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Over-Production of P60 Family Proteins, Glycolytic and Stress Response Proteins Characterizes the Autolytic Profile of *Listeria monocytogenes*

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ABSTRACT

Listeria monocytogenes is a foodborne pathogen capable of surviving under challenging conditions both outside and inside the host. During the transition from exponential to stationary phase it experiences a series of environmental changes that require an appropriate response to maintain cell viability. In this study the autolytic behaviour of a *L. monocytogenes* strain was investigated by two-dimensional electrophoresis. The study was done at the permissive autolysis temperature, 30˚C and at 20˚C, an autolysis non-permissive temperature. An autolytic strain proteome was also compared to a non-autolytic strain at the permissive autolysis temperature. The autolytic strain proteome at 30˚C in comparison to 20˚C evidenced increased synthesis of the P60 autolysin, glycolytic enzymes and proteins related with environmental stress responses. The over-production of P45 autolysin, was observed when the autolytic strain proteome was compared with the non-autolytic strain. The proteomes at the non-permissive temperature and the proteome of the non-autolytic strain were characterized by a diminished synthesis of several stress related proteins. The lack of autolysis seems to be associated to the over-production of proteins linked to fatty acid and amino acid synthesis, transcription regulation and cell morphogenesis as evidenced by the proteome at the non-permissive temperature and the non-autolytic strain. Autolysis proteome evidenced the over-production of P60 autolysins, glycolysis and stress proteins whereas the proteome obtained in conditions of absence of autolysis reveal a completely different group of proteins. Possible targets to activate listerial autolysis were identified.

Keywords: *Listeria monocytogenes*; Autolysis Proteome; Stress Proteins

1. Introduction

Autolysis in *Listeria monocytogenes*, a Gram-positive bacterium associated with the foodborne disease listeriosis, has been known for a long time [1,2]. The phenomenon occurs as a result of the activity of peptidoglycan hydrolases called autolysins [3]. These peptidoglycan hydrolases can participate in a significant number of important biological processes, namely in cell wall turnover, cell separation, cell division and their contribution in pathogenicity, either for Gram positive (e.g. *Streptococcus pneumoniae*, *Staphylococcus aureus*) and Gram negative bacteria (e.g. *Helicobacter pylori*) [4-6], also has been demonstrated. In *L. monocytogenes*, six autolysins have been identified P60, P45, Ami, Mur, Auto and the Lmo0327 [7-13] and at least three of these autolysins, P60, Ami and Auto are involved in survival and virulence of *L. monocytogenes* [8,14,15].

The peptidoglycan hydrolases constitute a distinctive family of enzymes whose principal function is to exactly cut the peptidoglycan in order that new murein strands can be inserted. Their activity must be firmly controlled to avoid the strand breakage resulting in cell lysis. The autolysis process is widespread amongst bacteria and other microorganisms, such as yeast and fungi (e.g. *Saccharomyces cerevisae* and *Aspergillus nidulans*) [16,17]. In Gram positive bacteria, teichoic acids are considered the principal regulators of the peptidoglycan hydrolases [18,19]. The binding of murein hydrolases to teichoic acids is mediated by repeat elements known as choline-binding domains [20] and the connection of murein hydrolases to the cell wall occurs through the modification of teichoic acids. The addition of D-alanine ester linkages to teichoic acids modifies the surface charge and protects the cell from autolysis [21]. Besides the require-

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ment for choline-binding domains and D-alanylation of teichoic acids, other studies have demonstrated that pyruvylation of unspecified carbohydrates (presumably teichoic acids) also contribute to the control of the murein hydrolases; namely a *Bacillus anthracis* mutant defective in *csaAB* genes, that are responsible for pyruvylation, displayed deficient cell division and autolysis [22]. Autolysis can be induced by the action of different factors, namely the dissipation of proton motive force (PMF) across the cell membrane, increase of cell wall pH, temperature mineral salts, ethanol and EDTA [23-25].

In our laboratory we found the process of autolysis in *L. monocytogenes* is more complex than may have been thought previously, with different strains of *L. monocytogenes* having very great differences in the pattern of their autolysis. For example, some *L. monocytogenes* isolates promptly and consistently undergo autolysis when grown in minimal medium, whereas other strains do not. Clearly there are differences in the control of autolysis in these strains but to date it is unknown how *L. monocytogenes* autolysins are regulated.

The objective of this study was to compare the intracellular proteome and the extracellular proteome of two *L. monocytogenes* strains that differ in the propensity to autolysis, one that had a propensity to autolyse and one which did not. Differences in the proteomes of these two strains provided important clues about this cellular process that can result in cell death and can be further explored to trigger listerial death in contaminated foods.

2. Material and Methods

2.1. Bacterial Strains and Growth Conditions

Two strains of *L. monocytogenes* were used in this study, C897 (a cheese isolate of 1/2a serotype prone to autolysis) and EGD (a clinical isolate of 1/2a serotype and a non-autolytic strain). For routine growth of listeria, tryptic soy agar medium (Merck, Darmstadt, Germany) was used at 30˚C. To study the protein synthesis under autolysis, the defined medium of Trivett and Meyer [26] (TM) was used. This medium has been used to investigate physiological responses in *L. monocytogenes* [1,27,28]. These cultures were incubated at two temperatures, at 30˚C at which the autolysis process is observed in C897 and at 20˚C at which autolysis is absent. The culture at both temperatures was done with shaking (120 rpm). All listeria cultures were inoculated to obtain an initial optical density at 600 nm (OD_{600nm}) of 0.02 - 0.05, using 16 h overnight cultures in TM as inocula.

2.2. Induction of Lysis by Penicillin G

Penicillin induction of lysis was done according to Fontana *et al.* [29]. Overnight listeria cultures were diluted in fresh medium to obtain an initial optical density at 600 nm (OD_{600nm}) of 0.02 - 0.05 and left to grow at 37˚C, with shaking, until they reached OD_{600nm} of 0.15 - 0.2. Penicillin G (100 U/ml) was added to the cultures and the OD_{600nm} was measured over 6 h and after 24 h. The viability was determined at the time of penicillin addition and at the end of 24 h using the drop method [30].

2.3. Protein Extracts and Protein Determination

Listeria cultures were grown until the end of exponential phase (OD_{600nm} = 0.5 - 0.6) and the cells were collected by centrifugation (3000 ×g, 10 min at 4˚C). The protein extraction from the supernatant was done according to Trost *et al.* [31]. Briefly, the listerial cultures were centrifuged to eliminate the bacterial cells and the supernatant was filtered using 0.22 μm filters. Protein precipitation was initiated by the addition of PMSF (0.2 mM) and sodium-deoxycholate (0.2 mg/ml) to the samples and incubation on ice for 30 min. Afterwards, TCA (7%, w/v) was added and the samples were incubated overnight at 4˚C. The samples were centrifuged at 4020 × g for 15 min at 4˚C. The supernatant was removed and the protein pellet was washed twice with cold acetone. The pellet was air-dried and the protein was dissolved in solubilisation buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 40 mM DTT, 0.8% (v/v) pharmalyte pH 4 - 7 (GE Healthcare). The samples were maintained at −80˚C until analyzed.

The protein extraction from the bacterial cells was done according to Folio *et al.* [32]. The collected cells were immediately washed after sampling with washing buffer (100 mM Tris-HCl pH 7.0, 100 mM EDTA and 0.1 ml 100× protease inhibitor mix (GE Healthcare, Madrid, Spain). Cell samples were resuspended in lysis buffer (25 mM Tris-HCl pH 7.0, 25 mM EDTA, 1% (v/v) DTT and 0.25 ml of 100× protease inhibitor mix) and were lysed by sonication on ice for 15 min. Contaminating nucleic acids were eliminated by the addition of 1 μl (1 unit) DNase RQ1 (Promega) and 5 μl RNase A (10 mg/ml, Promega). The samples were centrifuged at 3000 × g for 10 min at 4˚C and acetone (5× the volume of the supernatant) was added to each sample. The proteins were precipitated at −20˚C for 1 h and collected by centrifugation (18,000 × g for 30 min at 4˚C). Samples were air-dried and proteins were solubilised by the addition of 400 μl solubilisation buffer (7 M urea, 2 M thioureia, 4% (w/v) CHAPS, 40 mM DTT, 0.8% (v/v) Pharmalyte [GE Healthcare]). The total protein concentration was determined by using a Bio-Rad Protein Assay kit, according to the manufacturer's instructions. The protein samples were kept at −80˚C until use.

2.4. Two-Dimensional Gel Electrophoresis (2-DE) and Image Analysis

The protein profile of the cell extracts and the supernatant of the *L. monocytogenes* cultures were analyzed by 2-DE. Approximately 300 - 400 μg of protein was used. The protein samples were separated in the first dimension using an 18 cm pH 3 - 10 or pH 4 - 7 Immobiline Dry Strip (GE Healthcare). Rehydration and isoelectric focusing was done using an IPGphor Isoelectric Focusing System (GE Healthcare). The Immobiline strips were re-hydrated for 11 h at 20˚C, 30 V and 50 μA/strip. Proteins were focused at 20˚C, 50 μA/strip, 100 V for 1 h, 500 V for 1 h and then a gradient at 8000 V with a final voltage from 8000 V until 60,000 Vhr. After isoelectric focusing, the strips were maintained at −80˚C or were immediately placed in an equilibration buffer (6 M urea, 75 mM Tris-HCl pH 8.8, 2% (w/v) SDS, 29.3% (v/v) glycerol, 0.002% (w/v) bromophenol blue and 10 mg/ml DTT (GE Healthcare) for 20 min. After this first step, the strips were transferred to fresh equilibration buffer supplemented with 25mg/ml iodoacetamide (GE Healthcare). Protein separation in the second dimension was performed in 12.5% (w/v) SDS-polyacrylamide gels in an Ettan Dalt six apparatus (GE Healthcare). The gels were run in triplicate to confirm the reproducibility of the protein patterns. Protein spots were visualised by Coomassie Blue R-250 staining. The determination of the protein profiles was done using an Image Scanner II (GE Healthcare), in combination with computational image analysis done by using Image Master 2D Platinum software, version 6 (GE Healthcare). The statistical analysis was performed using the Student's t-test (confidence level 0.05 and 0.01). Mean normalized spot volume, standard deviation (SD) and coefficient of variance (CV) were determined for each spot.

2.5. Protein Identification

The spots of interest were manually excised from the stained 2-DE gels and analyzed by MALDI-TOF or LC-MS/MS by the Protein and Nucleic Acid Chemistry Laboratory at the University of Leicester, UK or Aberdeen Proteome Facility (extracellular spots) and proteins were identified using MASCOT software.

3. Results and Discussion

Listeria monocytogenes as many other bacterial pathogens meets a variety of challenging conditions either outside or inside the host, including insufficiency in nutrients (carbon, nitrogen, iron, and other nutrients), pH variations, oxidative stress and strong adverse conditions directed by the host innate and adaptive immune responses. The successful establishment of infection largely depends on the bacterial ability to modify and adjust their physiological state and virulence phenotype in agreement with the encountered challenging conditions. Bacterial cells exhibit growth-phase dependent physiological events and during the entry into the stationary phase an autolysis process may be trigger. The stationary growth phase is defined as the time that bacterial growth rate starts to decline and the development of multiple stress responses on promotion of survival can be activated. The comprehension of the cellular events at this time point of the growth phase is vital to fully understand the mechanisms of listerial response to environmental changes. The autolytic behaviour of *L. monocytogenes* C897 was observed when grown in the chemically defined TM medium at 30˚C, whereas autolysis was not seen with EGD in TM at 30˚C (**Figure 1(a)**). Consistent with this difference, C897 was more susceptible to lysis by penicillin (**Figure 1(b)**).

In contrast to the events at 30˚C, at lower growth temperature (20˚C) the autolysis process was not observed in C897 (**Figure 1(c)**). The generation time achieved by the autolytic strain and non-autolytic strain when grown at 30˚C was 2.33 ± 0.02 h and 2.08 ± 0.06 h, respectively and were not significantly different ($P < 0.05$).

To understand the events leading up to autolysis, two proteomic analysis were done: 1) the autolytic extracellular proteome (C897 grown at 30˚C) was compared to the non-autolytic extracellular proteome (C897 grown at 20˚C), and to the extracellular proteome of the non-autolytic strain EGD grown at 30˚C, 2) the autolytic intracellular proteome (C897 grown at 30˚C) was compared to the non-autolytic intracellular proteome (C897 grown at 20˚C) and to the non-autolytic intracellular proteome of EGD grown at 30˚C.

3.1. The Intracellular Proteome of the Autolytic Strain at Autolysis Condition

It was expected that the analysis of the intracellular proteome would reveal the cellular events that may be deregulated to allow the development of autolysis and also the cells' efforts to control this potentially lethal cellular event. To accomplish this the intracellular proteome of the autolytic strain C897 at an autolysis condition (30˚C) was compared to the intracellular proteome at a non-autolysis condition (20˚C) and to the intracellular proteome of the non-autolytic strain EGD (30˚C). Data are summarized in **Table 1**. A representative gel of each experiment is shown in **Figure 2(a)**. Twenty proteins were significantly more expressed ($P < 0.05$) by the autolytic strain, C897, at 30˚C in comparison to the intracellular proteome at the non-autolysis condition (20˚C). The identified proteins are distributed in ten functional categories, with some of these categories including at least three

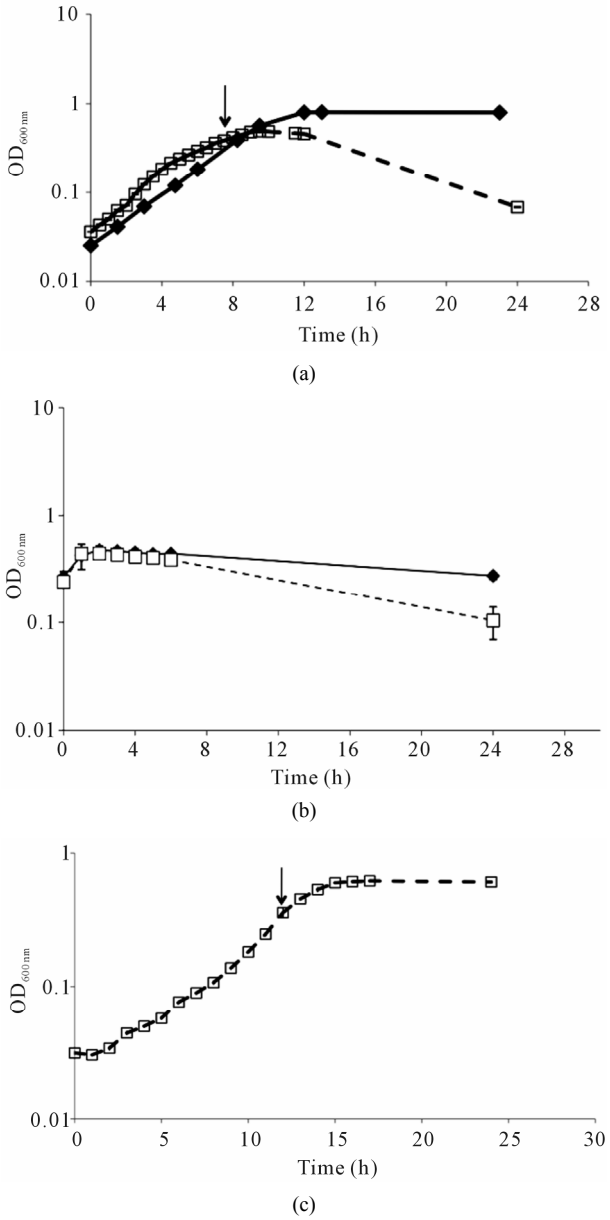

Figure 1. Autolysis behaviour of *Listeria monocytogenes* C897. (a) growth of *L. monocytogenes* C897 (□) and EGD (♦) in TM medium at 30˚C; (b) induction of lysis by penicillin G in *L. monocytogenes* C897 (□) and EGD (♦) cultures in BHI at 37˚C, (c) growth of *L. monocytogenes* C897 in TM medium at 20˚C. Data are the mean of three to four independent experiments. The standard error bars are within the area of the symbols in (a) and (c). The arrow indicates the time point at which samples were taken for 2-DE analysis.

proteins, which may indicate a higher importance of these cellular events in the autolysis process or its control. These categories are glycolysis, metabolism of amino acids and related molecules and the adaptation to stress conditions. A noteworthy aspect is the increased expression of six proteins of glycolysis, namely pyruvate kinase, phosphoglycerate kinase, enolase, glyceraldehyde 3-phosphate dehydrogenase (Gap protein), L-lactate dehydrogenase 1 (LDH-1) and Lmo 1634 (similar to alcohol dehydrogenase). The overproduction of six proteins involved in glucose metabolism by the autolytic strain indicates a strong need for energy.

Two proteins involved in the use of glucose were also over-produced by the C897 strain. These proteins, the phosphocarrier protein Hpr (spot 88) and the catabolite control protein CcpA (spot 31), are involved in the carbon catabolite repression (CCR) process (**Table 1**). The involvement of CcpA in stress responses of *L. monocytogenes* has been observed when listerial cells were exposed to salt stress [33]. Moreover the simultaneous overproduction of CcpA and the chaperones DnaK and GroEL (**Table 1**) was already reported for *Lactobacillus plantarum* [34] and *L. monocytogenes* [35] so is possible that CcpA may operate as a positive regulator of the two chaperones. Regarding the proteins involved in metabolism of amino acids and related molecules it is noteworthy that there was an increased synthesis of cysteine synthase (CysK) and D-alanine aminotransferase (DaaA). It is known that CysK is over-produced in *B. subtilis* under cold and oxidative stresses [36,37]. D-alanine is a key element in the synthesis of murein and one of the principal proteins involved in its synthesis is the D-alanine aminotransferase, which catalyzes the conversion of D-glutamic acid to α-ketoglutaric acid and D-alanine. The deletion of the genes that encode D-alanine aminotransferase (*dat* or *daaA*) and alanine racemase (*dal*) produces a phenotype that is entirely dependent on exogenous alanine and its absence induces lysis of *L. monocytogenes* cells in log phase of growth [38]. The over-expression of these two proteins under autolysis condition is indicative of the fatal murein break due to autolysis in C897 cells.

3.2. In Autolysis Condition the Autolytic Strain Shows a Stress Adaptation Response

The intracellular proteome of the C897 strain at 30˚C, in comparison to the intracellular proteome at the non-autolysis condition (20˚C), revealed the over-production of five proteins associated with adaptation to stress conditions (**Table 1**) and one of these were also over-produced by C897 at 30˚C, in comparison to EGD at 30˚C. Three proteins, in particular, that fit into the functional category of adaptation to atypical conditions were identified; namely the general stress protein Ctc, peroxide resistance protein Dpr and the Lmo1601 protein (stress protein-like protein). Two other protein spots related with stress response, the DnaK protein and the 60 KDa chaperonine, GroEL also were identified (**Table 1**).

The over-expression of Ctc in *L. monocytogenes* exposed to stress conditions, namely cold, salt and osmotic

Table 1. Differentially expressed intracellular proteins. Differential protein abundance is indicated as fold change (minimum 2-fold, P < 0.05).

Functional category or description*	Spot ID	Gene name	Locus name	UniProt ID	Protein name	Fold Change[a]
More abundant proteins in the autolytic strain C897 at 30˚C (autolysis condition) in comparison to 20˚C (non-autolysis condition)						
Transport/binding proteins and lipoproteins	88	*ptsH*	LMOf6854_1051	Q4ER91_LISMO	Phosphocarrier protein HPr	2.0
Specific pathways	3	*pykA*	lmo1570	Q8Y6W1_LISMO	Pyruvate kinase	3.6
Specific pathways	33	*ldh*1	LMOf2365_0221	LDH1_LISMF	L-lactate dehydrogenase 1	2.3
Specific pathways	91		lmo1634	Q8Y6Q0_LISMO	Lmo1634 protein (similar to alcohol dehydrogenase)	24.1
Main glycolytic pathways	16	*eno*	LMOh7858_2604	Q4EEP6_LISMO	Enolase	2.0
Main glycolytic pathways	32[b]	*gap*	lmo2459	Q8Y4I1_LISMO	Glyceraldehyde 3-phosphate dehydrogenase (Gap protein)	2.0
Main glycolytic pathways	196	*pgk*	lmo 2458	PGK_LISMO	Phosphoglycerate kinase	3.1
Metabolism of aminoacids and related molecules	13	*hom*	LMOf6854_2608	Q4EPH5_LISMO	Homoserine dehydrogenase	2.6
Metabolism of aminoacids and related molecules	40	*cysK*	lmo0223	Q8YAC3_LISMO	Cysteine synthase	2.0
Metabolism of aminoacids and related molecules	41	*dat*	lmo1619	DAAA_LISMO	D-alanine aminotransferase	2.1
Regulation	31	*ccpA*	LMOf6854_1652	Q4EP90_LISMO	Catabolite control protein A (Fragment)	2.3
Elongation	17	*tuf*	LMOh7858_2914	Q4EJM9_LISMO	Elongation factor Tu	2.0
Protein folding	4	*dnaK*	LMOh7858_1570	Q4EGL2_LISMO	Chaperone protein DnaK	2.5
Protein folding	5	*groEL*	LMOh7858_2197	Q4EEZ8_LISMO	60 kDa chaperonin, GroEL	3.6
Adaptation to atypical conditions	47	*ctc*	BSU00520	A1E155_LISMO	General stress protein Ctc	2.0
Adaptation to atypical conditions	77		LMOf6854_0989	Q4ERJ3_LISMO	Peroxide resistance protein Dpr	4.0
Adaptation to atypical conditions	104		lmo1601	Q9RQJ0_LISMO	Lmo1601 protein (Stress protein-like protein)	2.0
Formate-tetrahydrofolate ligase activity	11	*fhs*	LMOh7858_2002	Q4EH88_LISMO	Formate-tetrahydrofolate ligase	2.0
More abundant proteins in the autolytic strain C897 at 30˚C in comparison to the non autolytic strain EGD at 30˚C						
Specific pathways	3	*pykA*	lmo1570	Q8Y6W1_LISMO	Pyruvate kinase	6.3
Specific pathways	33	*ldh*1	LMOf2365_0221	LDH1_LISMF	L-lactate dehydrogenase 1	2.0
Main glycolytic pathways	196	*pgk*	lmo 2458	PGK_LISMO	Phosphoglycerate kinase	3.2
Metabolism of aminoacids and related molecules	13	*hom*	LMOf6854_2608	Q4EPH5	Homoserine dehydrogenase	2.0
Metabolism of aminoacids and related molecules	41	*daaA*	lmo1619	DAAA_LISMO	D-alanine aminotransferase	7.3
Metabolism of coenzymes and prosthetic groups	57	*thiD*	lmo0662	Q8Y971	Hypothetical protein lmo 0662; thiamin biosynthetic process	9.9
Regulation	31	*ccpA*	LMOf6854_1652	Q4EP90_LISMO	Catabolite control protein A (Fragment)	2.7
Protein modification	56		lmo1709	Q8Y6H5	Methionine aminopeptidase	2.7

Continued

Protein folding	4	*dnaK*	LMOh7858_1570	Q4EGL2_LISMO	Chaperone protein DnaK	2.0
Formate-tetrahydrofolate ligase activity	11	*fhs*	LMOh7858_2002	Q4EH88_LISMO	Formate-tetrahydrofolate ligase	3.9
More abundant proteins in the autolytic strain C897 at 20˚C in comparison to 30˚C						
Cell wall	141	*iap*	lmo0582	Q84DR8_LISMO	Invasion associated protein p60	4.0
Cell wall	**189**	*mbl*	lmo2525	Q8Y4C5_LISMO	Mbl protein	2.0
Transport/binding proteins and lipoproteins	37	*manL*	LMOf6854_0109	Q4EUJ0_LISMO	PTS system, mannose-specific, IIAB component	2.0
Membrane bioenergetics	**59**		LMOh7858_2963	Q4EJS8_LISMO	Oxidoreductase	2.0
Specific pathways	267	*pykA*	lmo1570	Q8Y6W1_LISMO	Pyruvate kinase	5.6
Main glycolytic pathways	2	*tkt*-2	LMOf6854_1347	Q4EPL3_LISMO	Transketolase	5.7
Main glycolytic pathways	108	*pdhA*	LMOf6854_1104	Q4ERT5_LISMO	Pyruvate dehydrogenase complex, E1 component	2.9
TCA cycle	22	*icd*	LMOf6854_1617	Q4EHL3_LISMO	Isocitrate dehydrogenase [NADP]	2.0
Metabolism of aminoacids and related molecules	20	*glyA*	LMOh7858_2691	Q4EED3_LISMO	Serine hydroxymethyltransferase	2.0
Metabolism of aminoacids and related molecules	21	*gdhA*	LMOh7858_0619	Q4EID3_LISMO	Glutamate dehydrogenase, NADP-specific	2.0
Metabolism of aminoacids and related molecules	**25**		LMOh7858_1711	Q4EEZ6_LISMO	Chorismate mutase/phospho-2-dehydro-3-deoxyh eptonate aldolase	2.0
Metabolism of aminoacids and related molecules	**26**	*trpB*	LMOf6854_1683	Q4EN62_LISMO	Tryptophan synthase, beta subunit	13.7
Metabolism of aminoacids and related molecules	**128**	*trpA*	LMOf6854_1682	Q4EN63_LISMO	Tryptophan synthase alpha chain	2.0
Metabolism of aminoacids and related molecules	**144**	*aroC*	LMOf6854_1987	Q4EN85_LISMO	Chorismate synthase	2.8
Metabolism of aminoacids and related molecules	233		lmo2414	Q8Y4M3_LISMO	Lmo2414 (iron-sulfur cluster assembly)	3.1
Metabolism of aminoacids and related molecules	**271**		LMOf6854_1956	Q4EQU8_LISMO	Aspartate aminotransferase, putative	3.1
Metabolism of nucleotides and nucleic acids	7	*guaA*	LMOh7858_1160	Q4EKN1_LISMO	GMP synthase	2.0
Metabolism of nucleotides and nucleic acids	**63**	*pyrE*	LMOf2365_1859	PYRE_LISMF	Orotate phosphoribosyltransferase	2.0
Metabolism of nucleotides and nucleic acids	132	*prs*1	LMOf6854_0208	Q4ERM9_LISMO	Ribose-phosphate pyrophosphokinase	5.8
Metabolism of nucleotides and nucleic acids	149	*purA*	LMOh7858_0068	Q4EK69_LISMO	Adenylosuccinate synthetase	3.1
Metabolism of nucleotides and nucleic acids	268	*pnp*	LMOf6854_1374	Q4ESF0_LISMO	Polyribonucleotide nucleotidyltransferase	13.2
Metabolism of lipids	**195**	*fabD*	lmo1808	Q8Y689_LISMO	FabD protein (Malonyl CoA-acyl carrier protein transacylase)	2.0
Metabolism of coenzymes and prosthetic groups	**139**	*nadA*	LMOh7858_2148	Q4EHC3_LISMO	Quinolinate synthetase complex, subunit A	2.0
DNA recombination	202	*recA*	lmo 1398	RECA_LISMO	Protein RecA	2.5
Regulation	**60**		LMOf6854_2133	Q4EST3_LISMO	Redox-sensing transcriptional repressor rex	3.5
Termination	72	*nusG*	LMOh7858_0272	Q4EDN9_LISMO	Transcription antitermination protein nusG	2.2

Continued

Ribosomal proteins	87	*rpsF*	LMOf6854_0054	Q4EUP5_LISMO	Ribosomal protein S6	2.0
Ribosomal proteins	99	*rplU*	LMOf6854_1590	Q4EMI9_LISMO	50S ribosomal protein L21	2.0
Ribosomal proteins	101	*rplQ*	LMOf6854_2725	Q4ENX9_LISMO	50S ribosomal protein L17	3.6
Ribosomal proteins	102	*rpsE*	LMOf6854_2735	Q4ENW9_LISMO	Ribosomal protein S5	3.8
Ribosomal proteins	105	*rplE*	LMOf6854_2740	Q4ENW4_LISMO	50S ribosomal protein L5	2.8
Ribosomal proteins	106	*rplA*	LMOh7858_0275	Q4EDU9_LISMO	Ribosomal protein L1	2.3
Elongation	1	*fusA*	LMOh7858_2915	Q4EJN0_LISMO	Translation elongation factor G	2.0
Detoxification	69	*sod*	LMOf6854_1482	Q4ETU5_LISMO	Superoxide dismutase	2.0
Detoxification	73		LMOf6854_1635	Q4EPZ2_LISMO	Thiol peroxidase	2.0
Detoxification	100	*isdG*	LMOf6854_0517	Q4EVM9_LISMO	Heme-degrading monooxygenase isdG	2.4
Terpenoid biosynthetic process	**61**		LMOf6854_1139	Q4EUU3_LISMO	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	2.0
GTPase activity	148		LMOf6854_1536	Q4ETZ9_LISMO	GTPase, putative	3.9
Cell redox homeostasis	205		LMOf6854_1107	Q4ERT8_LISMO	Dihydrolipoyl dehydrogenase	2.7
More abundant proteins in the non autolytic strain EGD at 30˚C in comparison to C897 at 30˚C						
Cell wall	**189**	*mbl*	lmo2525	Q8Y4C5_LISMO	Mbl protein	2.0
Cell wall	251	*murC*	LMOf6854_1658	Q4EP84_LISMO	UDP-N-acetylmuramate-alanine ligase	3.4
Membrane bioenergetics	**59**		LMOh7858_2963	Q4EJS8_LISMO	Oxidoreductase	3.0
Cell division	168	*minD*	LMOf6854_1593	Q4EQ78_LISMO	Septum site-determining protein MinD	2.0
Specific pathways	38	*pfkA*	LMOf2365_1593	K6PF_LISMF	6-phosphofructokinase	2.0
Main glycolytic pathways	54	*tpiA*-2	LMOh7858_2606	Q4EEP4_LISMO	Triosephosphate isomerase	2.0
Main glycolytic pathways	229		LMOf6854_1107	Q4ERT8_LISMO	Dihydrolipoyl dehydrogenase, Pyruvate dehydrogenase complex, E3 component	2.6
Metabolism of aminoacids and related molecules	**25**		LMOh7858_1711	Q4EEZ6_LISMO	Chorismate mutase/phospho-2-dehydro-3-deoxy heptonate aldolase	2.0
Metabolism of aminoacids and related molecules	**26**	*trpB*	LMOf6854_1683	Q4EN62_LISMO	Tryptophan synthase, beta subunit	2.7
Metabolism of aminoacids and related molecules	**128**	*trpA*	LMOf6854_1682	Q4EN63_LISMO	Tryptophan synthase alpha chain	2.6
Metabolism of aminoacids and related molecules	**144**	*aroC*	LMOf6854_1987	Q4EN85_LISMO	Chorismate synthase	2.0
Metabolism of aminoacids and related molecules	**271**		LMOf6854_1956	Q4EQU8_LISMO	Aspartate aminotransferase, putative	3.7
Metabolism of nucleotides and nucleic acids	9	*purH*	LMOf6854_1824	Q4ET64_LISMO	Phosphoribosylaminoimidazolecar-boxamide formyltransferase/IMP cyclohydrolase	2.3
Metabolism of nucleotides and nucleic acids	**63**	*pyrE*	LMOf2365_1859	PYRE_LISMF	Orotate phosphoribosyltransferase	2.0
Metabolism of nucleotides and nucleic acids	160	*purM*	LMOf6854_1826	Q4ET66_LISMO	Phosphoribosylformylglycinamidine cyclo-ligase	2.4

Continued

Metabolism of nucleotides and nucleic acids	179	*purQ*	LMOf6854_1829	Q4ET69_LISMO	Phosphoribosylformylglycinamidine synthase I	2.0
Metabolism of lipids	19	*fabF*	LMOf6854_2265	Q4EST8_LISMO	Beta-ketoacyl-acyl carrier protein synthase II	2.3
Metabolism of lipids	**195**	fabD	lmo1808	Q8Y689_LISMO	FabD protein (Malonyl CoA-acyl carrier protein transacylase)	3.2
Metabolism of lipids	23	*ackA*2	LMOf2365_1603	ACKA2_LISMF	Acetate kinase 2	23.9
Metabolism of coenzymes and prosthetic groups	42	*panB*	lmo1902	PANB_LISMO	3-methyl-2-oxobutanoate hydroxymethyltransferase	2.0
Metabolism of coenzymes and prosthetic groups	92	*thiD*-2	LMOf6854_0705	Q4EU28_LISMO	Phosphomethylpyrimidine kinase	3.3
Metabolism of coenzymes and prosthetic groups	**139**	*nadA*	LMOh7858_2148	Q4EHC3_LISMO	Quinolinate synthetase complex, subunit A	2.0
Metabolism of coenzymes and prosthetic groups	167	*menB*	lmo1673	Q8Y6L1_LISMO	Naphthoate synthase	2.2
Regulation	**60**		LMOf6854_2133	Q4EST3_LISMO	Redox-sensing transcriptional repressor rex	2.0
RNA modification	84		LMOf6854_2430	Q4ESL7_LISMO	S1 RNA binding domain protein	2.5
Ribosomal proteins	15		lmo1938	Q8Y5W7_LISMO	Lmo1938 protein	2.0
Ribosomal proteins	176	*rplA*	LMOh7858_0275	Q4EDU9_LISMO	Ribosomal protein L1	2.0
Terpenoid biosynthetic process	**61**		LMOf6854_1139	Q4EUU3_LISMO	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	2.0
Lipopolisacharide biosynthetic process	64	*rfbC*	LMOf6854_1135	Q4EUT9_LISMO	DTDP-4-dehydrorhamnose 3,5-epimerase	2.0
Proteins only expressed by the non-autolytic strain EGD						
Protein modification	275	*map*	LMOf6854_1768	Q4EMT5_LISMO	Methionine aminopeptidase	NA
Translation	277	*pheT*	lmo1607	Q8Y6S6_LISMO	PheT protein	NA
Quorum-sensing	280	luxS	LMOf6854_1330	Q4ENQ7_LISMO	Autoinducer-2 production protein LuxS	NA

[*]The functional category is indicated as at ListiList (http://genolist.pasteur.fr/ListiList/); [a]Fold changes in protein abundance (over-produced) are indicated as the ratio between the normalized spot volume from cells at tested conditions: C897 at 30˚C and 20˚C; C897 at 30˚C and EGD at 30˚C; C897 at 20˚C and C897 at 30˚C; EGD at 30˚C and C897 at 30˚C; [b]Three spots identified, spot 32, 75 and spot 203; ; underlined spots are over expressed by C897 at 30˚C in comparison to non-autolysis condition at 20˚C and to EGD (30˚C). Spots in bold are over-produced both by EGD at 30˚C and by C897 at 20˚C.

shock, already has been reported [33,35,39]. Likewise, in another Gram positive bacterium, *B. subtilis*, the over-expression of Ctc was observed when cells were under osmotic, heat and oxidative stresses and during glucose limitation [40]. Dpr that responds to peroxide stress has been associated with response to lactate exposition in *Lactobacillus plantarum* [41] and *Lactococcus lactis* [42] and was reported to protect *Streptococcus pyogenes* against several stresses [43]. It seems that the Lmo1601 protein (stress protein-like protein) may be involved in the maintenance of the redox balance of the cell because this protein has 59% similarity to the general stress protein YtxH from *B. subtilis*. Another over-produced protein worthy of note due to its involvement in stress response is the elongation factor Tu (EF-Tu). In *Escherichia coli* the EF-Tu seems to have a role additional to its translation elongation function, in that it may act as a chaperone-like protein protecting cells from stress [44]. It also has been reported to protect *L. monocytogenes* cells from salt and cold stress [33,35]. None of these proteins have previously been related to autolysis, except DnaK.

The heat shock proteins DnaK and GroEL were over-produced by C897 at 30˚C in comparison to 20˚C. The expression of these two proteins is elevated when bacterial cells are exposed to several environmental stress conditions and they play a crucial role in *L. monocytogenes in vivo* survival [45,46]. Besides their recognized value for cell protection under stress, an important role in protein folding has been attributed to GroEL even when bacterial cells grow at optimal temperature [47]. The association of induction of heat shock proteins with autolysis has been reported in *S. aureus* and *E. coli* [48,49]. Recently a *dnaK* mutant of *S. aureus* was described that showed a reduced autolysis rate in comparison to the wild type strain suggesting a possible role of DnaK on

autolysins activity [50]. Considering the results obtained by Singh *et al.* [50] it is tempting to hypothesise that DnaK alone, or in combination with GroEL activate the activity of P60 and/or P45.

The observation of the over-production of a significant number of stress response proteins by the C897 strain strongly suggests that it is stress that triggers autolysis.

3.3. The Intracellular Proteome of the Autolytic Strain at Non-Autolysis Condition

Thirty-night proteins over-expressed at the non-autolysis condition (20˚C) were identified. Data are summarized in the **Table 1**, Supplementary **Table A** and a representative gel is shown in **Figures 2(b)**. These proteins can be distributed among eighteen functional categories. The functional categories that have the highest number of spots are the metabolism of amino acids (8 proteins), metabolism of nucleotides and nucleic acids (5 proteins) and ribosomal proteins (6 proteins).

Two oxidative stress proteins were significantly expressed: superoxide dismutase and thiol peroxidase. A component of the SOS response, the activator RecA also was more expressed. The over-production of proteins involved in cell detoxification in non-autolysis and autolysis conditions indicates the higher sensitivity of the autolytic strain to oxidative stress.

At the non-autolysis condition, the intracellular over-expression of the autolysin P60 was observed. This autolysin was at lower levels in the extracellular proteome at 20˚C (see section 3.7) and thus we can hypothesize that intracellular/extracellular proteome ratio is an important factor in autolysis inhibition.

A significant set of over-produced proteins was also over-produced by the non-autolytic EGD and will be discussed in section 3.5. *Intracellular protein expressed in both strains in the absence of autolysis.*

3.4. The Intracellular Proteome of the Non-Autolytic Strain

Comparison of the intracellular proteomes of the non-autolytic EGD grown in conditions that induce autolysis of C897 can provide information on key elements of the autolysis inhibition process. Twenty-nine proteins that were over-produced in EGD were identified. Data are summarized in **Table 1** and a representative gel is shown in **Figure 2(c)**. The functional categories with the highest number of spots were the metabolism of amino acids (5 proteins), followed by the metabolism of coenzymes and prosthetic groups and the metabolism of nucleotides and nucleic acids (4 proteins) metabolism of lipids and main glycolytic pathways (3 proteins). The five proteins for the metabolism of amino acids and related molecules were common to proteins over-produced by the autolytic

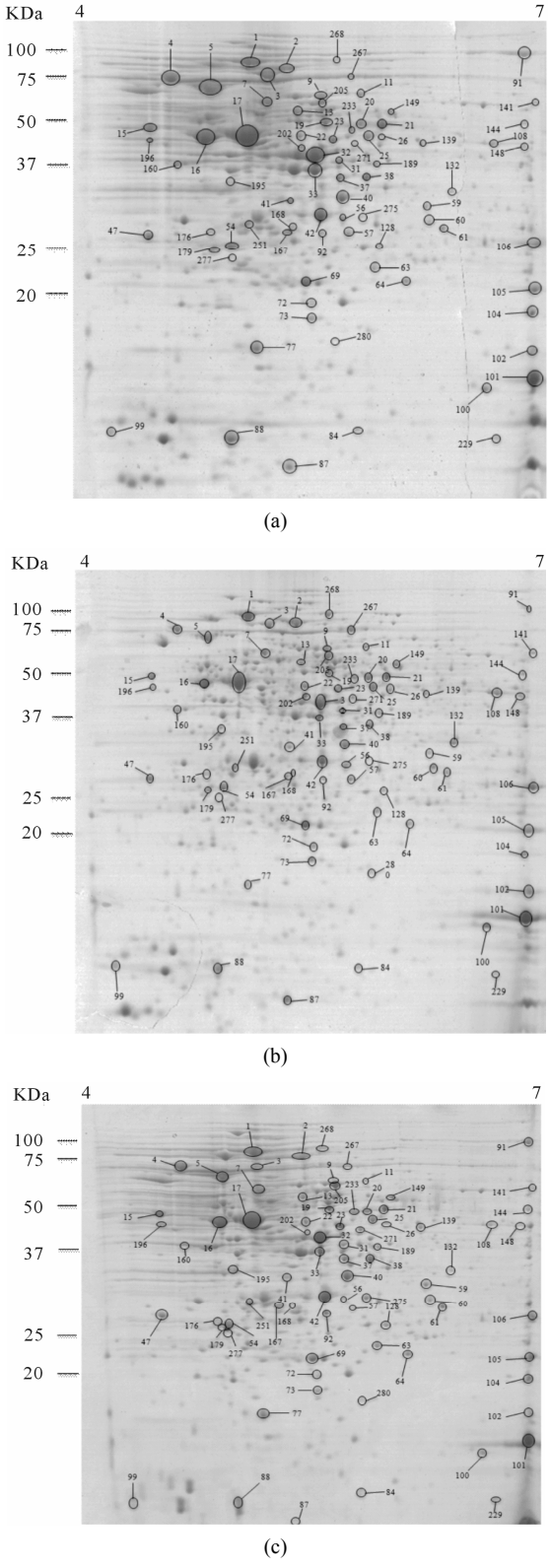

Figure 2. 2-DE maps of the intracellular proteome of the autolytic strain C897 grown at 30˚C (autolysis permissive temperature) (a) at 20˚C (non-autolysis permissive temperature); (b) and the non-autolytic strain EGD grown at 30˚C; (c) The identified spots are indicated.

strain at 20˚C in comparison to 30˚C (see following section). Two proteins related with the cell wall and one protein related with cell division, Mbl, UDP-N-acetylmuramate alanine ligase and the septum determining protein MinD, respectively were identified. UDP-N-acetylmuramate-alanine ligase (MurC) is one of the fundamental cytoplasmic peptidoglycan biosynthetic enzymes. This enzyme catalyzes the ATP-dependent ligation of L-alanine (Ala) and UDP-N-acetylmuramic acid (UNAM) to form UDP-N-acetylmuramyl-L-alanine (UNAM-Ala). The gene *minD* belongs to the minicell genetic locus constituted by other two genes *minC* and *minE* [51]. The investigation of the expression of different combinations of the *min* genes revealed that MinC is a division inhibitor and MinD induces MinC activity [51,52]. The possible role of Mbl will be discussed below, as is a common over produced spot by EGD and the autolytic strain at 20˚C.

The membrane lipid homeostasis and the capacity of bacterial cells to modify the lipid composition according to different environments may dictate bacterial survival. Consistent with the hypothesis that lipid composition influences autolysis, two proteins that are key elements of the fatty acid synthesis (β-ketoacyl-acyl carrier protein synthase II (FabF) and the FabD protein, Malonyl CoA-acyl carrier protein transacylase) were over-expressed in the non-autolytic strain and in the autolytic strain at 20˚C.

In contrast to over-production of several glycolytic enzymes in C897 at 30˚C, only two glycolytic proteins were higher in EGD at 30˚C (6-phosphofructokinase and triosephosphate isomerase). This is consistent with the previous suggestion that the requirement of glycolysis is an important part of the autolysis phenomenon.

The LuxS (S-ribosylhomocysteinase) protein was only found in the intracellular proteome of EGD (**Table 1**, Supplementary **Table A**, **Figure 2**). Autolysis is linked to quorum sensing in *S. pneumoniae* [53,54] which is regulated by the ComDE, which is generally considered to be a component of a quorum sensing mechanism [53]. The participation of quorum-sensing components in *L. monocytogenes* autolysis regulation has not been reported.

3.5. Intracellular Protein Expressed in Both Strain in the Absence of Autolysis

Analysis of data collected in the absence of autolysis (C897 at 20˚C and EGD at 30˚C) revealed a set of common proteins (**Table 1**, Supplementary **Table A**). From the 12 identified proteins five are involved in the metabolism of amino acids and two in the metabolism of coenzymes and prosthetic groups. The significance of increases in the abundance of proteins associated with the metabolism of the amino acids tyrosine, phenylalanine and tryptophan (chorismate mutase, chorismate synthase, tryptophan synthase, beta and alpha subunits) relies in the role of these aromatic amino acids in the maintenance of the structure and function of membrane proteins [55, 56]. An important clue to the inhibition of autolysis is the component of the cell wall, the Mbl protein (similar to MreB-like protein). Mbl is an isoform of MreB that, together with other proteins, provide a rod shape to bacterial cells. *B. subtilis* has three MreB isoforms (MreB, Mbl and MreBH) which seems to be responsible for the positioning of peptidoglycan synthases, a peptidoglycan hydrolase (LytE) and other membrane-associated cell morphogenesis proteins, such as MreC and MreD [57]. From these three MreB isoforms, Mre-BH seems to be responsible for the control of the autolytic activity by directing the localization of the cell wall hydrolase LytE [58]. Search of the *Listeria* genome indicates that only two MreB isoforms are present, the MreB and Mbl. So far the roles of MreB and its Mbl isoform in *L. monocytogenes* have not been clarified. From our data it is possible to propose that Mbl plays an important role in autolysis inhibition. The absence of the over-production of the glycolysis enzymes L-lactate dehydrogenase 1 (LDH-1), Lmo 1634 (similar to alcohol dehydrogenase) and glyceraldehyde 3-phosphate dehydrogenase (Gap protein) in the non-autolysis condition may be explained by the over-production of the redox-sensing transcriptional repressor rex (spot 60) which senses the NADH/NAD+ ratio in the cell and which has been shown in *Streptomyces coelicolor* and *Bacillus subtilis* to indirectly modulate the metabolism by regulation of genes encoding proteins of the respiratory chain [59,60], whereas in *Staph. aureus* it regulates the transcription process during the switch from aerobic to anaerobic growth [61]. In *Staph. aureus* the proteins Adh1, Ldh1 and GapA1 are Rex-regulated, but only Adh1 and Ldh1 have a Rex binding site [61]. The genes regulated by Rex and its role in the aerobic and anaerobic growth of *L. monocytogenes* remains to be clarified.

In both strains at absence of autolysis the 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase which, is involved in the terpenoid (or isoprenoid) biosynthetic process, was over-produced (**Table 1**, Supplementary **Table A**). Isoprenoids include a large number of compounds (more than 30,000) participating in a significant number of physiological processes either in eukaryotes and prokaryotes. In bacteria they have important roles in electron transport chains, with ubiquinone and menaquinone standing out in these roles. Bactoprenols function as carbohydrate carriers in the biosynthesis of peptidoglycan [62, 63].

3.6. The Extracellular Proteome of the Autolytic Strain at 30˚C Evidence Over-Production of P60 Family Proteins

Three spots were identified as being significantly ($P < 0.05$) more abundant in the culture supernatant of the autolytic strain C897 when grown at 30˚C, compared to growth at 20˚C. One of them is the autolytic enzyme P60 (invasion associated protein). Data are summarized in the **Table 2** and Supplementary Table A and a representative gel is shown in **Figure 3(a)**. Five proteins were identified in the extracellular proteome of the autolytic strain C897 grown at 30˚C compared to the extracellular proteome of the non-autolytic strain EGD grown at 30˚C. Among them is the P45 autolysin, an amino acid ABC transporter and Lmo1333 which is similar to the *B. subtilis* YqzC protein (**Table 2**, Supplementary **Table A**). P60 was equally abundant in the extracellular proteome of C897 and EGD at 30˚C.

The differences in the autolytic behaviour of C897 at 30˚C in comparison to the lack of autolysis at 20˚C could be linked to the higher abundance of P60 in the extracellular proteome of C897 at 30˚C whereas the higher abundance of P45 in the extracellular proteome of C897 in comparison to EGD could be linked to the different autolytic behaviour of these two strains.

P60 is classified as an endo-N-acetylmuramidase [15] and is implicated in cell separation because P60 depletion results on the formation of long chains of cells [14]. The P45 protein displays 55% similarity and 38% identity to P60 and also exhibits peptidoglycan hydrolase activity [13]. To date, and in contrast to reported phenotype alterations due to P60 depletion, the exact function of P45 has not been described. As these two autolysins were detected in higher quantities in the extracellular proteome of the autolytic strain at 30˚C, in comparison to the growth conditions in absence of autolysis (P60 linked to temperature and P45 linked to strain) become evident their impact on the autolysis process.

3.7. The Extracellular Proteome at Non-Autolysis Conditions (Temperature and Strain)

The analysis of the extracellular proteome at non-autolysis condition was done by collecting the supernatant of EGD at 30˚C and C897 at 20˚C at the end of exponential phase (an approximate OD_{600nm} of 0.5 - 0.6, **Figures 1(a)** and **(b)**). Seven extracellular proteins of the autolytic strain were identified as being expressed only at the non-autolytic condition (20˚C), (**Table 2**, Supplementary **Table A** and **Figure 3(b)**) and only two proteins belong to the same functional category (metabolism of nucleotides and nucleic acids). Among the eight proteins, a cell surface

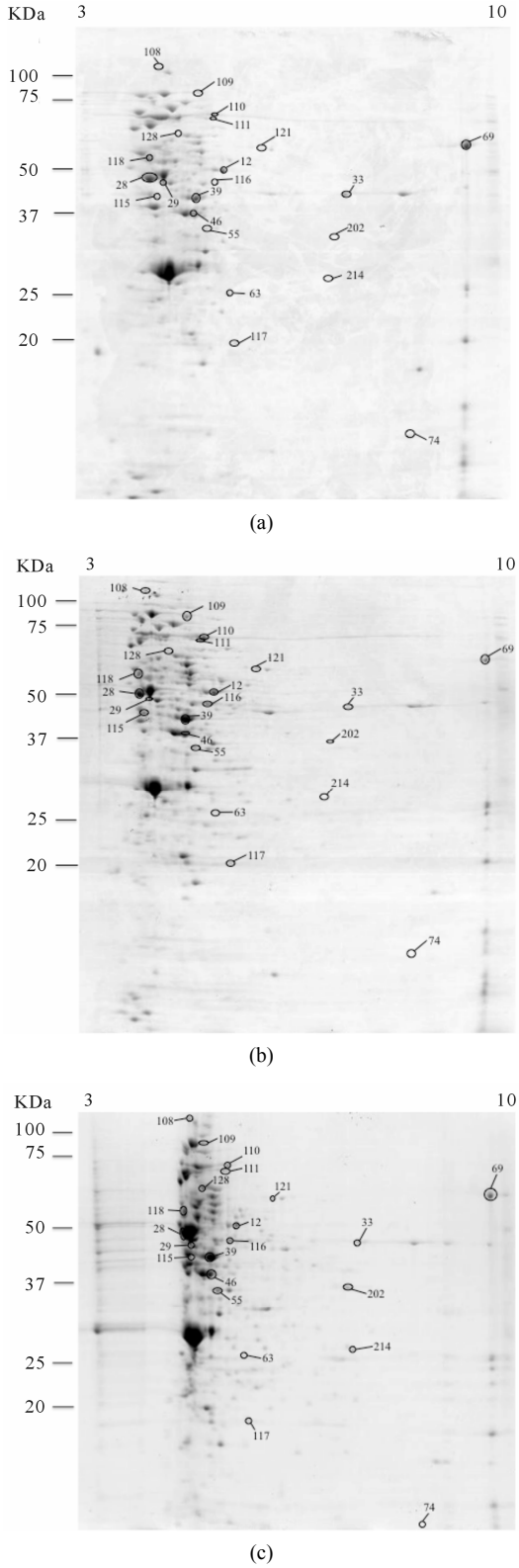

Figure 3. 2-DE maps of the extracellular proteome of the autolytic strain C897 grown at 30˚C (autolysis permissive temperature) (a) at 20˚C (non-autolysis permissive temperature); (b) and the non-autolytic strain EGD grown at 30˚C; (c) The identified spots are indicated.

protein (Lmo2185, SvpA, NEAr transporter) and a protein related with bioenergetics, an ATP synthase, were identified. The production of SvpA protein increases significantly when cells experience iron deprivation and studies on cellular fractions showed that in iron-rich media SvpA is entirely secreted into the culture supernatant [64]. The comparison of the extracellular proteome of C897 and EGD at 30˚C showed five over-expressed proteins of the extracellular proteome of the non-autolytic strain, EGD (**Table 2**, Supplementary **Table A** and **Figure 3(c)**). One of these spots belongs to the transport/binding lipoproteins and is an EII Mant PTS permease

Table 2. Proteins identified in the extracellular proteome of C897 and EGD strain.

Functional category or description*	Spot ID	Gene/Locus name	*Uniprot* ID	Uniprot protein name	Fold Change[a]
More abundant proteins secreted by the autolytic strain C897 at 30˚C in comparison to the non autolysis condition (20˚C)					
Cell wall	69	*iap*/LMOf2365_0611	Q722W8	Invasion associated protein P60	2.5
Transport/binding proteins and lipoproteins	63	LMOf2365_2319	Q71X80	Amino acid ABC transporter	2.0
Main glycolitic pathways	29	*pgk*/mo2458	Q8Y4I2	Phosphoglycerate kinase	23.7
More abundant proteins secreted by the autolytic strain C897 at 30˚C in comparison to the non autolytic strain EGD at 30˚C					
Cell wall	33[b]	*spl*/lmo2505	Q9RE04	Peptidoglycan lytic protein P45	3.7
Metabolism of amino acids and related molecules	12	lmo0560	Q8Y9G8	Lmo0560 protein, similar to NADP-specific glutamate dehydrogenase	2.0
Metabolism of nucleotides and nucleic acids	121	*guaB*/lin2901	Q7ANT6	Similar to inosine-monophosphate dehydrogenase	2.2
Specific pathways	110	*pykA*/lmo1570	Q8Y6W1	Pyruvate kinase	4.23
From other organisms (Regulation and sensing)	74	lmo1333	Q8Y7E9	Lmo1333, similar to *B. subtilis* YqzC protein	2.2
More abundant proteins secreted by the non-autolytic strain EGD at 30˚C in comparison to the autolytic strain EGD at 30˚C					
Cell wall	46	*mreB*/lmo1548	Q8Y6Y3	MreB protein	4.5
Transport/binding proteins and lipoproteins	55	*mptA*/lmo0096	Q8YAM2	EIIMant PTS permease IIAB subunit	3.0
Main glycolytic pathways	28	*eno*/lin2549	P64075	Hypothetical protein lin2549 (enolase)	5.6
Main glycolytic pathways	39	*gap*/lmo2459	Q8Y4I1	Glyceraldehyde-3-phosphate dehydrogenase	2.0
Main glycolytic pathways	128	*gpmI*/lmo2456	Q8Y4I4	Phosphoglyceratemutase	13.0
Proteins only detected in the extracellular proteome of the autolytic strain C897 at 20˚C					
Membrane bioenergetics	118	*atpD*2/lmo2529	Q8Y4C1	ATP synthase subunit beta 2	NA
Cell surface proteins	111	lmo2185	Q9KGV9	Lmo2185 protein (SvpA, P64 protein)	NA
Metabolism of amino acids and related molecules	116	lwe1614	A0AJ50	Chorismate mutase	NA
Metabolism of nucleotides and nucleic acids	108	*carB*/lmo1835	Q8Y665	Carbamoyl-phosphate synthase large chain	NA
Metabolism of nucleotides and nucleic acids	109	*pnp*/lmo1331	Q8Y7F1	Polyribonucleotide nucleotidyltransferase	NA
Elongation	115	*rpoA*/lin2755	P66700	DNA-directed RNA polymerase subunit alpha	NA
Ribosomal proteins	117	*rplF*/lmo2617	Q8Y444	50S ribosomal protein L6	NA
Proteins detected only in the secretome of the non-autolytic strain EGD at 30˚C					
Transport/binding proteins and lipoproteins	202	lmo2192	Q8Y581	Lmo2192 protein, similar to oligopeptide ABC transporter	NA
Metabolism of coenzymes and prosthetic groups	214	*nadC*/lmo2024	Q8Y5N3	NadC protein, similar to nicotinate-nucleotide pyrophosphorylase	NA

*The functional category is indicated as at ListiList (http://genolist.pasteur.fr/ListiList/); [a]Fold changes in protein abundance (over-produced) in the secretome are indicated as the ratio between the normalized spot volume from cells at tested conditions: C897 at 30˚C and 20˚C; C897 at 30˚C and EGD at 30˚C; EGD at 30˚C and C897 at 30˚C; [b]Two spots identified, spot 33 and spot 34.

IIAB subunit. An increase expression of this mannose-specific PTS enzyme IIAB also was detected in the intracellular proteome of *L. monocytogenes* LO28 when the bacterial cells were exposed to salt stress and this increase may be related to higher energy requirements [33].

4. Conclusions

Our results show that at the end of exponential phase the autolytic strain increases the production of a series of proteins with a recognized role in combating the action of environmental stressors. This does not occur with the non-autolytic EGD and suggest that C897 is more sensitive to stresses that eventually can trigger the autolysis process. At the autolysis permissive temperature (30˚C) the secretion of two autolysins (P60 and P45) was greater than at the non-autolysis permissive temperature (20˚C) and by the non-autolytic strain EGD. On other hand increased amounts of the P60 autolysin was observed in the intracellular proteome at non-permissive temperature. These findings suggest that at lower temperature the export of autolysins is inhibited and this may be due to alterations in the cell wall as evidenced by the over-production of proteins involved in cell wall synthesis. Differences in over-produced proteins of the glycolytic pathway, fatty acid and amino acids synthesis, transcription regulation, cell wall synthesis and morphogenesis are elements that may assure the less susceptible profile of EGD to autolysis. A significant number of proteins related to fatty acid and amino acid synthesis, transcription regulation and cell morphogenesis were commonly over-produced by the non-autolytic strain EGD and by the autolytic strain at autolysis non-permissive temperature, indicating a set of important cellular events important to the lack of autolysis.

From the collected data the cell shape Mbl protein and the transcriptional repressor Rex can constitute possible cell targets to trigger autolysis.

5. Acknowledgements

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Supplementary Table A. Identification of proteins differentially expressed under autolysis and non-autolysis condition.

Spot ID	UniProt ID[a]	Protein name	Mass (Da) (T/C)	pI (T/C)	Score	Peptide matching	Coverage (%)	Source
1	Q4EJN0_LISMO	Translation elongation factor G	76973/81510	4.85/5.10	2193	27/36	53	*L. monocytogenes* str. 4b H7858
2	Q4EPL3_LISMO	Transketolase	71831/78240	5.11/5.37	1789	19/26	50	*L.monocytogenes* str. 1/2a F6854
3	Q8Y6W1_LISMO	Pyruvate kinase	62673/75770	5.39/5.15	810	22/25	52	*L. monocytogenes* EGD
4	Q4EGL2_LISMO	Chaperone protein DnaK	66118/72340	4.57/4.57	1730	23/39	50	*L. monocytogenes* str. 4b H7858
5	Q4EEZ8_LISMO	60 kDa chaperonin, GroEL	57332/67860	4.72/4.80	2439	28/42	66	*L. monocytogenes* str. 4b H7858
7	Q4EKN1_LISMO	GMP synthase	57799/70490	4.95/5.19	1431	26/33	57	*L. monocytogenes* str. 4b H7858
9	Q4ET64_LISMO	Phosphoribosylaminoimidazolecar-boxamide formyltransferase/IMP cyclohydrolase	55022/75560	5.27/5.61	1530	20/27	53	*L.monocytogenes* str. 1/2a F6854
11	Q4EH88_LISMO	Formate-tetrahydrofolate ligase	60390/67750	5.45/5.55	1041	19/25	40	*L. monocytogenes* str. 4b H7858
13	Q4EPH5_LISMO	Homoserine dehydrogenase	46430/59120	5.13/5.25	548	7/14	24	*L.monocytogenes* str. 1/2a F6854
15	Q8Y5W7_LISMO	Lmo1938 protein	41406/57360	4.47/4.43	1092	19/30	55	*L. monocytogenes* EGD
16	Q4EEP6_LISMO	Enolase	46458/68940	4.70/4.82	2400	23/26	79	*L. monocytogenes* str. 4b H7858
17	Q4EJM9_LISMO	Elongation factor Tu	43429/51410	4.81/4.90	1873	29/36	82	*L. monocytogenes* str. 4b H7858
19	Q4EST8_LISMO	Beta-ketoacyl-acyl carrier protein synthase II	44423/63600	5.26/5.58	567	11/30	45	*L.monocytogenes* str. 1/2a F6854
20	Q4EED3_LISMO	Serine hydroxymethyltransferase	45178/58240	5.53/5.88	702	11/13	36	*L. monocytogenes* str. 4b H7858
21	Q4EID3_LISMO	Glutamate dehydrogenase, NADP-specific	49449/58800	5.54/6.00	1368	19/22	62	*L. monocytogenes* str. 4b H7858
22	Q4EHL3_LISMO	Isocitrate dehydrogenase [NADP]	46289/54480	5.24/5.47	610	12/15	31	*L.monocytogenes* str. 1/2a F6854
23	ACKA2_LISMF	Acetate kinase 2	43860/50290	5.31/5.65	97	10/31	25	*L.monocytogenes* str. 4b F2365
25	Q4EEZ6_LISMO	Chorismate mutase/phospho-2-dehydro-3-deoxyhep tonate aldolase	35826/52050	5.79/5.60	662	11/25	39	*L. monocytogenes* str. 4b H7858
26	Q4EN62_LISMO	Tryptophan synthase, beta subunit	43921/49070	5.50/5.98	680	11/12	38	*L.monocytogenes* str. 1/2a F6854
31	Q4EP90_LISMO	Catabolite control protein A (Fragment)	31566/43010	5.05/5.05	564	8/11	34	*L.monocytogenes* str. 1/2a F6854
32	Q8Y4I1_LISMO	Glyceraldehyde 3-phosphate dehydrogenase (Gap protein)	36435/45060	5.20/5.20	2353	14/20	71	*L. monocytogenes* EGD
33	LDH1_LISMF	L-lactate dehydrogenase 1	34284/42290	5.20/5.20	106	12/41	34	*L.monocytogenes* str. 4b F2365
37	Q4EUJ0_LISMO	PTS system, mannose-specific, IIAB component	34972/40190	5.33/5.33	767	13/23	60	*L.monocytogenes* str. 1/2a F6854
38	K6PF_LISMF	6-phosphofructokinase	34399/40280	5.46/5.46	203	17/30	52	*L.monocytogenes* str. 4b F2365
40	Q8YAC3_LISMO	Cysteine synthase	32198/34880	5.32/5.32	1257	12/18	66	*L. monocytogenes* EGD
41	DAAA_LISMO	D-alanine aminotransferase	32554/33400	5.12/5.12	153	11/22	50	*L. monocytogenes* EGD

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42	PANB_LISMO	3-methyl-2-oxobutanoate hydroxymethyltransferase	29836/31330	5.27/5.27	54	7/39	32	*L. monocytogenes* EGD
47	A1E155_LISMO	General stress protein Ctc	22641/27850	4.44/4.44	1287	11/15	61	*L. monocytogenes* EGD
54	Q4EEP4_LISMO	Triosephosphate isomerase	27073/25310	4.78/4.76	1287	8/11	33	*L. monocytogenes* str. 4b H7858
56	Q8Y6H5	Methionine aminopeptidase	27999/30960	5.32/5.32	69	5/13	32	*L. monocytogenes* EGD
57	Q8Y971	Hypothetical protein lmo 0662; thiamin biosynthetic process	28915/28360	5.25/5.25	127	9/10	45	*L. monocytogenes* EGD
59	Q4EJS8_LISMO	Oxidoreductase	32593/33540	5.87/5.87	1091	16/23	61	*L. monocytogenes* str. 4b H7858
60	Q4EST3_LISMO	Redox-sensing transcriptional repressor rex	24185/28590	5.78/5.78	427	9/19	56	*L.monocytogenes* str. 1/2a F6854
61	Q4EUU3_LISMO	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	26809/27670	5.71/5.71	719	14/19	57	*L.monocytogenes* str. 1/2a F6854
63	PYRE_LISMF	Orotate phosphoribosyltransferase	22849/22630	5,27/5,27	68	5/14	29	*L.monocytogenes* str. 4b F2365
64	Q4EUT9_LISMO	DTDP-4-dehydrorhamnose 3,5-epimerase	21149/21490	5.63/6.10	582	4/10	22	*L.monocytogenes* str. 1/2a F6854
69	Q4ETU5_LISMO	Superoxide dismutase	22617/19540	5.23/5.47	3310	10/12	60	*L.monocytogenes* str. 1/2a F6854
72	Q4EDN9_LISMO	Transcription antitermination protein nusG	19928/17160	5.16/5.51	487	6/15	44	*L. monocytogenes* str. 4b H7858
73	Q4EPZ2_LISMO	Thiol peroxidase	18208/15820	5.21/5.50	2484	6/10	52	*L.monocytogenes* str. 1/2a F6854
75	Q8Y4I1_LISMO	Glyceraldehyde 3-phosphate dehydrogenase (Gap protein)	36435/15810	5.20/5.08	583	7/11	25	*L. monocytogenes* EGD
77	Q4ERJ3_LISMO	Peroxide resistance protein Dpr	18036/13820	4.86/5.15	1492	11/13	93	*L.monocytogenes* str. 1/2a F6854
84	Q4ESL7_LISMO	S1 RNA binding domain protein	13011/7260	5.39/5.73	584	5/8	47	*L.monocytogenes* str. 1/2a F6854
88	Q4ER91_LISMO	Phosphocarrier protein HPr	9398/7740	4.81/4.96	736	7/13	56	*L.monocytogenes* str. 1/2a F6854
87	Q4EUP5_LISMO	Ribosomal protein S6	11500/**6**110	5.08/5.36	1740	7/19	74	*L.monocytogenes* str. 1/2a F6854
91	Q8Y6Q0_LISMO	Lmo1634 protein (similar to alcohol dehydrogenase)	95135/83280	6.48/6.90	2120	30/36	57	*L. monocytogenes* EGD
92	Q4EU28_LISMO	Phosphomethylpyrimidine kinase	28915/28250	5.25/5.55	879	12/13	57	*L.monocytogenes* str. 1/2a F6854
99	Q4EMI9_LISMO	50S ribosomal protein L21	11022/7390	9.64/4.21	989	5/15	58	*L.monocytogenes* str. 1/2a F6854
100	Q4EVM9_LISMO	Heme-degrading monooxygenase isdG	13777/10007	5.96/6.65	1313	4/12	34	*L.monocytogenes* str. 1/2a F6854
101	Q4ENX9_LISMO	50S ribosomal protein L17	15205/10970	10.27/6.92	1824	6/20	45	*L.monocytogenes* str. 1/2a F6854
102	Q4ENW9_LISMO	Ribosomal protein S5	17445/13140	9.43/6.92	1529	9/16	68	*L.monocytogenes* str. 1/2a F6854
104	Q9RQJ0_LISMO	Lmo1601 protein (Stress protein-like protein)	18435/17480	7.71/6.90	464	7/16	28	*L. monocytogenes* EGD
105	Q4ENW4_LISMO	50S ribosomal protein L5	19983/19540	9.02/6.93	661	9/13	65	*L.monocytogenes* str. 1/2a F6854
106	Q4EDU9_LISMO	Ribosomal protein L1	24531/26340	9.32/6.94	3412	13/22	54	*L. monocytogenes* str. 4b H7858
108	Q4ERT5_LISMO	Pyruvate dehydrogenase complex, E1 component	41241/51450	6.05/6.72	1498	18/22	51	*L.monocytogenes* str. 1/2a F6854

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128	Q4EN63_LISMO	Tryptophan synthase alpha chain	27930/24730	5,51/5,65	298	6/10	32	*L.monocytogenes* str. 1/2a F6854
132	Q4ERM9_LISMO	Ribose-phosphate pyrophosphokinase	35275/36490	5.83/6.45	1858	11/13	41	*L.monocytogenes* str. 1/2a F6854
139	Q4EHC3_LISMO	Quinolinate synthetase complex, subunit A	41591/49610	5.87/5.90	390	12/15	53	*L. monocytogenes* str. 4b H7858
141	Q84DR8_LISMO	Invasion associated protein p60	48162/64290	9.02/6.94	637	11/16	30	*L. monocytogenes* EGD
144	Q4EN85_LISMO	Chorismate synthase	42260/56430	6.59/6.35	899	13/19	49	*L.monocytogenes* str. 1/2a F6854
148	Q4ETZ9_LISMO	GTPase, putative	40616/50470	6.13/6.86	417	11/23	46	*L.monocytogenes* str. 1/2a F6854
149	Q4EK69_LISMO	Adenylosuccinate synthetase	47818/65310	5.57/6.06	2570	13/17	32	*L. monocytogenes* str. 4b H7858
160	Q4ET66_LISMO	Phosphoribosylformylglycinamidine cyclo-ligase	37490/44020	4.61/4.74	1295	13/24	70	*L.monocytogenes* str. 1/2a F6854
167	Q8Y6L1_LISMO	Naphthoate synthase	30176/29300	5.15/5.28	3614	13/15	49	*L. monocytogenes* EGD
168	Q4EQ78_LISMO	Septum site-determining protein MinD	29219/30590	5.03/5.29	1412	7/9	35	*L.monocytogenes* str. 1/2a F6854
176	Q4EDU9_LISMO	Ribosomal protein L1	24531/30330	9.32/4.72	2969	10/20	42	*L. monocytogenes* str. 4b H7858
179	Q4ET69_LISMO	Phosphoribosylformylglycinamidine synthase I	25170/24450	4.71/4.74	2491	8/18	35	*L.monocytogenes* str. 1/2a F6854
189	Q8Y4C5_LISMO	Mbl protein	35785/45850	5.45/5.86	745	12/21	51	*L. monocytogenes* EGD
195	Q8Y689_LISMO	FabD protein (Malonyl CoA-acyl carrier protein transacylase)	32970/38250	4.79/4.91	915	12/18	60	*L. monocytogenes* EGD
196	PGK_LISMO	Phosphoglycerate kinase	42135/50410	4.97/4.48	56	5/13	16	*L. monocytogenes* EGD
202	RECA_LISMO	Protein RecA	37971/49520	5.09/5.47	114	12/19	30	*L. monocytogenes* EGD
203	Q8Y4I1_LISMO	Glyceraldehyde 3-phosphate dehydrogenase (Gap protein)	36435/45060	5.20/5.42	1491	18/22	51	*L. monocytogenes* EGD
205	Q4ERT8_LISMO	Dihydrolipoyl dehydrogenase	49571/69160	5.24/5.62	4837	17/21	46	*L.monocytogenes* str. 1/2a F6854
229	Q4ERT8_LISMO	Dihydrolipoyl dehydrogenase, Pyruvate dehydrogenase complex, E3 component	49571/69160	5.24/6.63	1084	10/28	29	*L.monocytogenes* str. 1/2a F6854
233	Q8Y4M3_LISMO	Lmo2414 (iron-sulfur cluster assembly)	48072/55130	5.61/5.75	811	10/10	23	*L. monocytogenes* EGD
251	Q4EP84_LISMO	UDP-N-acetylmuramate-alanine ligase	50223/26650	5.47/4.92	1849	14/15	37	*L.monocytogenes* str. 1/2a F6854
267	Q8Y6W1_LISMO	Pyruvate kinase	62673/85320	5.39/5.76	3661	18/20	48	*L. monocytogenes* EGD
268	Q4ESF0_LISMO	Polyribonucleotide nucleotidyltransferase	79720/**1**04810	5.23/5.62	2042	11/11	19	*L.monocytogenes* str. 1/2a F6854
271	Q4EQU8_LISMO	Aspartate aminotransferase, putative	43388/48820	5.50/5.55	1155	15/21	50	*L.monocytogenes* str. 1/2a F6854
275	Q4EMT5_LISMO	Methionine aminopeptidase	27979/29560	5.39/4.95	360	7/10	48	*L.monocytogenes* str. 1/2a F6854
277	Q8Y6S6_LISMO	PheT protein	22581/22780	4.82/4.85	666	10/15	53	*L. monocytogenes* EGD
280	Q4ENQ7_LISMO	Autoinducer-2 production protein LuxS	17528/14210	5.32/5.45	219	3/11	20	*L.monocytogenes* str. 1/2a F6854

Continued

		Extracellular proteins						
12	Q8Y9G8	Lmo0560 protein, similar to NADP-specific glutamate dehydrogenase	49464/51880	5.54/5.40	1224	32/33	64	*L. monocytogenes* EGD
28	P64075	Hypothetical protein lin2549 (enolase)	46444/49940	4.70/4.65	2010	50/52	76	*L. innocua* str. Clip 11262
	Q8YAW1_LISMO	DNA polimerase III subunit beta	42403	4.70	482	15/16	35	*L. monocytogenes* EGD
29*	Q8Y4I2	Phosphoglycerate kinase	42135/46850	4.97/4.85	1418	42/44	71	*L. monocytogenes* EGD
33*	Q9RE04	Peptidoglycan lytic protein P45	42743/44570	8.56/6.20	1223	32/34	45	*L. monocytogenes* EGD
34*	Q9RE04	Peptidoglycan lytic protein P45	42743/44570	8.56/6.6	1349	35/38	48	*L. monocytogenes* EGD
39	Q8Y4I1	Glyceraldehyde-3-phosphate dehydrogenase	36435/43750	5.20/5.10	1350	43/48	71	*L. monocytogenes* EGD
46	Q8Y6Y3	MreB protein	35595/39800	5.16/5.20	743	14/15	42	*L. monocytogenes* EGD
55	Q8YAM2	EIIMant PTS permease IIAB subunit	34972/36200	5.33/5.30	781	23/28	76	*L. monocytogenes* EGD
63*	Q71X80	Amino acid ABC transporter	29614/24780	6.64/5.45	1028	33/39	61	*L. monocytogenes* str. 4b F2365
69*	Q722W8	Invasion associated protein P60	48162/59780	9.23/9.50	1461	52/63	62	*L. monocytogenes* EGD
74*	Q8Y7E9	Lmo1333, similar to *B. subtilis* YqzC protein	17884/11030	9.11/7.80	176	9/9	23	*L. monocytogenes* EGD
108*	Q8Y665	Carbamoyl-phosphate synthase large chain	118272/96280	4.79/4.75	1709	38/40	38	*L. monocytogenes* EGD
109	Q8Y7F1	Polyribonucleotide nucleotidyltransferase	79780/82620	5.23/5.25	1771	42/48	55	*L. monocytogenes* EGD
110*	Q8Y6W1	Pyruvate kinase	62673/73100	5.39/5.37	1552	41/45	65	*L. monocytogenes* EGD
111*	Q9KGV9	Lmo2185 protein (SvpA, P64 protein)	63341/71620	6.44/5.30	644	16/17	42	*L. monocytogenes* EGD
115*	P66700	DNA-directed RNA polymerase subunit alpha	34941/46190	4.80/4.75	807	23/26	50	*L. innocua* str. Clip 11262
116*	A0AJ50	Bifunctional 3-deoxy-7-phosphoheptulonate synthase/Chorismate mutase	39832/49110	5.25/5.60	1125	27/31	47	*L. welshimeri* serovar 6b str. SLCC5334
		Bifunctional 3-deoxy-7-phosphoheptulonate synthase/Chorismate mutase	39831	5.41	1100	27/30	47	*L. monocytogenes* EGD
117*	Q8Y444	50S ribosomal protein L6	19388/19210	9.75/5.50	516	13/15	57	*L. monocytogenes* EGD
121*	Q7ANT6	Similar to inosine-monophosphate dehydrogenase	52637/58580	6.06/5.50	550	13/13	27	*L. innocua* str. Clip 11262
128*	Q8Y4I4	Phosphoglyceratemutase	56104/65100	4.73/5.45	1250	29/33	48	*L. monocytogenes* EGD
202*	Q8Y581	Lmo2192 protein, similar to oligopeptide ABC transporter	36712/37240	6.85/6.25	874	19/22	47	*L. monocytogenes* EGD
214*	Q8Y5N3	NadC protein, similar to nicotinate-nucleotide pyrophosphorylase	30585/25990	7.02/6.40	608	17/17	54	*L. monocytogenes* EGD

[a]For each spot identification the hit with the top score is indicated and where applicable the hit for *L. monocytogenes* EGD is shown; *protein spots identified by LC/MS-MS, the remaining were identified by MALDI-TOF.

