene expression at fixed time intervals (log and stationary phases) of *L. monocytogenes* growth when cultured in broth at 4°C and 37°C under given conditions. However, there are no studies directed towards understanding the time-dependent gene expression pattern of *L. monocytogenes* in biofilm. For the first time, we have demonstrated specific genes that are upregulated or overexpressed and downregulated or poorly expressed during particular stages of biofilm growth. We have also tried to ascertain the generic function of genes active at a particular stage of biofilm formation. To represent the gene expression pattern, we have used *L. monocytogenes* J0161 gene expression over 24 hours in broth culture as a reference.

As compared with *L. monocytogenes* J0161 gene expression after 24 hours of broth culture, about 159 gene transcripts were specifically upregulated after 4 hours of biofilm formation. Upregulation by more than two-fold was observed in only three of the total number of genes upregulated, and among them were two gene transcripts that were related to oxidative stress at hour 4 of incubation. Oxidative stress in biofilm has been directly linked to diversity within the biofilm in *Pseudomonas aeruginosa*.[28] The mechanism of

Table 2 List of genes and their annotations upregulated at 4 hours of incubation as biofilms

S no	Gene	Fold expression values	No of gene transcripts	Annotations
1	LMOG_00557T0	2.3	3	Hypothetical protein
2	LMOG_00491T0	2.1		Thioredoxin
3	LMOG_02373T0	2.0		Hypothetical protein
4	LMOG_01789T0	2.0	92	Hypothetical protein
5	LMOG_01659T0	2.0		Thioredoxin
6	LMOG_01058T0	1.9		Hypothetical protein
7	LMOG_01215T0	1.9		Cold shock protein CspB
8	LMOG_01361T0	1.8		Ribonuclease HI
9	LMOG_00723T0	1.8		Hypothetical protein
10	LMOG_00716T0	1.8		YkuJ protein
11	LMOG_00589T0	1.8		Glyoxalase
12	LMOG_01063T0	1.7		Ribonucleotide reductase-associated flavodoxin
13	LMOG_01728T0	1.7		Cellobiose-specific PTS system IIA component
14	LMOG_02751T0	1.7		Adenylosuccinate lyase
15	LMOG_01776T0	1.7		6-phosphogluconolactonase
16	LMOG_01615T0	1.7		Cytochrome aa3 quinol oxidase subunit IV
17	LMOG_02706T0	1.6		Hypothetical protein
18	LMOG_00859T0	1.5		Transcriptional regulator
19	LMOG_01707T0	1.5		Catalase
20	LMOG_02774T0	1.4		Hypothetical protein
21	LMOG_01796T0	1.4		Hypothetical protein
22	LMOG_01729T0	1.4		ROK family protein
23	LMOG_01748T0	1.4		General stress protein 26
24	LMOG_02173T0	1.3		Phospholipase/carboxylesterase
25	LMOG_02549T0	1.3		Maltose/maltodextrin transport ATP-binding protein MalK
26	LMOG_00928T0	1.3		Carbonic anhydrase
27	LMOG_00980T0	1.3		Hypothetical protein
28	LMOG_01932T0	1.3		Hypothetical protein
29	LMOG_01799T0	1.3		Oxidoreductase
30	LMOG_00343T0	1.3		Cold shock protein CspB
31	LMOG_00429T0	1.3		Succinyl-CoA synthetase
32	LMOG_03195T0	1.3		Pyridine nucleotide-disulfide oxidoreductase
33	LMOG_02413T0	1.3		Preprotein translocase SecG subunit
34	LMOG_02841T0	1.3		Hypothetical protein
35	LMOG_01669T0	1.3		Hypothetical protein
36	LMOG_02457T0	1.3		FeS assembly protein SufB
37	LMOG_00881T0	1.3		Hypothetical protein
38	LMOG_00965T0	1.3		Glyoxalase
39	LMOG_02813T0	1.3		Hypothetical protein
40	LMOG_00126T0	1.3		FxsA
41	LMOG_03310T0	1.3		Methionine-R-sulfoxide reductase
42	LMOG_01767T0	1.3		HAD-superfamily hydrolase
43	LMOG_03193T0	1.3		Hypothetical protein
44	LMOG_03055T0	1.3		Virulence regulatory factor *Prf*A
45	LMOG_00673T0	1.2		Molybdenum cofactor biosynthesis protein B
46	LMOG_02319T0	1.2		Serine hydroxymethyltransferase
47	LMOG_02232T0	1.2		Sulfate transporter
48	LMOG_00834T0	1.2		Phosphoserine phosphatase rsbX
49	LMOG_00749T0	1.2		Esterase
50	LMOG_02014T0	1.2		Peptidoglycan binding protein
51	LMOG_01697T0	1.2		Phosphosugar-binding transcriptional regulator
52	LMOG_00671T0	1.2		Hypothetical protein
53	LMOG_02538T0	1.2		Two-component system response regulator
54	LMOG_03015T0	1.2		Hypothetical protein
55	LMOG_02092T0	1.2		Hypothetical protein
56	LMOG_01199T0	1.2		Yggt family protein

(*Continued*)

Table 2 (*Continued*)

S no	Gene	Fold expression values	No of gene transcripts	Annotations
57	LMOG_02221T0	1.2		Transcriptional regulator
58	LMOG_02393T0	1.1		NADPH dehydrogenase NamA
59	LMOG_00954T0	1.1		Hypothetical protein
60	LMOG_00829T0	1.1		Hypothetical protein
61	LMOG_02637T0	1.1		Hypothetical protein
62	LMOG_00750T0	1.1		Acetyltransferase
63	LMOG_03030T0	1.1		Cysteine synthase A
64	LMOG_00650T0	1.1		Hypothetical protein
65	LMOG_00080T0	1.1		Hypothetical protein
66	LMOG_02827T0	1.1		Hypothetical protein
67	LMOG_01064T0	1.1		Thioredoxin
68	LMOG_00272T0	1.1		Proton-coupled thiamine transporter YuaJ
69	LMOG_00007T0	1.1		l,4-dihydroxy-2-naphthoate octaprenyltransferase
70	LMOG_02601T0	1.1		Glutamyl-tRNA synthetase
71	LMOG_02324T0	1.1		ATP synthase subunit A
72	LMOG_02080T0	1.1		Acetyltransferase
73	LMOG_01159T0	1.1		Chaperonin GroL
74	LMOG_03033T0	1.1		ATP-dependent metalloprotease FtsH
75	LMOG_00144T0	1.1		Valyl-tRNA synthetase
76	LMOG_00787T0	1.1		Peroxide resistance protein Dpr
77	LMOG_01502T0	1.1		Lipase
78	LMOG_00466T0	1.1		Hypothetical protein
79	LMOG_00068T0	1.1		Dipeptidase PepV
80	LMOG_02678T0	1.1		Alpha-mannosidase
81	LMOG_00219T0	1.1		30S ribosomal protein S20
82	LMOG_01068T0	1.1		Hypothetical protein
83	LMOG_02994T0	1.1		Transcriptional regulator
84	LMOG_01037T0	1.1		Hypothetical protein
85	LMOG_02681T0	1.1		Fructose-specific IIA PTS system component
86	LMOG_00814T0	1.1		Cellobiose-specific PTS system IIB component
87	LMOG_01393T0	1.1		Manganese-binding lipoprotein mntA
88	LMOG_02095T0	1.0		Transcriptional regulator
89	LMOG_00813T0	1.0		IIC component PTS system
90	LMOG_01434T0	1.0		Acyl carrier protein
91	LMOG_01007T0	1.0		Hypothetical protein
92	LMOG_01004T0	1.0		Hypothetical protein
93	LMOG_03216T0	1.0		General stress protein 13
94	LMOG_00431T0	1.0		Aldose epimerase
95	LMOG_003 60T0	1.0	64	Glycine cleavage system T protein
96	LMOG_00624T0	1.0		Monooxygenase
97	LMOG_01803T0	1.0		Dihydroxyacetone kinase L subunit
98	LMOG_01631T0	1.0		DNA polymerase III beta subunit
99	LMOG_01730T0	1.0		Cellobiose-specific PTS system IIC component
100	LMOG_01377T0	1.0		Hemolysin-3
101	LMOG_02218T0	1.0		Thermostable carboxypeptidase 1
102	LMOG_03160T0	1.0		Hypothetical protein
103	LMOG_03041T0	1.0		Peptidyl-tRNA hydrolase
104	LMOG_01834T0	1.0		Transcriptional regulator
105	LMOG_01167T0	1.0		MOSC domain-containing protein
106	LMOG_02679T0	1.0		Fructose-specific PTS system fructose-specific II component
107	LMOG_00257T0	1.0		Transmembrane protein
108	LMOG_01358T0	1.0		Chitinase
109	LMOG_00743T0	1.0		Glutathione peroxidase
110	LMOG_00994T0	1.0		Hypothetical protein
111	LMOG_01952T0	1.0		Mannose-specific PTS system IID component
112	LMOG_02094T0	0.9		Acetyltransferase

(*Continued*)

Table 2 (*Continued*)

S no	Gene	Fold expression values	No of gene transcripts	Annotations
113	LMOG_00329T0	0.9		6-phosphogluconate dehydrogenase
114	LMOG_02252T0	0.9		Hypothetical protein
115	LMOG_00998T0	0.9		Hypothetical protein
116	LMOG_01839T0	0.9		PTS system IIA 2 domain-containing protein
117	LMOG_00821T0	0.9		Phosphoglycerate mutase
118	LMOG_01699T0	0.9		Hypothetical protein
119	LMOG_01797T0	0.9		Recombination protein RecR
120	LMOG_03303T0	0.9		UTP-glucose-1-phosphate uridylyltransferase
121	LMOG_01024T0	0.9		Competence negative regulator mecA
122	LMOG_00479T0	0.9		Phosphoglycerate mutase
123	LMOG_02332T0	0.9		ATP synthase F1 epsilon subunit
124	LMOG_01692T0	0.9		Oxidoreductase
125	LMOG_01858T0	0.9		Transcriptional antiterminator
126	LMOG_02001T0	0.9		LacI family transcription regulator
127	LMOG_00748T0	0.9		Branched-chain amino acid aminotransferase
128	LMOG_01947T0	0.9		Amino acid permease
129	LMOG_02435T0	0.9		Hypothetical protein
130	LMOG_00444T0	0.9		rnhB
131	LMOG_00769T0	0.9		Ilm protein
132	LMOG_01087T0	0.9		APC family amino acid-polyamine-organocation transporter
133	LMOG_01691T0	0.9		N-acetylmannosamine-6-phosphate 2-epimerase
134	LMOG_03200T0	0.9		4-hydroxybenzoyl-CoA thioesterase domain-containing protein
135	LMOG_00987T0	0.9		Hypothetical protein
136	LMOG_02680T0	0.9		Fructose-specific PTS system IIB component
137	LMOG_01478T0	0.9		Acetyltransferase
138	LMOG_00421T0	0.9		Glycerophosphoryl diester phosphodiesterase
139	LMOG_01624T0	0.9		Spermidine N1-acetyltransferase
140	LMOG_03027T0	0.9		DNA-directed RNA polymerase delta subunit
141	LMOG_01094T0	0.9		Maltose/maltodextrin ABC transporter
142	LMOG_00797T0	0.9		Membrane protein
143	LMOG_01802T0	0.9		Dihydroxyacetone kinase phosphotransfer subunit
144	LMOG_00158T0	0.8		Glycerol kinase
145	LMOG_00167T0	0.8		Preprotein translocase
146	LMOG_01870T0	0.8		Heptaprenyl diphosphate synthase component I
147	LMOG_01148T0	0.8		M22 family peptidase
148	LMOG_00124T0	0.8		6-phosphofructokinase
149	LMOG_01255T0	0.8		Hypothetical protein
150	LMOG_00352T0	0.8		Translation elongation factor P
151	LMOG_01162T0	0.8		Hypothetical protein
152	LMOG_01618T0	0.8		Quinol oxidase AA3
153	LMOG_02561T0	0.8		Phosphoglycerate mutase
154	LMOG_02517T0	0.8		Hypothetical protein
155	LMOG_02819T0	0.8		Methionine aminopeptidase type I
156	LMOG_01213T0	0.8		PAP2 family protein
157	LMOG_01842T0	0.8		Galactitol-specific PTS enzyme IIC component
158	LMOG_01956T0	0.8		Hypothetical protein
159	LMOG_00953T0	0.8		Intracellular protease I

diversity caused by oxidative stress is attributed to double-stranded DNA breaks that cause breaks in the whole genome and repair thereafter, leading to mutagenic variants.[23] Oxidative stress has also been attributed to increased antibiotic resistance in *P. aeruginosa*. Even though we could not observe any discernible increase in antibiotic resistance in terms of related gene expression patterns from our data, two oxidative stress genes showing the highest upregulation further corroborate earlier established links between oxidative stress and biofilm physiology. *prf*A (positive [virulence] regulator factor) is another gene of significance that we observed to be upregulated, specifically at hour 4 of incubation. Lemon et al[29] have suggested that *prf*A positively regulates biofilm formation. The expression of *prf*A increased

Table 3 List of genes and their annotations upregulated at 12 hours of incubation as biofilms

S no	Gene	Fold expression values	No of gene transcripts	Annotations
1	LMOG_01126T0	3.4	1	M protein trans-acting positive regulator
2	LMOG_01128T0	2.7	2	PTS system galactitol-specific enzyme IIB component
3	LMOG_02496T0	2.7		Hypothetical protein
4	LMOG_01127T0	1.7	15	PTS system galactitol-specific enzyme IIA component
5	LMOG_02429T0	1.7		Glyoxalase/bleomycin resistance protein/dioxygenase
6	LMOG_00058T0	1.6		Indole-3-glycerol phosphate synthase
7	LMOG_02409T0	1.5		Phosphopyruvate hydratase
8	LMOG_02376T0	1.4		Hypothetical protein
9	LMOG_01132T0	1.4		Hypothetical protein
10	LMOG_01556T0	1.3		Hypothetical protein
11	LMOG_00585T0	1.3		Propanediol utilization polyhedral body protein PduU
12	LMOG_02404T0	1.2		Transcriptional regulator
13	LMOG_01131T0	1.2		Hypothetical protein
14	LMOG_01129T0	1.2		Galactitol-specific PTS system IIC component
15	LMOG_02407T0	1.1		Triosephosphate isomerase
16	LMOG_02408T0	1.1		2,3-bisphosphoglycerate-independent phosphoglycerate mutase
17	LMOG_00151T0	1.1		Septum site-determining protein MinC
18	LMOG_00275T0	1.1		Osmotically activated L-carnitine/choline ABC transporter
19	LMOG_00231T0	1.0	22	GatB/Yqey domain-containing protein
20	LMOG_02178T0	1.0		Transcriptional regulator
21	LMOG_02220T0	1.0		Glycosyl hydrolase, family 4
22	LMOG_01105T0	1.0		Bacitracin export ATP-binding protein BceA
23	LMOG_00328T0	0.9		Two-component system response regulator
24	LMOG_01310T0	0.9		2-heptaprenyl-l,4-naphthoquinone methyltransferase
25	LMOG_00956T0	0.9		Hydrolase
26	LMOG_01454T0	0.9		STAS domain-containing protein
27	LMOG_02302T0	0.9		Fructose-16-bisphosphate aldolase class II
28	LMOG_01059T0	0.9		Metallo-beta-lactamase
29	LMOG_00205T0	0.9		MTA/SAH nucleosidase
30	LMOG_01322T0	0.9		Zn-dependent protease
31	LMOG_03179T0	0.9		YbbK protein
32	LMOG_02234T0	0.8		Phosphosugar-binding transcriptional regulator
33	LMOG_00079T0	0.8		Glutamyl aminopeptidase
34	LMOG_00742T0	0.8		LytTr DNA-binding domain family
35	LMOG_01227T0	0.8		Acetolactate synthase catabolic
36	LMOG_01900T0	0.8		Adenylate kinase
37	LMOG_02465T0	0.8		Hypothetical protein
38	LMOG_00039T0	0.8		Hypothetical protein
39	LMOG_01873T0	0.8		Sex pheromone cADI
40	LMOG_02142T0	0.8		Internalin

by 1.26-fold at 4 hours and thereafter declined by 0.56-fold at 12 hours and 0.20-fold at 24 hours, strongly suggesting that expression of this gene is only necessary in the initial stages of biofilm formation.

At hour 12 of biofilm formation, only about 40 gene transcripts were upregulated. Expression of the upregulated genes except for M-protein transacting factor was less than three-fold. M-protein transacting factor has a probable role in regulation of surface protein expression, and was the only significantly upregulated gene at hour 12 of biofilm formation. At later stages of biofilm formation, cell-cell communication is largely mediated via signal transduction mechanisms, and surface proteins are key players. We speculate that upregulation of a gene involved in expression of surface proteins establishes its role in biofilm cell communication. Further studies involving knockout of these genes will help broaden our understanding of their role(s) in biofilm formation.

In our study, 20 genes showed more than a two-fold increase in expression at 24 hours of biofilm formation. Of these 20 genes, nine were glycoproteins and five were hypothetical proteins. Among the four genes that showed more than a three-fold increase in expression, two were hypothetical proteins, and one was *gp43*. Apart

Table 4 List of genes and their annotations upregulated at 24 hours of incubation as biofilms

S no	Gene	Fold expression values	No of gene transcripts	Annotations
1	LMOG_01638T0	5.2	1	Hypothetical protein
2	LMOG_01387T0	3.5	3	Copper-translocating P-type ATPase
3	LMOG_03098T0	3.1		gp43
4	LMOG_01262T0	3.1		Hypothetical protein
5	LMOG_02415T0	3.0	20	Carboxylesterase
6	LMOG_02195T0	2.9		Magnesium transporter
7	LMOG_03129T0	2.6		gp11
8	LMOG_03157T0	2.4		Transmembrane protein
9	LMOG_03097T0	2.4		gp37
10	LMOG_03100T0	2.3		gp39
11	LMOG_03095T0	2.3		Hypothetical protein
12	LMOG_01388T0	2.3		Heavy metal-binding protein
13	LMOG_03133T0	2.3		gp15
14	LMOG_03099T0	2.2		gp44
15	LMOG_03119T0	2.2		Hypothetical protein
16	LMOG_03102T0	2.2		Hypothetical protein
17	LMOG_03116T0	2.2		gp64
18	LMOG_01660T0	2.1		Nitroreductase
19	LMOG_00387T0	2.1		YlxR
20	LMOG_03115T0	2.1		gp63
21	LMOG_02114T0	2.1		Methyltransferase
22	LMOG_02100T0	2.1		Glyoxalase
23	LMOG_03140T0	2.0		gp22
24	LMOG_01461T0	2.0		Zinc ABC transporter
25	LMOG_01002T0	2.0	114	Acetyltransferase
26	LMOG_02801T0	1.9		ABC transporter ATP-binding protein
27	LMOG_00254T0	1.9		Zinc ABC transporter
28	LMOG_01450T0	1.8		Methyltransferase
29	LMOG_02416T0	1.8		Ribonuclease R
30	LMOG_00014T0	1.8		Laminin-binding surface protein
31	LMOG_02807T0	1.8		Outer surface protein
32	LMOG_03300T0	1.8		N-acetylmuramoyl-L-alanine amidase, family 4
33	LMOG_01386T0	1.8		YvgZ
34	LMOG_01493T0	1.8		Hypothetical protein
35	LMOG_03132T0	1.7		gp14
36	LMOG_03106T0	1.7		Predicted protein
37	LMOG_03085T0	1.7		gp33
38	LMOG_02115T0	1.7		Rrf2 family protein
39	LMOG_03101T0	1.7		Predicted protein
40	LMOG_03111T0	1.7		Hypothetical protein
41	LMOG_00784T0	1.7		Late competence protein ComEC
42	LMOG_03128T0	1.7		gp10
43	LMOG_00385T0	1.6		Translation initiation factor IF-2
44	LMOG_01448T0	1.6		Hypothetical protein
45	LMOG_00438T0	1.6		Tyrosine recombinase XerC
46	LMOG_00135T0	1.6		Primosomal protein DnaI
47	LMOG_01359T0	1.6		SSU ribosomal protein S14p
48	LMOG_02803T0	1.6		Helicase domain-containing protein
49	LMOG_00253T0	1.6		Zinc ABC transporter
50	LMOG_03127T0	1.5		gp9
51	LMOG_02768T0	1.5		Glutamyl-tRNA(Gln) amidotransferase (chain C)
52	LMOG_00450T0	1.5		Trigger factor tig
53	LMOG_01426T0	1.5		DAK2 domain-containing protein
54	LMOG_01413T0	1.5		Guanylate kinase
55	LMOG_01263T0	1.5		Hypothetical protein
56	LMOG_00427T0	1.5		DNA topoisomerase IV A subunit

(*Continued*)

Table 4 (*Continued*)

S no	Gene	Fold expression values	No of gene transcripts	Annotations
57	LMOG_00440T0	1.5		tRNA:m(5)U-54 methyltransferase
58	LMOG_02572T0	1.5		Hypothetical protein
59	LMOG_02463T0	1.4		SMC domain-containing protein
60	LMOG_01855T0	1.4		Translation elongation factor G
61	LMOG_03110T0	1.4		gp54
62	LMOG_00436T0	1.4		Heat shock protein HslVU ATPase subunit HslU
63	LMOG_00490T0	1.4		Excinuclease ABC C subunit
64	LMOG_00774T0	1.4		Hypothetical protein
65	LMOG_00263T0	1.3		Penicillin-binding protein
66	LMOG_00446T0	1.3		Signal peptidase I
67	LMOG_02170T0	1.3		Translocase subunit secA 2
68	LMOG_01003T0	1.3		Hydrolase
69	LMOG_00112T0	1.3		N-6DNAmethylase
70	LMOG_01888T0	1.3		30S ribosomal protein S17
71	LMOG_03066T0	1.3		4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase
72	LMOG_00043T0	1.3		Helicase
73	LMOG_02806T0	1.3		Beta-glucoside-specific PTS system IIA component
74	LMOG_02765T0	1.3		ATP-dependent DNA helicase PcrA
75	LMOG_00384T0	1.3		YlxP
76	LMOG_00378T0	1.3		Polyribonucleotide nucleotidyltransferase
77	LMOG_00441T0	1.3		DNA topoisomerase I
78	LMOG_02145T0	1.3		ABC transporter
79	LMOG_02140T0	1.3		Transcriptional regulator
80	LMOG_01484T0	1.3		Phage holin
81	LMOG_02766T0	1.3		DNA ligase NAD-dependent
82	LMOG_02146T0	1.3		Transcriptional regulator
83	LMOG_02417T0	1.3		SsrA-binding protein
84	LMOG_01884T0	1.3		50S ribosomal protein L22
85	LMOG_00101T0	1.3		Aminotransferase
86	LMOG_01890T0	1.3		50S ribosomal protein L24
87	LMOG_02589T0	1.3		50S ribosomal protein L11
88	LMOG_01489T0	1.3		Hypothetical protein
89	LMOG_02594T0	1.2		RNA polymerase sigma-30 factor
90	LMOG_01879T0	1.2		50S ribosomal protein L3
91	LMOG_01889T0	1.2		50S ribosomal protein L14
92	LMOG_01449T0	1.2		Hypothetical protein
93	LMOG_01949T0	1.2		Sigma54-associated activator ManR
94	LMOG_03270T0	1.2		Lysyl-tRNA synthetase
95	LMOG_01490T0	1.2		Hypothetical protein
96	LMOG_03113T0	1.2		Predicted protein
97	LMOG_01483T0	1.2		N-acetylmuramoyl-L-alanine amidase
98	LMOG_00212T0	1.2		Hypothetical protein
99	LMOG_00456T0	1.2		Helix-turn-helix domain-containing protein
100	LMOG_03125T0	1.2		Phage capsid protein
101	LMOG_00045T0	1.2		STAS domain-containing protein
102	LMOG_03131T0	1.2		gp13
103	LMOG_02605T0	1.2		DNA repair protein RadA
104	LMOG_00395T0	1.2		Di-trans,poly-cis-decaprenylcistransferase
105	LMOG_00153T0	1.2		Ribonuclease
106	LMOG_01319T0	1.2		TPR domain-containing protein
107	LMOG_03120T0	1.2		Phage terminase small subunit
108	LMOG_03130T0	1.2		Major tail shaft protein
109	LMOG_02588T0	1.2		50S ribosomal protein LI
110	LMOG_03117T0	1.2		gp65
111	LMOG_00695T0	1.2		Zn-dependent hydrolase
112	LMOG_01488T0	1.1		Hypothetical protein

(*Continued*)

Table 4 (*Continued*)

S no	Gene	Fold expression values	No of gene transcripts	Annotations
113	LMOG_01896T0	1.1		30S ribosomal protein S5
114	LMOG_00255T0	1.1		zurR
115	LMOG_00512T0	1.1		Transcriptional regulator
116	LMOG_00172T0	1.1		Adenine phosphoribosyltransferase
117	LMOG_00213T0	1.1		Iojap protein 155
118	LMOG_03123T0	1.1		Minor capsid protein
119	LMOG_00383T0	1.1		Ribosome-binding factor A
120	LMOG_00373T0	1.1		5-formyltetrahydrofolate cyclo-ligase
121	LMOG_03156T0	1.1		Rrf2 family protein
122	LMOG_03109T0	1.1		Hypothetical protein
123	LMOG_01195T0	1.1		Cell division protein FtsA
124	LMOG_01852T0	1.1		30S ribosomal protein S12
125	LMOG_00408T0	1.1		Cell division suppressor protein yneA
126	LMOG_00209T0	1.1		Shikimate 5-dehydrogenase
127	LMOG_00500T0	1.1		ABC transporter permease
128	LMOG_00171T0	1.1		Single-stranded-DNA-specific exonuclease recJ
129	LMOG_03139T0	1.1		Short tail fiber
130	LMOG_00102T0	1.1		Thiamine biosynthesis/tRNA modification protein ThiI
131	LMOG_01901T0	1.1		Hypothetical protein
132	LMOG_01495T0	1.1		Prophage LambdaLm01
133	LMOG_03122T0	1.0		Phage portal protein
134	LMOG_03121T0	1.0		Phage terminase large subunit
135	LMOG_02452T0	1.0		Hypothetical protein
136	LMOG_02335T0	1.0		MreB-like protein
137	LMOG_03308T0	1.0		Translation initiation factor IF-3
138	LMOG_01869T0	1.0		Heptaprenyl diphosphate synthase component II
139	LMOG_00486T0	1.0	47	Ribonuclease PH
140	LMOG_00240T0	1.0		DNA repair protein RecO
141	LMOG_01892T0	1.0		30S ribosomal protein S14p/S29e
142	LMOG_00132T0	1.0		Dephospho-CoA kinase
143	LMOG_02609T0	1.0		Transcriptional regulator CtsR
144	LMOG_00437T0	1.0		ATP-dependent protease hslV
145	LMOG_01482T0	0.9		Hypothetical protein
146	LMOG_00074T0	0.9		Phosphotransferase enzyme family protein
147	LMOG_03142T0	0.9		Phage holin
148	LMOG_03090T0	0.9		gp37
149	LMOG_03126T0	0.9		gp8
150	LMOG_01486T0	0.9		Hypothetical protein
151	LMOG_00759T0	0.9		Inorganic polyphosphate/ATP-NAD kinase
152	LMOG_02356T0	0.9		Cell division ATP-binding protein FtsE
153	LMOG_00501T0	0.9		ABC transporter ATP-binding protein
154	LMOG_00611T0	0.9		Hypothetical protein
155	LMOG_00703T0	0.9		Ribonucleic acid-binding domain-containing protein
156	LMOG_02007T0	0.9		Hypothetical protein
157	LMOG_03273T0	0.9		Dihydroneopterin aldolase
158	LMOG_00804T0	0.9		ABC transporter permease
159	LMOG_00183T0	0.9		ATPase
160	LMOG_01902T0	0.9		Translation initiation factor IF-1
161	LMOG_03238T0	0.9		50S ribosomal protein L35
162	LMOG_03034T0	0.9		tilS/hprT
163	LMOG_03070T0	0.9		YabE protein
164	LMOG_00100T0	0.9		Septation ring formation regulator EzrA
165	LMOG_00717T0	0.9		Hypothetical protein
166	LMOG_01264T0	0.9		Pentitol PTS system enzyme II B component
167	LMOG_00445T0	0.9		Ribosome biogenesis GTP-binding protein YlqF

(*Continued*)

Table 4 (*Continued*)

S no	Gene	Fold expression values	No of gene transcripts	Annotations
168	LMOG_01378T0	0.9		DegV family protein
169	LMOG_03271T0	0.8		Dihydrouridine synthase
170	LMOG_00220T0	0.8		GTP-binding protein LepA
171	LMOG_00011T0	0.8		Hydrolase
172	LMOG_02658T0	0.8		Transcriptional regulator
173	LMOG_01491T0	0.8		Hypothetical protein
174	LMOG_02291T0	0.8		Hypothetical protein
175	LMOG_03065T0	0.8		Hypothetical protein
176	LMOG_00404T0	0.8		Predicted protein
177	LMOG_03124T0	0.8		Scaffolding protein
178	LMOG_02655T0	0.8		PRD/PTS system IIA 2 domain-containing regulatory protein
179	LMOG_00339T0	0.8		DNA repair protein RecN
180	LMOG_01302T0	0.8		Ribosome-associated GTPase EngA
181	LMOG_02304T0	0.8		Glycosyltransferase
182	LMOG_00122T0	0.8		Acetyl-CoA carboxylase carboxyl transferase alpha subunit
183	LMOG_00218T0	0.8		DNA polymerase III delta subunit
184	LMOG_02522T0	0.8		Hypothetical protein
185	LMOG_01494T0	0.8		Prophage LambdaLm01

from the genes that were upregulated specifically at fixed time intervals, we also report on the genes that showed a pattern of fluctuating levels of upregulation. Some genes were either not upregulated or minimally upregulated at 4 hours of growth, but were increasingly upregulated after 12 and 24 hours. Similarly, a group of genes was markedly upregulated at 4 hours, but was downregulated after 12 and 24 hours.

An interesting observation in this respect is the expression pattern for glycoproteins, which have been reported to have varied functions, ranging from cell adhesion to cell-to-cell signaling.[30,31] Glycoprotein expression, as observed in our study, is of significance for the reason that a set of 12 glycoproteins showed an ascending pattern of upregulation over time. For instance, gp49, a putative gene transcript with an unknown function, is expressed with an ascending order of more than six-fold variation from 4 to 24 hours. The glycoprotein expression pattern specifically in biofilm but not in broth culture signifies a clear role of glycoproteins in biofilms. Glycoproteins have been reported to play a role in cell-to-cell signaling and communication. The ascending trend of glycoprotein expression in biofilm is suggestive of a similar role of cell-to-cell communication/signaling. Cell-to-cell communication for quorum sensing in biofilms is critical. Though we report specific genes that were upregulated at a particular time of biofilm growth, we could not fully understand or implicate the role of these genes in biofilm formation. Knockout studies would help to clarify the role of these genes further.

Table 5 Number of genes upregulated with a ascending pattern from 4 to 24 hours of biofilms growth as compared to the 24 hours broth culture of *L. moncytogenes* J0161.

Fold variation	Number of genes	% of genes
More than 6 fold variation	1	0.1
More than 5 fold variation	8	1
More than 4 fold variation	20	2.4
More than 3 fold variation	44	5.3
More than 2 fold variation	86	10.3
More than 1 fold variation	330	39.5
Less than 1 fold variation	347	41.5

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Table 6 List of genes and their annotations upregulated with a ascending pattern from 4 to 24 hours of biofilms growth as compared to the 24 hours broth culture of *L. monocytogenes* J0161

Gene	Variation (ascending) in fold expression from 4 to 24 hours of biofilm	Gene description
LMOG_03104T0	Above 6.0	gp49
LMOG_03102T0	5.0 to 6.0	Hypothetical protein
LMOG_02195T0		Magnesium transporter
LMOG_01461T0		Zinc ABC transporter
LMOG_03098T0		gp43
LMOG_03101T0		Predicted protein
LMOG_03111T0		Hypothetical protein
LMOG_03129T0		gp11
LMOG_03115T0		gp63
LMOG_03099T0	4.0 to 5.0	gp44
LMOG_03113T0		Predicted protein
LMOG_03097T0		gp37
LMOG_03100T0		gp39
LMOG_03106T0		Predicted protein
LMOG_03116T0		gp64
LMOG_03140T0		gp22
LMOG_03133T0		gp15
LMOG_03090T0		gp37
LMOG_03120T0		Phage terminase small subunit
LMOG_02114T0		Methyltransferase
LMOG_01262T0		Hypothetical protein
LMOG_03107T0		gp51
LMOG_02115T0		Rrf2 family protein
LMOG_01386T0		YvgZ
LMOG_00932T0		Spermidine/putrescine import ATP-binding protein potA
LMOG_01462T0		Zinc ABC transporter
LMOG_01652T0		Inner membrane ABC transporter permease YcjP
LMOG_03122T0		Phage portal protein
LMOG_01650T0		ABC-type sugar transport system periplasmic binding protein YcjN

Table 7 Number of genes upregulated with a descending pattern from 4 to 24 hours of biofilms growth as compared to the 24 hours broth culture of *L. monocytogenes* J0161

Fold variation	Number of genes	% of genes
More than 6 fold variation	2	0.2
More than 5 fold variation	6	0.7
More than 4 fold variation	19	2.2
More than 3 fold variation	34	4
More than 2 fold variation	120	14.2
More than 1 fold variation	296	34.9
Less than 1 fold variation	370	43.7

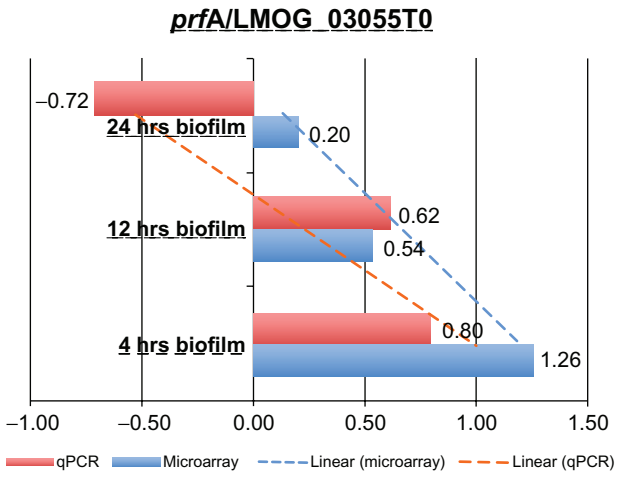

Figure 3 Comparison of expression data of *prf*A from microarray and qPCR.

Table 8 List of genes and their annotations upregulated with a descending pattern from 4 to 24 hours of biofilms growth as compared to the 24 hours broth culture of *L. monocytogenes* JO161

Gene	Variation (descending) in fold expression from 4 to 24 hours of biofilm formation	Gene description
LMOG_02681T0	Above 6.0	Fructose-specific IIA PTS system component
LMOG_02679T0		Fructose-specific PTS system fructose-specific II component
LMOG_01728T0	5.0 to 6.0	Cellobiose-specific PTS system IIA component
LMOG_02680T0		Fructose-specific PTS system IIB component
LMOG_01844T0		Alcohol dehydrogenase
LMOG_01843T0		Alcohol dehydrogenase
LMOG_01845T0		Ribose 5-phosphate isomerase B
LMOG_00557T0		Hypothetical protein
LMOG_01848T0	4.0 to 5.0	Ribulose-phosphate 3-epimerase
LMOG_01842T0		Galactitol-specific PTS enzyme IIC component
LMOG_01839T0		PTS system IIA 2 domain-containing protein
LMOG_02678T0		Alpha-mannosidase
LMOG_01846T0		Ribulose-phosphate 3-epimerase
LMOG_01215T0		Cold shock protein CspB
LMOG_01841T0		Galactitol-specific PTS enzyme IIB component
LMOG_00362T0		Competence protein ComGA
LMOG_01840T0		Galactitol-specific PTS system IIA component
LMOG_01729T0		ROK family protein
LMOG_02677T0		PRD/PTS system IIA 2 domain-containing protein
LMOG_01162T0		Hypothetical protein
LMOG_01996T0		Glycosyl hydrolase family 1 subfamily
LMOG_00980T0		Hypothetical protein
LMOG_01371T0		DedA family protein
LMOG_02000T0		D-allulose-6-phosphate 3-epimerase
LMOG_01731T0		Cellobiose-specific PTS system IIB component
LMOG_01094T0		Maltose/maltodextrin ABC transporter
LMOG_01732T0		Beta-glucosidase

Table 9 List of genes unregulated commonly at all the three time intervals

S no	Gene	Annotation
1	LMOG_02396T0	Clp protease
2	LMOG_00591T0	Clp protease
3	LMOG_02411T0	Hypothetical protein
4	LMOG_02477T0	NifU family protein
5	LMOG_00277T0	Nramp family mn2+/fe2+ transporter
6	LMOG_02607T0	ATP:guanido phosphotransferase
7	LMOG_00679T0	Molybdopterin biosynthesis protein MoeA
8	LMOG_01005T0	ATP-dependent chaperone ClpB
9	LMOG_02567T0	Internalin C2
10	LMOG_01012T0	Transcriptional regulator
11	LMOG_02442T0	ArsC family protein
12	LMOG_00358T0	Glycine dehydrogenase
13	LMOG_02443T0	Glycine cleavage system H protein
14	LMOG_01666T0	Phosphoserine aminotransferase
15	LMOG_01498T0	Helix-turn-helix domain-containing protein
16	LMOG_03035T0	Hypothetical protein
17	LMOG_00763T0	Hypothetical protein
18	LMOG_00674T0	Molybdenum cofactor biosynthesis protein A
19	LMOG_00286T0	Hydroxymethylglutaryl-CoAsynthase
20	LMOG_01784T0	GW repeat-containing protein
21	LMOG_00411T0	LexA repressor
22	LMOG_00675T0	Molybdenum cofactor biosynthesis protein C
23	LMOG_01667T0	D-isomer specific 2-hydroxy acid dehydrogenase
24	LMOG_01701T0	Chromosome partitioning protein parA/ Sporulation initiation inhibitor protein Soj
25	LMOG_01013T0	OsmC/Ohr family protein
26	LMOG_00309T0	3-ketoacyl-(acyl-carrier-protein) reductase
27	LMOG_00287T0	Acetyl-CoA acetyltransferase
28	LMOG_02412T0	Carboxylesterase
29	LMOG_00049T0	Muramoyl-tetrapeptide carboxypeptidase family
30	LMOG_00321T0	Isopentenyl-diphosphate delta-isomerase
31	LMOG_01783T0	Peptidoglycan bound protein
32	LMOG_00234T0	HDIG domain-containing protein
33	LMOG_00237T0	Cytidine deaminase
34	LMOG_00225T0	Co-chaperone GrpE

Table 9 (*Continued*)

S no	Gene	Annotation
35	LMOG_00667T0	Pyruvate dehydrogenase complex, E1 component, pyruvate dehydrogenase beta subunit
36	LMOG_01759T0	CBS domain-containing protein
37	LMOG_00224T0	Heat-inducible transcription repressor HrcA
38	LMOG_00304T0	RecA protein
39	LMOG_00236T0	Diacylglycerol kinase
40	LMOG_01170T0	Protoheme IX farnesyltransferase
41	LMOG_00661T0	Thioredoxin family protein
42	LMOG_01018T0	Oligopeptide ABC transporter oligopeptide-binding protein
43	LMOG_02468T0	Hypothetical protein
44	LMOG_02473T0	HAD-superfamily subfamily IIA hydrolase
45	LMOG_02386T0	Thioredoxin-disulfide reductase
46	LMOG_00946T0	HAD-superfamily hydrolase
47	LMOG_00233T0	PhoH family protein
48	LMOG_00676T0	Molybdopterin converting factor subunit 1
49	LMOG_00322T0	Hypothetical protein
50	LMOG_01573T0	Accessory regulator protein C
51	LMOG_01006T0	Phosphoglycerate mutase
52	LMOG_02471T0	Ser/Thr protein phosphatase
53	LMOG_01702T0	Partition protein ParB homolg
54	LMOG_02604T0	Pin/tram domain-containing protein
55	LMOG_00133T0	Transcriptional regulator NrdR
56	LMOG_00325T0	Hypothetical protein
57	LMOG_00843T0	CopG family transcriptional regulator
58	LMOG_00130T0	DNA polymerase I
59	LMOG_00128T0	Methylcitrate synthase
60	LMOG_02374T0	Excinuclease ABC B subunit
61	LMOG_01572T0	Accessory regulator protein A
62	LMOG_00100T0	Septation ring formation regulator EzrA
63	LMOG_00235T0	Hypothetical protein
64	LMOG_00824T0	Hypothetical protein
65	LMOG_00095T0	Hypothetical protein
66	LMOG_00662T0	Hypothetical protein
67	LMOG_01019T0	Oligopeptide ABC transporter permease
68	LMOG_02999T0	Hypothetical protein
69	LMOG_00623T0	PTS system IIB component
70	LMOG_00694T0	Hypothetical protein
71	LMOG_00326T0	OxaA-like protein
72	LMOG_02171T0	p60
73	LMOG_00448T0	Signal peptidase I
74	LMOG_00406T0	Transketolase
75	LMOG_00048T0	DNA-3-methyladenine glycosylase I
76	LMOG_00091T0	General stress protein
77	LMOG_00211T0	Nicotinate nucleotide adenylyltransferase
78	LMOG_01200T0	S4 domain-containing protein
79	LMOG_01622T0	Mevalonate kinase
80	LMOG_00713T0	Transcriptional regulator
81	LMOG_00840T0	RsbS
82	LMOG_00131T0	Formamidopyrimidine-DNA glycosylase
83	LMOG_02455T0	Carbon-sulfur lyase
84	LMOG_00371T0	Hypothetical protein
85	LMOG_01574T0	Accessory regulator protein D
86	LMOG_00312T0	ABC transport system permease
87	LMOG_01136T0	Lipase
88	LMOG_02347T0	Transcriptional regulatory protein DegU
89	LMOG_00020T0	Peptidoglycan linked protein
90	LMOG_00302T0	Hypothetical protein
91	LMOG_01907T0	50S ribosomal protein L17
92	LMOG_00839T0	Serine/threonine-protein kinase rsbT
93	LMOG_00315T0	Surface antigen
94	LMOG_00301T0	Acetyltransferase
95	LMOG_01281T0	Fur protein
96	LMOG_00842T0	PemK family transcriptional regulator
97	LMOG_00665T0	Dihydrolipoyl dehydrogenase
98	LMOG_02735T0	Hypothetical protein
99	LMOG_00663T0	Lactate/malate dehydrogenase
100	LMOG_01172T0	Regulatory protein YlbF
101	LMOG_01261T0	DNApolymerase IV
102	LMOG_01173T0	Hypothetical protein
103	LMOG_00011T0	Hydrolase
104	LMOG_00188T0	TPR domain-containing protein
105	LMOG_03307T0	dTDP-4-dehydrorhamnose 3,5-epimerase
106	LMOG_02392T0	Hypothetical protein
107	LMOG_02474T0	Acetyltransferase
108	LMOG_00841T0	Modulator protein RsbR
109	LMOG_00083T0	tRNA binding domain-containing protein
110	LMOG_00771T0	Glucosamine-6-phosphate isomerase
111	LMOG_03305T0	Minor teichoic acids biosynthesis protein GgaB
112	LMOG_01099T0	Hypothetical protein
113	LMOG_02767T0	CamS sex pheromone cAM37
114	LMOG_01188T0	Cell division protein ftsL
115	LMOG_00389T0	Hypothetical protein
116	LMOG_00349T0	General stress protein
117	LMOG_02571T0	DNA-directed RNA polymerase beta subunit
118	LMOG_02771T0	Hypothetical protein
119	LMOG_01340T0	Pantoate-beta-alanine ligase
120	LMOG_00204T0	Hypothetical protein
121	LMOG_00207T0	HAD superfamily phosphatase
122	LMOG_00699T0	Potassium transport system NAD-binding component
123	LMOG_01621T0	Diphosphomevalonate decarboxylase
124	LMOG_00802T0	Sulfatase
125	LMOG_00299T0	YmcA
126	LMOG_02353T0	Preprotein translocase SecA subunit
127	LMOG_00191T0	DNA-binding response regulator
128	LMOG_02456T0	NifU family SUF system FeS assembly protein
129	LMOG_00823T0	Hypothetical protein

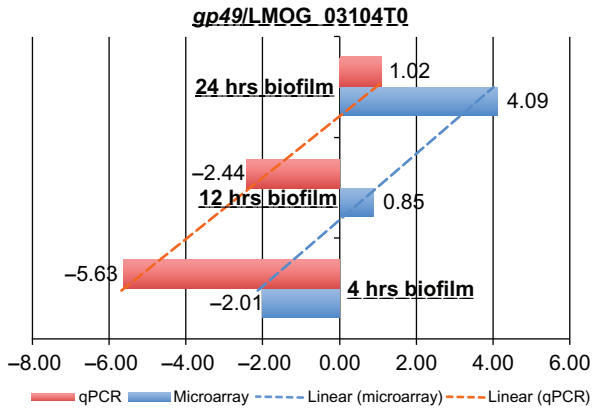

Figure 4 Comparison of expression data of *gp49* from microarray and qPCR.

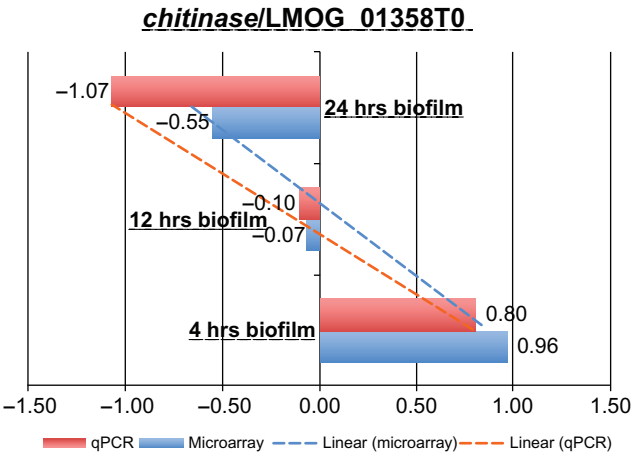

Figure 5 Comparison of expression data of *chitinase* from microarray and qPCR.

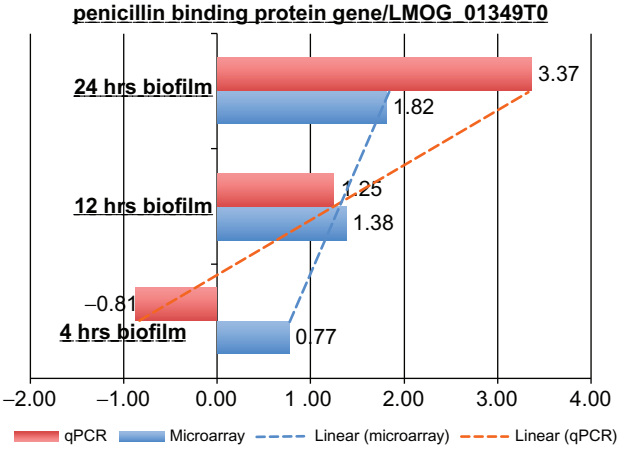

Figure 6 Comparison of expression data of penicillin binding protein from microarray and qPCR.

Disclosure

The authors report no conflicts of interest in this work.

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