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Carbon Catabolite Control is Important for *Listeria monocytogenes* Biofilm Formation in Response to Nutrient Availability

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Abstract The foodborne pathogen *Listeria monocytogenes* has the ability to develop biofilm in food-processing environment, which becomes a major concern for the food safety. The biofilm formation is strongly influenced by the availability of nutrients and environmental conditions, and particularly enhanced in poor minimal essential medium (MEM) containing glucose rather than in rich brain heart infusion (BHI) broth. To gain better insight into the conserved protein expression profile in these biofilms, the proteomes from biofilm- and planktonic-grown cells from MEM with 50 mM glucose or BHI were compared using two-dimensional polyacrylamide gel electrophoresis followed by MALDI-TOF/TOF analysis. 47 proteins were successfully identified to be either up (19 proteins) or down (28 proteins) regulated in the biofilm states. Most (30 proteins) of them were assigned to the metabolism functional category in cluster of orthologous groups of proteins. Among them, up-regulated proteins were mainly associated with the pentose phosphate pathway and glycolysis, whereas a key enzyme CitC involved in tricarboxylic acid cycle was down-regulated in biofilms compared to the planktonic states. These data implicate the importance of carbon catabolite control for *L. monocytogenes* biofilm formation in response to nutrient availability.

Qingchun Zhou and Xiaoqin Feng contributed equally to this work and should be considered as co-first authors.

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Introduction

Biofilms are densely packed multicellular communities of microorganisms attached to a biotic or abiotic surface. It is well known that bacterial cells within biofilms are highly resistant to antibiotics, UV light, acid exposure, dehydration, and phagocytosis in comparison to their free-living (planktonic) forms [12] and are metabolically different from their free-living forms. *Listeria monocytogenes*, a Gram-positive food borne pathogen responsible for listeriosis, has the ability to form biofilms on food-processing surfaces, which is a major concern for the food safety because bacteria from biofilms can contaminate food products during processing and packaging [7]. Earlier studies and our present data showed that this bacterium preferably produced biofilm in nutrient-limited essential medium rather than the nutrient-rich medium, although the growth rates of cells were dominantly higher in the rich medium [19, 20]. However, the molecular mechanism of nutrient availability as the specific environmental signal to promote biofilm formation is not clear.

To understand the underlying mechanism of biofilm formation of *L. monocytogenes* adapting to the different nutrient conditions, the differentially expressed proteins obtained from both brain heart infusion (BHI) broth and poor minimal essential medium (MEM) containing 50 mM glucose were investigated in this study using two-dimensional polyacrylamide gel electrophoresis (2-DE) followed by MALDI-TOF/TOF analysis. Our results indicated that carbon catabolite control might be a critical regulatory

determinant of bacterial biofilm formation under a variety of cultivation conditions.

Materials and Methods

Bacterial Cultures and Growth of Biofilms

Listeria monocytogenes EGDe (serotype 1/2a) is a gift from Prof. W. Goebel of Department of Microbiology in Wuerzburg University in Germany. For all experiments, individual colonies of the stock culture were inoculated into 5 mL of BHI broth (Difco, Sparks, MD) and incubated at 37 °C with shaking (180 rpm) for overnight, then transferred either directly into the fresh BHI broth with 1:50 dilution, or after three times of washing with the MEM, the pellets were resuspended with 250 mL of MEM [13] and grown at 37 °C with shaking until the optical density at 600 nm reached 0.2, followed by dispensing into microtiter plates or sterile plastic petri dishes.

Microtiter Plate Assay and Fluorescence Microscopic Observation

The microtiter plate assay described previously by Zhou et al. [23] was used to quantify biofilm production of *L. monocytogenes* EGDe cultured in BHI or MEM at 37 °C after 24, 48, and 72 h.

For fluorescence microscopic observation, biofilm was grown on the glass cover slips which were submerged in a six-well plate (NestBiotech Co., Ltd, Wuxi, China) containing 6 mL of bacterial cultures prepared as described above. The plate was incubated statically at 37 °C for 3, 24, 48 h or 5 days. After the indicated incubation period, slips were taken out carefully and rinsed thrice with phosphate-buffered saline (PBS; pH 7.2) to remove loosely adhered bacteria, and then immersed into 0.1 % fluorescein isothiocyanate (FITC; Sigma-Aldrich) in the dark for 30 min at room temperature to stain the sessile cells in the biofilms. Following staining, slips were rinsed thrice with PBS. Biofilms formed were examined and photographed using Nikon fluorescence microscope E400 (Nikon company, Japan).

Protein Extraction, Two-Dimensional Polyacrylamide Gel Electrophoresis (2-DE)

Biofilms for proteomic analysis were grown on 20 sterile plastic petri dishes (10 cm in diameter) filled with 20 mL of BHI or MEM statically at 37 °C for 48 h. For collection of biofilm-grown cells, the media were discarded and then the dishes were carefully washed thrice with Tris buffer (pH 7.2) to remove loosely adhered bacteria. The cells attached to the dishes were scraped off using a sterile razor and put into 10 mL of Tris buffer and then pelleted by centrifugation (4,000 rpm, 15 min). The planktonic-grown cells (PC) from BHI or MEM medium were grown at 37 °C with shaking (180 rpm) until the optical density at 600 nm reached 0.5. After centrifugation, the pellets were washed thrice with Tris buffer to remove thoroughly the chemical components. For protein extraction, the cell pellets were resuspended in 1.2 mL of pre-cooled sonication buffer containing 7 mol/L urea (Biosharp), 2 mol/L thiourea (Biosharp), 2 % CHAPS (Sigma), 65 mmol/L DTT (Sigma), 0.5 % pharmalyte 3–10 (Bio-Rad), 0.5 mg/mL DNase I (Bio-Rad), 70 U RNase A (Sigma) and 100 mmol/L PMSF (Bio-Rad), 5 μL of protease inhibitor cocktail (Bio-Rad) and 3 g of sterilized silica sands (Sinopharm Chemical Reagent Co. Ltd, China). Cells were sonicated with a UP200S sonifier (Hielscher, Germany) on ice bath eight times for 40 s (20 s pause in between) at power level 5 and 30 % of the active cycle. The cell pellets and silica sands were eliminated by centrifugation at 4 °C for 60 min (13,000×*g*). The supernatant was collected and stored at −80 °C in aliquots until use. Protein concentrations were determined using Bradford protein assay according to the manufacturer's instructions (Bio-Rad).

Protein extracts (690 μg) were cup-loaded onto 17-cm strips with a pH range of 4 to 7 (Bio-Rad) for isoelectric focusing, which was performed using a flat-bed Multiphor II (Amersham Biosciences), and then separated on a 12 % SDS-polyacrylamide gel using a Protean II Xi cell (Bio-Rad) as previously reported by Dumas et al. [5]. The gels were stained with Coomassie Brilliant Blue G-250 (Bio-Rad) and scanned on a PowerLook 2100XL scanner (UMAX Technologies).

Image Analysis of the 2-DE Gels and Protein Identification

Image analysis of the 2-DE gels was performed using the PD Quest 7.3.0 software (Bio-Rad) and gel comparisons were performed through a master gel that was a synthetic image containing all the protein spots of all the gels to be analyzed. Spot intensities were normalized to the sum of intensities of all valid spots in the master gel. Each protein spot was thus identified by a unique master number. Only reliable protein spots were retained for analysis. A protein spot was considered reliable when it was present or absent at least in two gels out of three repetitive gels for a strain. For analysis of changes in protein expression in the biofilm or planktonic states under BHI or MEM media, a protein was considered differentially produced when changes in normalized spot intensities were of least twofold at a significance level of $P < 0.05$ (Student's t test for paired samples). Three independent biological replicates were carried out under the same condition.

Spots of interest were subjected to tryptic in-gel digestion and analyzed by a MALDI-TOF/TOF mass spectrometer (ABI 4700 Proteomics Analyzer; Applied Biosystems). For data acquisition and export, two software packages, 4000 Series software and GPS Explorer™ v3.5, provided by the ABI were used. After data acquisition, files were uploaded for searching the NCBI (nr) *L. monocytogenes* EGD-e database with the following selection parameters: trypsin digestion allowing up to one missed cleavage, methionine oxidation, cysteine carbamidomethyl and peptide tolerance of ±0.1 Da. Only proteins of their protein score CI % ≥95% were considered to be correctly identified.

Results

Formation of Biofilm was Enhanced in Glucose Minimal Essential Medium

To investigate whether the culture media has an impact on biofilm formation, *L. monocytogenes* EGDe was cultured in both in rich BHI and poor glucose MEM media. As shown in Fig. 1b and C, *L. monocytogenes* EGDe was able to develop biofilm with meshwork of bacterial aggregates in those media. Dense localized biofilm structures formed within 24 h and became denser after 24 h. Thicker and complex biofilm structures were formed after 48 h of incubation (Fig. 1c). However, biofilm biomass generated in MEM was much greater than that in a parallel BHI culture inoculated at the same period, while the growth rates of cells in BHI were actually higher than those in MEM (Fig. 1a, b). The fluorescence microscopy also showed that the biofilm formed in MEM had more clumped and aggregated cells than that in BHI even in the early phase of biofilm development (3 h), which were well consistent with the quantitative estimations of biofilms.

Identification of Proteins Differentially Expressed Under Biofilm Formation Conditions

Given that *L. monocytogenes* can develop biofilm in different nutrient conditions, we therefore propose that a conserved signaling pathway for nutrient metabolism exists in *L. monocytogenes* biofilm formation under different environments, in which the capability of biofilm development in different nutrient conditions would be due to differential regulation of this conserved signal pathway. To test this hypothesis, the differentially expressed proteins common in two biofilm states cultivated under rich BHI broth and poor MEM supplemented with 50 mM glucose were investigated in the present study using 2-dimensional polyacrylamide gel electrophoresis. Figure 2a–d shows representatives of 2-DE patterns for *L. monocytogenes* wild-type strain EGDe grown in MEM or BHI under biofilm and planktonic states. A total of 47 proteins were successfully identified to be differentially expressed in both biofilm states (BHI and MEM) compared to their corresponding planktonic states, 19 of which were up-regulated or only detected in the biofilm states, and 28 were down-regulated or not found in the protein profiles from biofilm-grown cells (Fig. 1; Table 1). Meanwhile, five proteins identified were found in more than one spot, indicating that the protein isoforms or posttranslational modification events occurred.

Based on analysis of the cluster of orthologous groups of proteins (COG) obtained from the MicrobesOnline Comparative Genomics Database (MOCGD) (http://www.microbesonline.org/), these proteins can be classified into 11 functional categories (Table 1), and most of them (30 proteins) were involved in energy production (7 proteins), metabolism of amino acid (7 proteins), nucleotide (6 proteins), carbohydrate (9 proteins), and lipid (1 protein). Remarkably, several key enzymes involved in the pentose phosphate pathway (6-phosphogluconate dehydrogenase encoded by *lmo1376*, 6-phosphogluconolactonase encoded by *pgl* and transketolase encoded by *tkt*) and the 6-phosphofructokinase encoded by *pfkA* in glycolysis were present at a higher level in biofilms, while the isocitrate dehydrogenase encoded by *citC*, which converts isocitrate to alpha ketoglutarate in the tricarboxylic acid (TCA) cycle, was down-regulated in biofilm-growth cells compared with planktonic-growth cells. These data suggest that TCA activity may be present to a low level in *L. monocytogenes* biofilms, so that energy will be supplied from glycolysis and the pentose phosphate pathways (see "Discussion"). Interestingly, expression of two enzymes, Sod (superoxide dismutase encoded by *lmo1439*) and MsrA (methionine sulfoxide reductase A encoded by *lmo1860*), which play a role in cellular defense against oxidative stress, was also repressed in biofilms. Since an increased ratio of TCA cycle to respiratory activity boosts the production of reactive oxygen species (ROS) such as O^{2-} and subsequently H_2O_2, i.e., oxidative stress, low expression level of Sod and MrsA may be due to relatively low TCA activity. In fact, Nguyen et al. [11] reported that the activity of Sod was reduced in response to the repressed respiration in oral Streptococci biofilm and considered the low respiratory activity would facilitate bacterial cells to survive in the crowded biofilm condition. Moreover, enzymes related to degradation of protein, peptides, glycopeptides (Lmo2188, Lmo1611, and Lmo1138), and mRNA (PnpA) were repressed in biofilms, while those involved in protein synthesis were up-regulated, particularly, in cell wall biogenesis, including elongation factors G and Ts, 50S ribosomal protein L10, Lmo0197, and

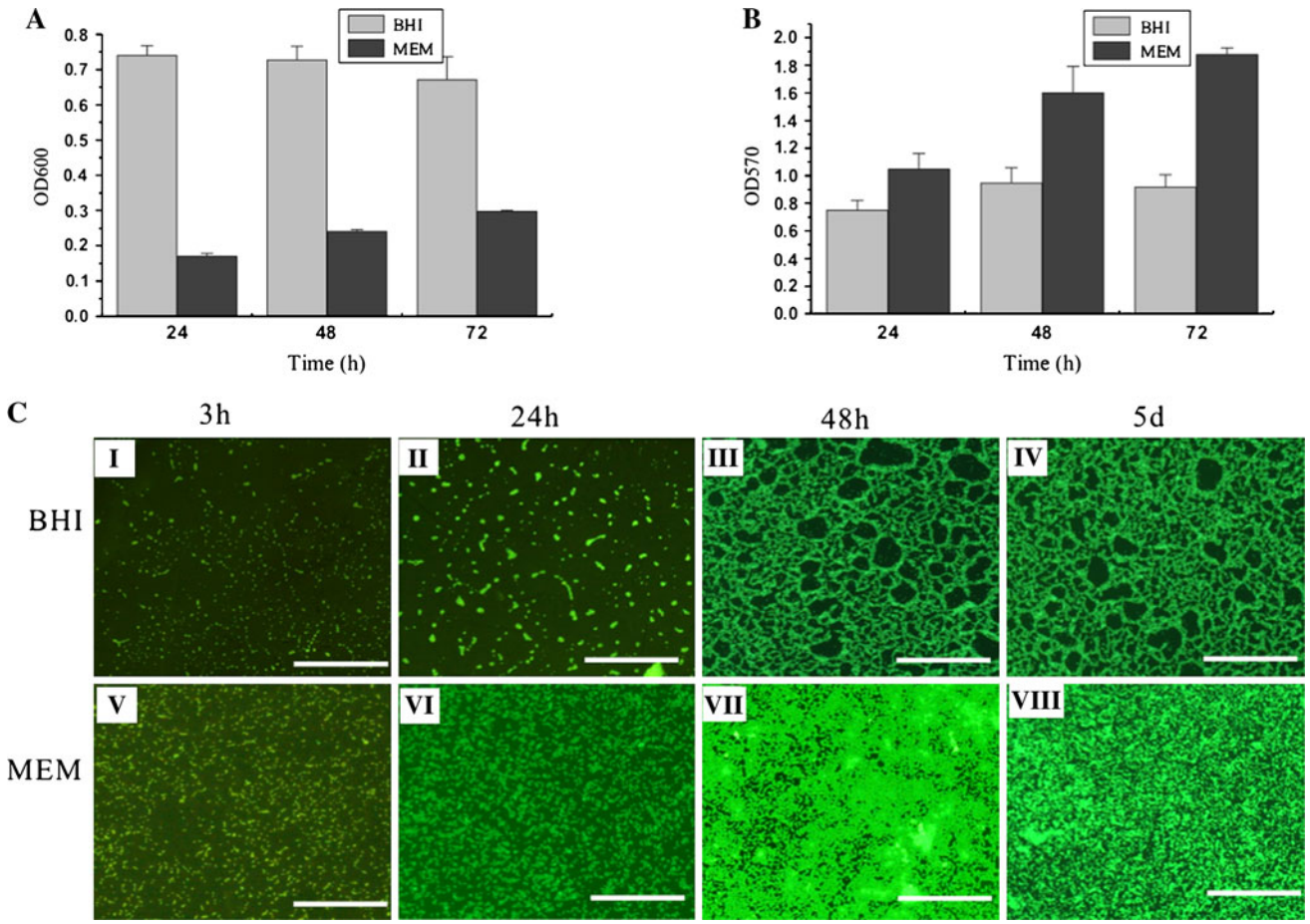

Fig. 1 Biofilm formation by *L. monocytogenes* EGDe in BHI and MEM with 50 mM glucose. **a** Cell densities after 24, 48, and 72 h incubation. **b** Biofilm productions measured by microtiter plate assay after 24, 48, and 72 h incubation. **c** Fluorescence microscopy observation of *L. monocytogenes* EGDe biofilm formation. The strain was grown at 37 °C in BHI (*I–IV*) or MEM with 50 mM glucose (*V–VIII*) on glass slips for 3, 24, 48 h, and 5 days. The *error bars* represent the standard deviations of triplicate experiments (when not visible, *error bars* are smaller than the thickness of the bar border). *Bars* equal 0.1 mm

Lmo2391. Furthermore, a glutamine synthetase encoded by *glnA* was also up-regulated. Glutamine synthetase necessary for cell wall resistance and biofilm formation of *Mycobacterium bovis* [2] has been found to be synthesized in higher amounts in the proteomic study of *Staphylococcus aureus* biofilm cells [14]. In addition, two proteins GuaB and Upp were detected to be up-expressed in *L. monocytogenes* biofilm in this study. GuaB that converts IMP into GMP was important for the production of *Mycobacterium avium* biofilm [22], and Upp that catalyzes uracil to UMP was required to biofilm development in *Pseudomonas aeruginosa* through control of quorum-sensing (QS) pathways [21]. Upp was aslo found to be induced by glucose starvation in *L. monocytogenes* biofilm [8]. These differentially expressed proteins have also been identified as differentially expressed during biofilm formation by other bacteria, suggesting that there may be widely conserved responses of bacterial cells to biofilm formation.

Discussion

In this study, proteomics was applied to gain insight into key cellular events that allow *L. monocytogenes* to develop mature biofilms under nutrient-rich (BHI) and poor (MEM) conditions. We observed that the biofilm-forming processes mainly affected metabolic pathways for energy production and transport as well as metabolism of amino acid, nucleotide, and carbohydrate. Interestingly, many proteins related to the pentose phosphate pathway and glycolysis were found to present in a higher level, while a key enzyme CitC involved in TCA cycle were down-regulated in biofilms compared to the planktonic states. The enzymatic activity of the TCA cycle has been found to be negatively associated with the synthesis of the biofilm-promoting exopolysaccharide polysaccharide intercellular adhesin (PIA) in *S. aureus* and *S. epidermidis*, in which the activated TCA cycle decreased PIA synthesis, reduced biofilm formation, and significantly attenuated virulence in

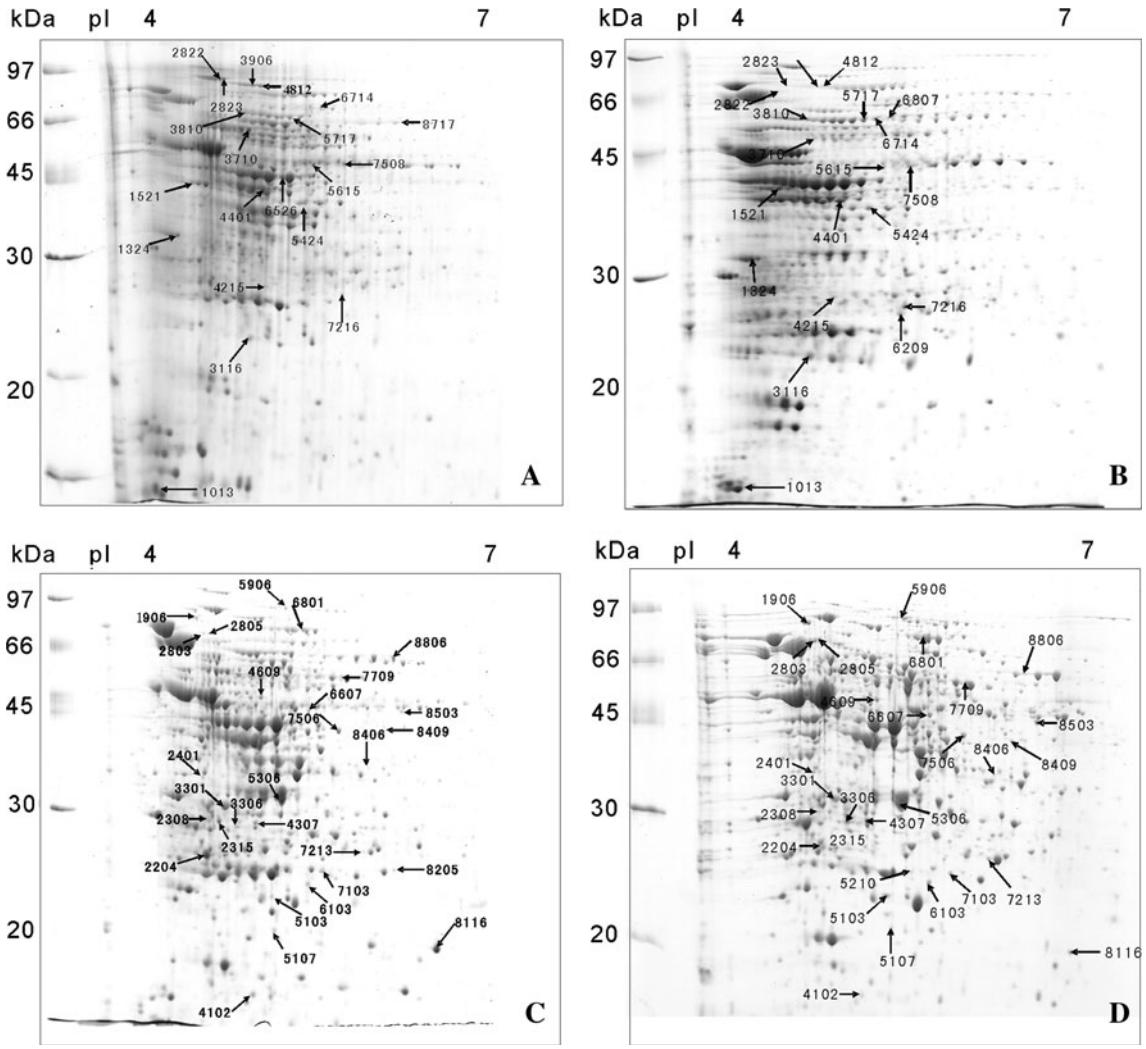

Fig. 2 2-DE gels of cytoplasmatic extracts of *L. monocytogenes* EGDe from biofilm-grown cells in MEM with 50 mM glucose (**a**) or BHI (**b**), and planktonic-grown cells cultured in MEM with 50 mM glucose (**c**) or BHI (**d**). *Spots* identified by mass spectrometry are labeled and their identification is listed in Table 1

an *in vivo* biofilm model [16, 24]. This negative regulation of entrance to the TCA cycle was considered to allow the biofilm-growth cells to avoid the production of excess ATP as long as they can obtain enough ATP through glycolysis and the pentose phosphate pathways (i.e., substrate level phosphorylation) [16, 24]. In addition, while some specific determinants including flagella proteins [9] and the Agr system for the putative QS peptide [15] for *L. monocytogenes* biofilm formation were not detected in this study, this might be due to the detection limit of the proteomic technique and nonoptimal temperature (37 °C) for the expression of listerial flagella proteins used in this study. However, the transcription and/or stability of RNAIII, part of the Agr QS system, was increased in an *S. aureus* TCA cycle mutant [24], raising the possibility that decreased TCA cycle activity could increase bacteria biofilm development via alteration of RNAIII transcription levels.

A significant number of reports have demonstrated that a specific environmental signal nutrient availability plays a critical role in promotion of *L. monocytogenes* biofilm formation [3, 4]. Specifically, bacterial biofilm mass is enhanced during growth in minimal essential media containing glucose [3, 19], suggesting the global catabolite regulation (CR) may influence bacterial biofilm formation. Similar to other low G + C content gram-positive bacteria, such as *Bacillus subtilis* and *S. aureus*, in *L. monocytogenes*, the predominant mechanism of CR involves transcriptional repression mediated by the catabolite control protein A (CcpA) [1]. After formation of a complex with the phosphorylated co-repressors, HPr, one of the components of the phosphoenolpyruvate:sugar phosphotransferase (PTS) sugar uptake system, CcpA becomes active and blocks transcription initiation via binding to the catabolite responsive elements (cre) located in or near carbon catabolite repressed

Table 1 Proteins of *L. monocytogenes* EGDe identified to be expressed differently between biofilm and planktonic states in BHI or MEM medium

Functional category[a]	Locus[b]	Putative function[c]	Spot no.[d]	MM/pI[e]	Protein score	Fold change of BC vs. PC[f] in	
						MEM	BHI
Energy production and conversion (C)	lmo1052 (pdhA)	Highly similar to pyruvate dehydrogenase (E1 alpha subunit)	7508	44/5.61	126	+	+
	lmo1053 (pdhB)	Highly similar to pyruvate dehydrogenase (E1 beta subunit)	1521	41.1/4.69	210	+	+
	lmo1055 (pdhD)	Highly similar to dihydrolipoamide dehydrogenase (E3 subunit of pyruvate dehydrogenase complex)	5717	58.7/5.38	143	+	+
			6714	58.6/5.45	108	+	+
	lmo1054 (pdhC)	Highly similar to pyruvate dehydrogenase (dihydrolipoamide acetyltransferase E2 subunit)	1906	82.4/4.72	110	−	−
	lmo1566 (citC)	Isocitrate dehydrogenase	4609	46.8/5.18	79.6	0.18	–
	lmo0485	Hypothetical protein (COG778 (NfnB) Nitroreductase)	8205	24.7/6.13	159	−	−
	lmo0913	Similar to succinate semialdehyde dehydrogenase	8806	59.5/6.12	167	0.02	−
Amino acid transport and metabolism (E)	lmo1299 (glnA)	Highly similar to glutamine synthetases	3810	60.1/5.01	141	14.18	+
			3906	75.7/5.08	154	3.36	+
	lmo1350	Glycine dehydrogenase subunit 2	6807	68.7/5.52	125	3.15	+
	lmo2188	Similar to oligoendopeptidase	2803	72.0/4.77	99.7	−	−
			2805	72.3/4.80	99.9	−	−
	lmo1011	Similar to tetrahydrodipicolinate succinylase	3301	30.2/4.93	150	0.03	0.11
	lmo1897 (aspB)	Aspartate aminotransferase	6607	43.6/5.52	145	−	−
	lmo1813	Similar to phosphoglycerate dehydrogenase	7213	26.2/5.91	167	−	−
	lmo1436	Aspartate kinase I	8503	43.6/6.18	95.9	−	−
Nucleotide transport and metabolism (F)	lmo2538 (upp)	Uracil phosphoribosyltransferase	6209	26.0/5.57	102	+	+
	lmo2758 (guaB)	Similar to inosine-monophosphate dehydrogenase	8717	57.5/5.95	132	+	3.24
	lmo1313 (pyrH)	Uridylate kinase	3306	28.3/5.01	108	−	−
	lmo2611 (adk)	Adenylate kinase	4307	28.6/5.14	109	−	0.08
	lmo0055 (purA)	Adenylosuccinate synthetase	7709	53.6/5.75	134	−	−
	lmo0199 (prs)	Ribose-phosphate pyrophosphokinase	8406	35.1/5.94	146	−	−
Carbohydrate transport and metabolism (G)	lmo1376	6-Phosphogluconate dehydrogenase	3710	53.1/5.05	196	2.22	2.59
	lmo1305 (tkt)	Highly similar to transketolase	4812	75.5/5.11	132	+	+
	lmo1571 (pfkA)	6-Phosphofructokinase	5424	37.4/5.34	133	+	2.08
	lmo0558 (pgl)	6-Phosphogluconolactonase	5615	44.1/5.45	144	+	+
	lmo2674	Ribose-5-phosphate isomerase B	4102	16.2/5.12	119	−	0.17
	lmo2556 (fbaA)	Similar to fructose-1,6-bisphosphate aldolase	5306	30.9/5.33	123	0.04	0.08
	lmo1570 (pykA)	Pyruvate kinase	6801	74.3/5.48	195	−	−
	lmo1611	Similar to aminopeptidase	7506	40.4/5.73	156	0.08	0.15
	lmo0783 (mpoB)	Similar to mannose-specific phosphotransferase system (PTS) component IIB	8116	19.1/6.4	154	0.01	−

Table 1 continued

Functional category[a]	Locus[b]	Putative function[c]	Spot no.[d]	MM/pI[e]	Protein score	Fold change of BC vs. PC[f] in	
						MEM	BHI
Lipid metabolism (I)	lmo0344	Short chain dehydrogenase	4215	27.5/5.17	128	+	+
Translation, ribosomal structure and biogenesis (J)	lmo2654 (fus)	Elongation factor G	2822	80.5/4.86	205	+	+
			2823	79.2/4.89	276	+	+
	lmo0250 (rplJ)	50S ribosomal protein L10	3116	22.7/5.02	105	+	+
	lmo1657 (tsf)	Elongation factor Ts	4401	38.6/5.14	208	2.78	3.02
	lmo1331 (pnpA)	Polynucleotide phosphorylase/polyadenylase	5906	89.1/5.36	111	−	0.07
Transcription (K)	lmo0246 (nusG)	Transcription antitermination protein NusG	5103	22.7/5.26	121	−	−
Cell envelope biogenesis, outer membrane (M)	lmo0197	Similar to *B. subtilis* SpoVG protein	1013	14.4/4.63	93.6	+	+
	lmo2391	Conserved hypothetical protein similar to *B. subtilis* YhfK protein	7216	26.5/5.65	104	+	+
	lmo1082	Similar to dTDP-sugar epimerase	7103	24.3/5.66	149	0.21	−
Posttranslational modification, protein turnover, chaperones (O)	lmo2415	Similar to ABC transporter, ATP-binding protein	1324	31.0/4.64	170	+	+
	lmo1138	ATP-dependent Clp protease proteolytic subunit	2204	25.9/4.84	118	−	0.09
	lmo1860 (msrA)	Methionine sulfoxide reductase A	6103	23.6/5.51	191	−	−
Inorganic ion transport and metabolism (P)	lmo0811	Similar to carbonic anhydrase	2308	28.9/4.81	141	−	−
			2315	28.8/4.88	191	−	−
	lmo2601 (cbiO)	Cobalt transporter ATP-binding subunit	2401	33.8/4.77	92.3	−	−
	lmo1439 (sod)	Superoxide dismutase	5210	24.6/5.39	94.2	−	−
General function prediction only (R)	lmo2648	Similar to Phosphotriesterase	6526	41.4/5.53	116	+	2.67
	lmo1240	Conserved hypothetical protein, similar to *B. subtilis* YsnB protein	5107	20.4/5.28	97.9	−	−
	lmo1858	Similar to dehydogenases and hypothetical proteins	8409	40.3/6.05	134	−	−

[a] Functional category was according to the cluster of orthologous genes using the MicrobesOnline Comparative Genomics Database (MOCGD) (http://www.microbesonline.org/)

[b] Locus tag was obtained from NCBI (nr) *Listeria monocytogenes* EGD-e database with the common name in brackets

[c] Putative functions were assigned from NCBI (nr) *Listeria monocytogenes* EGD-e database

[d] Spots numbers refer to the proteins excised from gels and labeled in Fig. 2

[e] Theoretical molecular masses (expressed in kDa) and isoelectric point (expressed in pI)

[f] Changes in protein levels was reported as the ratio between the normalized protein spot volume derived from biofilm-grown cells (BC) of *L. monocytogenes* EGDe cultured in MEM with 50 mM glucose or in BHI and the PC cultured in the same media. Only Change fold greater than 2 or less than 0.5 and statistically significant results ($P < 0.05$) are presented. +, novel spot. −, not detected

promoters. The phosphorylation of Hpr is controlled by the activity of a kinase that responds to the intracellular concentration of fructose 1-6-bisphosphate (FBP), which varies based on the rate of glucose transport into the cell [1]. High concentration of FBP can activate the ATP-dependent HPr kinase to phosphorylate HPr and form a complex with CcpA. CcpA has been shown to coordinate central metabolism and biofilm formation in some gram-positive bacteria, such as *B. subtilis*, *S. aureus*, and *S. epidermidis*. In *B. subtilis*, CcpA enhances pyruvate assimilation and shut-down of the TCA cycle and respiration [6]. In *S. aureus* and *S. epidermidis*, CcpA functions as a regulator of the TCA cycle, deletion of *ccpA* derepressed TCA cycle activity and inhibited biofilm formation [16, 17]. In this study, two important proteins closely related to up-regulation of CcpA activity were detected: the 6-phosphofructokinase encoded by *pfkA* which catalyzes the formation FBP from D-fructose 6-phosphate, was up-regulated; and Lmo0783 (MpoB), a putative mannose-specific PTS component IIB, which is one of components of the major glucose uptake systems in *L. monocytogenes* [18] and negatively controlled by CcpA [10], was down-regulated. These results indicated that the activity of CcpA in the mature biofilms might be higher than that in the planktonic state. Hence, the observation that formation of biofilm by *L. monocytogenes* was enhanced during growth in glucose MEM appear partially due to the relatively higher level of CcpA-mediated CR in MEM than that in BHI, since the growth of *L. monocytogenes* in nutrient-rich media depends only in part on CcpA-regulated carbohydrate metabolism [18]. Therefore, although the impact of up-expression of the pyruvate dehydrogenase complex (except PdhC; this complex was encoded by *pdhABCD* operon that oxidates pyruvate to acetyl-CoA) in biofilms remains to be unclear and needs to be further investigated, given the importance of coordination of carbon source utilization and regulation of metabolic pathways, it is reasonable to assume that CcpA could have a positive role in regulation of genes that are important for biofilm formation by *L. monocytogenes*. This hypothesis needs to be further investigated, while the absence of CcpA protein identified in this study can be due to the detection limit of the proteomic technique. We are currently working on this to confirm our proposal.

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