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

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Received April 11, 2012; revised April 27, 2012; accepted May 10, 2012

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] and have 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 cerevisiae 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-
ment for choline-binding domains and D-alanylation of teichoic acids, other studies have demonstrated that pyruvlation 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 pyruvlation, 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 (OD600nm) 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 (OD600nm) of 0.02 - 0.05 and left to grow at 37°C, with shaking, until they reached OD600nm of 0.15 - 0.2. Penicillin G (100 U/ml) was added to the cultures and the OD600nm 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 (OD600nm = 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 thiourea, 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 IPIGphor 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 V hr. 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 Etta 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-autolysic 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-autolysic 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
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 conditions, which may indicate a higher importance of these cellular events in the autolysis process or its control.
Table 1. Differentially expressed intracellular proteins. Differential protein abundance is indicated as fold change (minimum 2-fold, P < 0.05).

<table>
<thead>
<tr>
<th>Functional category or description*</th>
<th>Spot ID</th>
<th>Gene name</th>
<th>Locus name</th>
<th>UniProt ID</th>
<th>Protein name</th>
<th>Fold Changea</th>
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<tbody>
<tr>
<td>More abundant proteins in the autolytic strain C897 at 30°C (autolysis condition) in comparison to 20°C (non-autolysis condition)</td>
<td>88</td>
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<td>Phosphocarrier protein HPr</td>
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<td>Specific pathways</td>
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<td>pykA</td>
<td>lmo1570</td>
<td>Q8Y6W1_LISMO</td>
<td>Pyruvate kinase</td>
<td>3.6</td>
</tr>
<tr>
<td>Specific pathways</td>
<td>33</td>
<td>ldh1</td>
<td>LMO2365_0221</td>
<td>LDH1_LISMF</td>
<td>L-lactate dehydrogenase 1</td>
<td>2.3</td>
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<td>Specific pathways</td>
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<td>lmo1634</td>
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<td>Lmo1634 protein (similar to alcohol dehydrogenase)</td>
<td>24.1</td>
<td></td>
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<tr>
<td>Main glycolytic pathways</td>
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<td>eno</td>
<td>LMOh7858_2604</td>
<td>Q4EEP6_LISMO</td>
<td>Enolase</td>
<td>2.0</td>
</tr>
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<td>Main glycolytic pathways</td>
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<td>gap</td>
<td>lmo2459</td>
<td>Q8Y411_LISMO</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase (Gap protein)</td>
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<td>Main glycolytic pathways</td>
<td>196</td>
<td>pgk</td>
<td>lmo 2458</td>
<td>PGK_LISMO</td>
<td>Phosphoglycerate kinase</td>
<td>3.1</td>
</tr>
<tr>
<td>Metabolism of aminoaids and related molecules</td>
<td>13</td>
<td>hom</td>
<td>LMO6854_2608</td>
<td>Q4EPH5_LISMO</td>
<td>Homoserine dehydrogenase</td>
<td>2.6</td>
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<tr>
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<td>Q8YAC3_LISMO</td>
<td>Cysteine synthase</td>
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<td>lmo1619</td>
<td>DAAA_LISMO</td>
<td>D-alanine aminotransferase</td>
<td>2.1</td>
</tr>
<tr>
<td>Regulation</td>
<td>31</td>
<td>ccpA</td>
<td>LMO6854_1652</td>
<td>Q4EP90_LISMO</td>
<td>Catabolite control protein A (Fragment)</td>
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<td>Elongation</td>
<td>17</td>
<td>tuf</td>
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<td>Q4EJM9_LISMO</td>
<td>Elongation factor Tu</td>
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<td>Protein folding</td>
<td>4</td>
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<td>Q4EGL2_LISMO</td>
<td>Chaperone protein DnaK</td>
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<tr>
<td>Protein folding</td>
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<td>groEL</td>
<td>LMOh7858_2197</td>
<td>Q4EZE8_LISMO</td>
<td>60 kDa chaperonin, GroEL</td>
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<td>ctc</td>
<td>BSU00520</td>
<td>A1E155_LISMO</td>
<td>General stress protein Ctc</td>
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<td>Adaptation to atypical conditions</td>
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<td>Adaptation to atypical conditions</td>
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<td>Lmo1601 protein (Stress protein-like protein)</td>
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<tr>
<td>Formate-tetrahydrofolate ligase activity</td>
<td>11</td>
<td>fhs</td>
<td>LMOh7858_2002</td>
<td>Q4EH88_LISMO</td>
<td>Formate-tetrahydrofolate ligase</td>
<td>2.0</td>
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Table 2. Differentially expressed intracellular proteins. Differential protein abundance in the autolytic strain C897 at 30°C in comparison to the non-autolytic strain EGD at 30°C.

<table>
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<tr>
<th>Functional category or description*</th>
<th>Spot ID</th>
<th>Gene name</th>
<th>Locus name</th>
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<th>Protein name</th>
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<tr>
<td>More abundant proteins in the autolytic strain C897 at 30°C in comparison to the non autolytic strain EGD at 30°C</td>
<td>2</td>
<td>pykA</td>
<td>lmo1570</td>
<td>Q8Y6W1_LISMO</td>
<td>Pyruvate kinase</td>
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<td>Specific pathways</td>
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<td>ldh1</td>
<td>LMO2365_0221</td>
<td>LDH1_LISMF</td>
<td>L-lactate dehydrogenase 1</td>
<td>2.0</td>
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<tr>
<td>Main glycolytic pathways</td>
<td>196</td>
<td>pgk</td>
<td>lmo 2458</td>
<td>PGK_LISMO</td>
<td>Phosphoglycerate kinase</td>
<td>3.2</td>
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<td>Metabolism of aminoaids and related molecules</td>
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<td>LMO6854_2608</td>
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<td>Homoserine dehydrogenase</td>
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<td>Metabolism of coenzymes and prosthetic groups</td>
<td>57</td>
<td>thiD</td>
<td>lmo0662</td>
<td>Q8Y971</td>
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<td>Catabolite control protein A (Fragment)</td>
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<td>Protein modification</td>
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<td>Methionine aminopeptidase</td>
<td>2.7</td>
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### 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

<table>
<thead>
<tr>
<th>Category</th>
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<th>Accession</th>
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<td>Cell wall</td>
<td>iap</td>
<td>lmo0582</td>
<td>Invasion associated protein p60</td>
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<td>mbl</td>
<td>lmo2525</td>
<td>Mbl protein</td>
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<tr>
<td>Transport/binding proteins and lipoproteins</td>
<td>manL</td>
<td>LMOf6854_0109</td>
<td>PTS system, mannose-specific, IIAB component</td>
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</tr>
<tr>
<td>Membrane bioenergetics</td>
<td>glyA</td>
<td>LMOh7858_2691</td>
<td>Serine hydroxymethyltransferase</td>
<td>2.0</td>
</tr>
<tr>
<td>Specific pathways</td>
<td>pykA</td>
<td>lmo1570</td>
<td>Pyruvate kinase</td>
<td>5.6</td>
</tr>
<tr>
<td>Main glycolytic pathways</td>
<td>tkr-2</td>
<td>LMOf6854_1347</td>
<td>Pyruvate dehydrogenase complex, E1 component</td>
<td>5.7</td>
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<td>Main glycolytic pathways</td>
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<td>Isocitrate dehydrogenase [NADP]</td>
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<td>TCA cycle</td>
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<td>Serine hydroxymethyltransferase</td>
<td>2.0</td>
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<td>Chorismate mutase/phospho-2-dehydro-3-deoxyxepotonate aldolase</td>
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<td>Quinolinate synthetase complex, subunit A</td>
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<td>lmo 1398</td>
<td>Protein RecA</td>
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<td>Redox-sensing transcriptional repressor rex</td>
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<td>Q4EDU9_LISMO</td>
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<td>Q4EJN0_LISMO</td>
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<td>Superoxide dismutase</td>
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<td>Q4EPM2_LISMO</td>
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<td>Q4EVM9_LISMO</td>
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<td>Cell wall</td>
<td>189</td>
<td>mbl</td>
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<td>Q8Y4C5_LISMO Mbl protein</td>
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<td>Membrane bioenergetics</td>
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<td>minD</td>
<td>LMOh7858_2963</td>
<td>Q4EJS8_LISMO Oxidoreductase</td>
<td>3.0</td>
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<tr>
<td>Cell division</td>
<td>168</td>
<td>minD</td>
<td>LMOf6854_1593</td>
<td>Q4EQ78_LISMO Septum site-determining protein MinD</td>
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<td>Specific pathways</td>
<td>38</td>
<td>pfkA</td>
<td>LMO2365_1593</td>
<td>K6PF_LISMF 6-phosphofructokinase</td>
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<tr>
<td>Main glycolytic pathways</td>
<td>54</td>
<td>tpiA-2</td>
<td>LMOh7858_2606</td>
<td>Q4EEP4_LISMO Triosephosphate isomerase</td>
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<td>Main glycolytic pathways</td>
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<td>Q4EEZ6_LISMO</td>
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<td>trpB</td>
<td>LMOf6854_1683</td>
<td>Q4EN62_LISMO Tryptophan synthase, beta subunit</td>
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<td>Metabolism of aminoaicids and related molecules</td>
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<td>trpA</td>
<td>LMOf6854_1682</td>
<td>Q4EN63_LISMO Tryptophan synthase alpha chain</td>
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<td>LMOf6854_1987</td>
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<td>Q4EQU8_LISMO</td>
<td>Aspartate aminotransferase, putative</td>
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<td>Metabolism of nucleotides and nucleic acids</td>
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<td>parH</td>
<td>LMOf6854_1824</td>
<td>Q4ET64_LISMO Phosphoribosylaminomimidazolecarboxamide formyltransferase/IMP cyclohydrolase</td>
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<td>Metabolism of nucleotides and nucleic acids</td>
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<td>LMO2365_1859</td>
<td>PYRE_LISMF Orotate phosphoribosyltransferase</td>
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<td>Metabolism of nucleotides and nucleic acids</td>
<td>160</td>
<td>parM</td>
<td>LMOf6854_1826</td>
<td>Q4ET66_LISMO Phosphoribosylformylglycinamidine cyclo-ligase</td>
<td>2.4</td>
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</table>
Continued

| Metabolism of nucleotides and nucleic acids | 179 | purQ | LMO6854_1829 | Q4ET69_LISMO | Phosphoribosylformylglycinamidine synthase I | 2.0 |
| Metabolism of lipids | 19 | fabF | LMO6854_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 | ackA2 | LMO2365_1603 | ACKA2_LISMO | 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 | LMO6854_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 | | LMO6854_2133 | Q4EST3_LISMO | Redox-sensing transcriptional repressor rex | 2.0 |
| RNA modification | 84 | | LMO6854_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 | | LMO6854_1139 | Q4EUU3_LISMO | 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase | 2.0 |
| Lipopolisacharide biosynthetic process | 64 | rfbC | LMO6854_1135 | Q4EU79_LISMO | DTDP-4-dehydrorhamnose 3,5-epimerase | 2.0 |

**Proteins only expressed by the non-autolytic strain EGD**

| Protein modification | 275 | map | LMO6854_1768 | Q4EMT5_LISMO | Methionine aminopeptidase | NA |
| Translation | 277 | pheT | lmo1607 | Q8Y6S6_LISMO | PheT protein | NA |
| Quorum-sensing | 280 | luxS | LMO6854_1330 | Q4ENQ7_LISMO | Autoinducer-2 production protein LuxS | NA |

*The functional category is indicated as at ListiList (http://genolist.pasteur.fr/ListiList/); *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; C897 at 20°C and C897 at 30°C; EGD at 30°C and C897 at 30°C. Spots in bold are over-produced both by EGD at 30°C and by C897 at 20°C.*

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 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].
E. PINTO ET AL.

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-acetyl-muramate alanine ligase and the septum determining protein MinD, respectively were identified. UDP-N-acetyl-muramate-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 OD600nm 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

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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/lipoproteins and is an EII Mant PTS permease.

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

<table>
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<th>Spot ID</th>
<th>Gene/Locus name</th>
<th>Uniprot ID</th>
<th>Uniprot protein name</th>
<th>Fold Change</th>
<th>Description</th>
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</thead>
<tbody>
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<td><strong>More abundant proteins secreted by the autolytic strain C897 at 30°C in comparison to the non autolysis condition (20°C)</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell wall</td>
<td>69</td>
<td>iap/LMO2365_0611</td>
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<td>pgk/lmo2458</td>
<td>Q8Y4I2</td>
<td>Phosphoglycerate kinase</td>
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<td><strong>More abundant proteins secreted by the autolytic strain C897 at 30°C in comparison to the non autolytic strain EGD at 30°C</strong></td>
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<td>Cell wall</td>
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<td>lmo0560</td>
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<td>Q7ANT6</td>
<td>Similar to inosine-monophosphate dehydrogenase</td>
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<td>Specific pathways</td>
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<td>Pyruvate kinase</td>
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<td>From other organisms (Regulation and sensing)</td>
<td>74</td>
<td>lmo1333</td>
<td>Q8Y7E9</td>
<td>Lmo1333, similar to B. subtilis YqC protein</td>
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<td><strong>More abundant proteins secreted by the non-autolytic strain EGD at 30°C in comparison to the autolytic strain EGD at 30°C</strong></td>
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<td>mreB/lmo1548</td>
<td>Q8Y6Y3</td>
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<td><strong>Proteins detected only in the secretome of the non-autolytic strain EGD at 30°C</strong></td>
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<td>NadC protein, similar to nicotinate-nucleotide pyrophosphorylase</td>
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</table>

1 The functional category is indicated as at ListiList (http://genolist.pasteur.fr/ListiList/); 2 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; 3 Two spots identified, spot 33 and spot 34.

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IIAB subunit. An increase expression of this mannose-
specific PTS enzyme IIAB also was detected in the in-
tracellular 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 sensi-
tive 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 in-
creased amounts of the P60 autolysin was observed in the
intracellular proteome at non-permissive temperature.
These findings suggest that at lower temperature the ex-
port of autolysins is inhibited and this may be due to al-
terations in the cell wall as evidenced by the over-pro-
duction of proteins involved in cell wall synthesis. Dif-
fences in over-produced proteins of the glycolytic
pathway, fatty acid and amino acids synthesis, transcrip-
tion 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, transcrip-
tion regulation and cell morphogenesis were commonly
over-produced by the non-autolytic strain EGD and by the
autolytic strain at autolysis non-permissive tempera-
ture, indicating a set of important cellular events impor-
tant 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

This work was supported by Fundação para a Ciência e a
Tecnologia (PTDC/AGRI-ALI/2006 and IBB/CBME, LA,
FEDER/POCI). ML Faleiro is grateful for the grant
SFRH/BSAB/859/2008.

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doi:10.1111/j.1365-2958.2007.05854.x

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doi:10.1093/emboj/19.17.4473


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doi:10.1046/j.1365-2958.2000.02004.x


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

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<th>UniProt ID*</th>
<th>Protein name</th>
<th>Mass (Da) (T/C)</th>
<th>pI (T/C)</th>
<th>Score</th>
<th>Peptide matching</th>
<th>Coverage (%)</th>
<th>Source</th>
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<td>1</td>
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### Extracellular proteins

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<td>Q8Y4U4</td>
<td>Phosphoglycerate mutase</td>
<td>56104/65100, 4.73/5.45, 1250, 29/33, 48</td>
<td>L. monocytogenes EGD</td>
</tr>
<tr>
<td>202</td>
<td>Q8Y581</td>
<td>Lmo2192 protein, similar to oligopeptide ABC transporter</td>
<td>36712/37240, 6.85/6.25, 874, 19/22, 47</td>
<td>L. monocytogenes EGD</td>
</tr>
<tr>
<td>214</td>
<td>Q8Y5N3</td>
<td>NadC protein, similar to nicotinate-nucleotide pyrophosphorylase</td>
<td>30585/25990, 7.02/6.40, 608, 17/17, 54</td>
<td>L. monocytogenes EGD</td>
</tr>
</tbody>
</table>

*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.