Comparison of Listeria monocytogenes Exoproteomes from biofilm and planktonic state

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Comparison of *Listeria monocytogenes* exoproteomes from biofilm and planktonic state: Lmo2504 a protein associated with biofilms

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Abstract

The foodborne pathogen *Listeria monocytogenes* is the causative agent of the severe human and animal disease listeriosis. The persistence of this bacterium in food processing environments is mainly attributed to its ability to form biofilms. The search for proteins associated with biofilm formation is an issue of great interest, with most studies targeting the whole bacterial proteome. Nevertheless, exoproteins constitute an important class of molecules participating in various physiological processes such as cell signaling, pathogenesis and matrix remodeling. The aim of this work was to quantify differences in protein abundance between exoproteomes from biofilm and from planktonic state. For this, two field strains previously evaluated as good biofilm producers (3119 and J311) were used, and a procedure for the recovery of biofilm exoproteins was optimized. Proteins were resolved by 2D-DIGE and identified by ESI-MS/MS. One of the proteins identified in higher abundance in the biofilm exoproteomes of both strains was the putative cell wall binding protein Lmo2504. A deletion mutant strain on this gene was produced (3119Δ*lmo2504*) and its biofilm forming ability compared to the wild type using the crystal violet and the ruthenium red assays, as well as scanning electron microscopy. The results confirmed the involvement of Lmo2504 in biofilm formation, as strain 3119Δ*lmo2504* showed significantly ($p < 0.05$) lower biofilm forming ability, compared to the wild type. The identification of additional exoproteins associated with biofilm formation may lead to new strategies for controlling this pathogen in food processing facilities.

Keywords: *Listeria monocytogenes*, exoproteome, biofilm, planktonic, Lmo2504 (putative cell wall binding protein).

1. Introduction

Listeria monocytogenes is a foodborne pathogen able to persist in food processing environments. It is the causative agent of the severe human and animal disease, invasive listeriosis, whose features frequently include meningitis or sepsis. Listeriosis has a mortality rate of 23.7 % (1) and is especially severe in the elderly and in immunocompromised persons. In the case of pregnant women it may lead to premature birth, abortion or stillbirth.

The ability of these bacteria to form biofilms is often associated with their ability to survive adverse conditions in food processing environments. Its biofilm forming ability has been evaluated by several methods more or less correlated to the conditions found in the food industry, namely the type of surface material (2). In addition to microbial cells, the biofilm matrix is composed by exopolysaccharides, lipids, glycolipids, DNA and proteins (3). The evaluation of biofilm forming ability may be performed by several methods that target different biofilm components. Methods such as the crystal violet assay (4) are directed towards the viable cells within the biofilm, and methods such as the ruthenium red assay (4) target the biofilm matrix exopolysaccharides. A more reliable comparison between strains is obtained when agreement between such different approaches is verified.

The role of proteins within the biofilm matrix has also been demonstrated by the decrease in adhesion which occurs after treatment of *L. monocytogenes* cells with proteases (5).

The cells within biofilms are characterized by gene expression patterns different from their planktonic counterparts. Bacterial transcriptomic analysis does not always correlate with detected proteins and their functional activity. The proteomic approach allows a glimpse into the presence of functional molecules (6).

The aim of this work was to evaluate differences in exoproteins from biofilm cells *versus* its planktonic counterpart by using two-dimensional difference gel electrophoresis (2D DIGE) and electrospray tandem mass spectrometry (ESI-MS/MS) identification. We have developed a suitable method to obtain an appropriate amount of biofilm exoproteins for analysis and to minimize the contamination

with proteins from planktonic cells. Protein Lmo2504, putative cell wall binding, was one of the most abundant proteins in the biofilm exoproteomes. A deletion mutant on the gene coding for this protein was constructed and its biofilm forming ability compared with the wild type. This allowed to experimentally confirm, for the first time, the involvement of this protein in biofilm formation.

2. Materials and methods

2.1. Strains

Two field strains of *L. monocytogenes,* previously identified as good biofilm producers (2), were selected for this study: strain J311 (serovar 4b) was isolated from raw chicken (7) and strain 3119 (serovar 1/2b) was isolated from cheese (8). To construct a mutant of strain 3119, *Escherichia coli* strain DH5α carrying pAUL-A (9) was used. For mutant construction, *E. coli* strain was grown at 37 °C with shaking in LB medium or on LA plates and when required, erythromycin was added to a final concentration of 250 μg ml^{-1}. *L. monocytogenes* strains were grown at 37 °C with shaking in BHI broth or on BHI plates and when required, erythromycin was added to a final concentration of 5 μg ml^{-1}. Cultures were stored at -80 °C in Tryptic Soy Broth or LB with 15% (w/v) glycerol, until use.

2.2. Growth of biofilm and planktonic cultures and protein secretion

Cultures from -80 ºC were struck onto TSA-YE (Oxoid, Hampshire, UK) and incubated overnight at 25 ºC. For each isolate, 10 ml of Modified Welshimer's broth (MWB) (10) was inoculated and incubated 24 hours at 25 ºC to obtain pre-inocula.

For planktonic growth, 1 ml of each pre-inoculum was used to inoculate 30 ml of MWB and incubated for 24 h at 25 ºC at 150 rpm. The cultures were then centrifuged at 3000 g for 20 minutes and the supernatant fluid discarded. Cells were resuspended in 30 ml of fresh MWB and centrifuged again to

discard the supernatant fluid. Thirty ml of fresh MWB was used to resuspend the pellet and incubation proceeded for 6 hours at 25 ºC for protein secretion.

Biofilms were grown on stainless steel coupons prepared as described by Lourenco et al. (2).Disposable test tubes with 24 ml of MWB were inoculated with 1 ml of each pre-inoculum and incubated for 24 h at 25 ºC. After this period, 14 stainless steel coupons were immersed in the cultures and incubated for 4 hours at 25 ºC to allow attachment. After this contact period, unattached cells were removed by replacing the media with 25 ml of fresh MWB. The biofilm was then allowed to form by incubating 48 h at 25 ºC followed by media renewal and subsequent incubation of another 48 hours. After biofilm formation, the biofilm was rinsed with 25 ml of fresh MWB to remove planktonic cells. Twenty five ml of fresh MWB was placed in contact with the biofilm, for 6 hours at 25 ºC, to allow for protein secretion. Three biological replicates were obtained for each strain-condition combination.

2.3. Protein precipitation and quantification

Proteins were precipitated from culture supernatant fluids as described by Cabrita et al. (11). Just before use, protein samples were dissolved in 300 µl of buffer solution **A** (8 M urea, 2 M thiourea, 4 % CHAPS, 20 mM Tris pH 8.5, 0.2% SDS). Two µl of Benzonase® nuclease (Novagen, Madison WI, USA) and MgCl (final concentration of 5 mM) were then added. The protein concentration in the samples was determined using the EZQ™ Protein Quantitation Kit according to manufacturer instructions (Invitrogen, Ltd., USA).

2.4. Two-dimensional Difference Gel Electrophoresis (2-D DIGE)

A total of 35 µg of protein from each sample was labeled with a different CyDye according to the three dye protocol for minimal labeling (GE Healthcare Inc., USA). Briefly, adding dye solution (400 pmol/µl), vortexing, centrifuging and incubating on ice, in the dark, for 30 min. The labeling reaction was then stopped by adding 1 µl of 10 mM lysine and incubation on ice, in the dark, for 10 min. An

internal standard pool was created from all of the samples and 35 µg was also labeled for use in each gel. Dye swapping between experimental samples was performed to control dye-specific artifacts. In each gel, 120 µg of unlabeled protein was added. The total volume of the samples was normalized to 500 µl by adding Dithiothreitol (DTT) solution to a final concentration of 100 mM, IPG buffer pH 4-7 and 2 µl of Orange G dye (Fisher Scientific, Fair Lawn NJ, USA) and buffer solution A. The samples were loaded on IPG strips (24 cm pH 4-7) (GE Healthcare Biosciences AB, Uppsala, Sweden) and rehydrated overnight (16 -18 hours). An Ettan IPGphor 3 (GE Healthcare) was used for isoelectric focusing. The proteins on IPG strips were then reduced and alkylated in NuPAGE® LDS Sample Buffer (Invitrogen) with 100 mM DTT and then 2.5 % iodoacetamide. The strips were subsequently loaded on 8.0 % to 16.0 % Tris-Glycine gradient, 1 mm thick gels (Jule Inc. Biotechnologies, Milford CT, USA). A 25 -225 kDa ladder (GE Healthcare) was loaded in each gel. The electrophoresis of the six gels was performed simultaneously in an Ettan DALTsix (GE Healthcare) at 12 ^{0}C with an initial step of 80 V for one hour at 1 W/gel followed by an overnight run at 150 V and 2 W/gel.

2.5. Gel analysis

Gels were scanned with a Thyphoon Trio + scanner (GE Healthcare) with a pixel size of 100 µm using the appropriate excitation lasers and emission filters depending on the dye according to manufacturer (GE Healthcare). The gel images were analyzed using DeCyder™ image analysis software, Biological Variation Analysis (BVA) module (GE Healthcare). Spots were selected based on a fold difference higher than 1.5 and with a significance of $p < 0.05$ (Student's t test for paired samples) excised using Investigator™ ProPic protein picking robot (Genomic Solutions Ltd, Ann Arbor MI, USA) and combined. The samples were destained twice with 50 % (v/v) ACN, and 50 mM ammonium bicarbonate (ABC) (Fisher Scientific) solution. The solution was then removed and dried using a speed vaccum centrifuge (Labconco Corporation, Kansas City MO, USA).

The samples were rehydrated in 100 µl of 45 mM DDT at 55 ^{0}C for 45 min. The tubes were chilled to room temperature, DTT quickly removed and replaced by 100 µl of 100 mM iodoacetamide (IAA)

followed by incubation in the dark at room temperature, for 45 minutes. IAA was removed and the samples washed three times with 1:1 ACN:ABC solution. The gel pieces were then dried down in speed vaccum for 15 minutes. Protein in-gel digestion was obtained using 10 µl of a 12.5 ng/µl solution of porcine trypsin (Promega, Madison WI, USA) and covering it with 30 µl of ABC to keep the gel pieces immersed throughout the digestion of 12 hours at 37 ^{0}C. Peptides were extracted by removing the supernatant fluid to fresh tubes. Digestion tubed were washed 200 µl of 80 % ACN, 0.1 % formic acid (FA) in order to increase the peptide recovery. The samples were s speed dried.

For mass spectrometry the peptides were solubilized in 11 µl of a solution 30 % ACN, 1 M FA. The samples were then sonicated for two minutes and vortexed. The samples were subsequently washed using Zip Tip® Pipette tips C18 (Millipore, Billerica MA USA) with 0.1 % FA solution, and eluted from the Zip tips using 5 µl of a 20 % isopropyl alcohol (IPA), 30 % ACN, and 0.1 % FA solution.

After prediction of the volume of protein present in each sample, the ones with higher concentration were identified in ESI-MS/MS through direct infusion. The less concentrated samples were dried and solubilized in 50% ACN, 0.1% FA for high performance liquid chromatography – electrospray tandem mass spectrometry (HPLC-ESI-MS/MS).

2.6. Peptide sequencing by ESI-MS/MS

LC-MS/MS: The enzymatically digested samples were injected onto a C18 trap column (SGE Incorporated, TX) and desalted for 5 min with a flow rate 3 µl/min of 0.1% v/v formic acid. The samples were loaded onto an LC Packing® C18 Pep Map nanoflow HPLC column. The elution gradient of the HPLC column started at 3 % solvent B, 97 % solvent C and finished at 50% solvent B, 40% solvent C for 20 min then brought back to initial conditions for protein identification. Solvent B consisted of 0.1% v/v formic acid, 96.9% v/v ACN, and 3% v/v H_2O. Solvent C consisted of 0.1% v/v formic acid, 3% v/v ACN, and 96.9% v/v H_2O.

A LTQ Orbitrap XL mass spectrometer (ThermoFisher Scientific, West Palm Beach, FL) was used for LC-MS/MS analysis. The ion spray voltage was set to 2200 V. Full MS scans were acquired with a

resolution of 60,000 in the orbitrap from m/z 300–2000.The ten most intense ions were fragmented by collision induced dissociation (CID). Dynamic exclusion was set to 60 seconds.

2.7. Protein Search Algorithm

MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.2.2). Mascot was set up to search a *L. monocytogenes* database extracted from NCBI assuming digestion with trypsin. Mascot was searched with a fragment ion mass tolerance of 0.8 Da and a parent ion tolerance of 15 ppm. Iodoacetamide derivative of Cys, deamidation of Asn and Gln, oxidation of Met, were specified in Mascot as variable modifications. Scaffold (version Scaffold-3.3.2, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (12). Protein identifications were accepted if they established at greater than 99.0% probability and they contained, at least, two identified unique peptides. Protein probabilities were assigned by the Protein Prophet algorithm (13).

2.8. Construction of deletion mutant strain (*L. monocytogenes* 3119*Δlmo2504*)

Genome sequence of strain FSL J1-175 (from the same serovar 1/2b) provided by the BROAD Institute (http://www.broad.mit.edu) was used for primer design to generate the isogenic in-frame deletion mutant of strain 3119. Two flanking regions, upstream and downstream of the gene homologous to *lmo2504* in strain EGDe, with approximately 700 basepair (bp) each, were generated by PCR from strain 3119 genomic DNA, using primers P1 together with P2 and P3 together with P4 (Table 1), respectively. The PCR product obtained by using primers P1 and P4 and using as templates the up and downstream regions digested with *Bam* HI and *Eco* RI, was ligated to the temperature-sensitive suicide vector pAUL-A (9) previously linearized with the same enzymes. The resulting plasmid (pFARRUQ) was electroporated using Gen Pulser® (Biorad) set at 400 Ω, 10 kV/cm,

196 25 µF, into strain 3119, integrated and excised as described by Arnaud et al. (14) for homologous
197 recombination.
198 The resulting mutant strain (3119Δ*lmo2504)* was confirmed by PCR analysis using primers P5
199 together with P6 (flanking the gene), P7 together with P8 (intragenic region) and also P8 together with
200 P5 and P7 together with P6 (Table 1).
201

202 **2.9. Evaluation of biofilm forming ability: crystal violet, ruthenium red and scanning**
203 **electron microscopy (SEM)**

204 For SEM, biofilms of wild type and mutant strain 3119Δ*lmo2504* were grown on 1×1 cm stainless
205 steel coupons as described in section 2.2. After biofilm formation, the coupons were washed with
206 phosphate buffer, fixed with gluteraldehyde 2.5 % (v/v) for 3 hours and washed by overnight
207 immersion in phosphate buffer. Biofilms were then dehydrated in a 10 minutes steps gradient of 30-
208 50-70-90-100 % (v/v) ethanol. Each coupon was attached to a stub and coated with gold using a
209 JEOL JFC-1200 Sputter Coater. The samples were observed in a JEOL JSM-5200LV electron
210 microscope operating at 25 kV and with an analysis distance of 10/20 mm. Digital images were
211 directly captured.
212 Biofilm-forming ability was evaluated using the crystal violet (CV) and the ruthenium red (RR) assays
213 according to the procedures described by Lourenco et al. (2) and Borucki et al. (4), respectively, with
214 MWB as growth media.
215 Conformance to normality of the data generated by CV and RR assays was checked using the
216 Anderson-Darling test and conformance to homogeneity of variance was determined using Levene's
217 test. The comparison between wild type and mutant was then performed by ANOVA Least Significant
218 Differences (LSD) post hoc multiple comparison test by running the software Statistica® version 7.0
219 (Statsoft, Tulsa, OK, USA).
220

3. Results

Preliminary 2-D gel electrophoresis analysis of the exoproteome from planktonic and biofilm cells, using a wide range pH gradient (3 - 10), showed that the majority of the proteins had their isoelectric point (pI) in the acidic region (data not shown), therefore the analysis performed in this study used IPG strips pH 4-7. Figure 1 shows representative gel images of three biological replicas for the two strains in bioflm and planktonic form. The exoproteins from biofilms of strains *L. monocytogenes* 3119 and J311 are shown in Fig. 1A and Fig. 1C, respectively. The corresponding exoproteins from planktonic state are shown in Fig. 1B and Fig. 1D. Approximately 472 spots were matched. The protein spots that were significantly more intense in the biofilm state, for the joint analysis of the strains, are numbered in the Figure 1 according to table 2.

For strain 3119, statistical analysis of the images indicated 40 spots (23 identified) with significant higher intensity in biofilm state when compared with the planktonic state (average ratio > 1.5 and $p < 0.05$, Table 2). In the same analysis, 39 spots (29 identified) were significantly less intense in the biofilm state. For strain J311, 32 spots (18 identified) had a significant higher intensity in the biofilm state when compared with the planktonic state. Twenty eight spots (21 identified) were detected has having significantly less intensity in the biofilm than in the planktonic state (Table 2).

Data from both strains was combined to strengthen the comparison between biofilm and planktonic growth states. The joint analysis allowed the detection of 26 spots (16 identified) has having significantly higher intensity in the biofilm state than in the planktonic state and 12 (10 identified) were significantly more intense in the planktonic state.

The identification of these proteins indicated that PlcA, Lmo2504 and ActA were the proteins with significantly higher intensity in the biofilm state compared with planktonic state, either for both strains individually as for the joint analysis. On the other hand, for both strains individually and for the joint analysis, antigen A and internalin C were among the proteins with the more negative ratios (Table 2).

With strain 3119, compared with strain J311, more spots, either with a positive or with a negative ratio, were detected. Consequently, more differences between biofilm and planktonic state were observed. In fact, when the spots with significant intensity and a positive ratio are compared, strain 3119 has 10 spots that were not considered on strain J311, whereas only five spots were not significant for strain 3119. Likewise, on the negative ratio, strain 3119 has 13 spots that were not considered on strain J311, whereas only five spots were not significant for strain 3119 (Table 2). It can also be seen that, for strain 3119, the absolute values of the ratios are generally higher than for strain J311, making strain 3119 a better candidate for the construction of a deletion mutant.

A deletion mutant on the gene coding for the protein with the second highest ratio between biofilm and planktonic state (Lmo2504) was subsequently obtained on strain 3119. The resulting mutant strain (3119Δlmo2504) and the wild type strain were grown separately in triplicate in MWB at 25 ^{0}C and growth rates (μmax) were for both strains approximately 0.072 h^{-1}.

The results obtained with crystal violet and ruthenium red assays for the mutant strain 3119Δ*lmo2504* and its wild type are presented in Table 3. For both assays, either after 24 or 48 hours of growth, the values obtained for the wild type strain were significantly higher than for the deletion mutant strain. Figure 2 shows SEM images of 3119 and 3119Δ*lmo2504*. The wild type strain produced more numerous and larger microlonies as compared to the mutant.

4. Discussion

In this study some proteins were identified in more than one spot. This same effect has been verified by other authors. Dumas *et al.* (15) stated that PlcB and InlC, from *L. monocytogenes* were present in five and four spots, respectively, with different pIs and Mrs. These authors explained this effect by the possible presence of protein orthologues with different Mrs, pIs, and/or posttranslational modifications (isoforms). Trost *et al.* (16) on the analysis of extracellular and intracellular proteomes of *Listeria* reported that following 2-DE, 120 spots were identified as belonging to 58 different proteins.

Posttranslational modifications or isoelectric focusing artefacts due to TCA precipitation or desulphuration were pointed out by the authors as explanatory.

The major sources for protein isoforms include: variable spliced forms of the same gene product, (ii) N- and C-terminal truncations, (iii) co-and post-translational modifications (includes modifications that influence the charge of the protein such as phosphorylation, deamidation, glycosylation, and N-terminal acetylation), (iv) endogenous protein degradation, and (v) oligomerisation (reviewed by Harry *et al*., 17). Protein modification by proteases that remove short peptides from either end of the protein such as the cleavage and the removal of specific signal peptides will result in a unique migration position on a 2-D gel. A narrow-range IPG allows the separation of isoforms that differ only by a single amino acid substitution (reviewed by Harry *et al*., 17). In this work, the narrow- range of IPG (pH 4-7) used may also account for these results.

By comparing the exoproteomes of *L. monocytogenes* from biofilms and planktonic cells we have detected and identified, for the two strains, 16 protein spots that were significantly more intense in the biofilm state (Fig.1 and Table 2). Within this group of proteins were: phospholipase PlcA (Lmo0201), flagellin (FlaA), a putative penicillin-binding protein (Lmo1438), an actin-assembly inducing protein (ActA) and a putative cell wall binding protein (Lmo2504).

PlcA, phosphatidylinositol-specific phospholipase C is a *L. monocytogenes* virulence factor that synergizes with listeriolysin O (LLO) and another phospholipase, PlcB, for the destabilization of the membranes of primary and secondary phagosomes. PlcA, secreted by the Sec system, is positively regulated by the positive regulatory factor A (PrfA) (18). Lemon et al. (19) have shown that this virulence regulator has a significant role in biofilm formation. The transcription of *prfA* is under the control of three promoters, including P*plcA* that is autoregulated by PrfA and results in a bicistronic transcript for both *plcA* and *prfA*. Nevertheless, Lemon et al. (19) reported that a double mutant lacking *plcA* and *plcB* unlike the Δ*prfA* mutant did not show defective biofilm formation.

Flagellin is a protein monomer secreted by the flagella export apparatus (FEA) that polymerizes to form the flagella. The importance of flagella for biofilm establishment and development has been

296 reported (20, 21, 22). In a comparison of the whole proteome of *L. monocytogenes* cells from
297 planktonic and biofilm state, Tremoulet et al. (23) reported that flagellin was the only protein that
298 decreased in biofilm, compared to planktonic state. Nevertheless, Hefford et al. (24) observed higher
299 amounts of flagellin in biofilm grown cells than in planktonic cells. In the present study, the joint
300 analysis, with input from the two strains showed a significantly higher abundance of flagellin in the
301 biofilm exoproteome. However, when the comparison between growth states was performed
302 separately for each strain, only strain 3119 had a significantly ($p < 0.05$) and valid higher abundance
303 (average ratio > 1.5).
304 In both strains, a putative penicillin-binding protein (PBP) was observed at greater levels in the
305 exoproteomes of biofilm compared to the ones from planktonic state. The PBP's are a group of
306 proteins responsible for the final steps of peptidoglycan exoskeleton synthesis. In *L. monocytogenes*
307 EGDe 10 putative genes coding for PBP's have been identified (25). The protein identified in our
308 study is similar to the one coded by *lmo1438* of strain EGDe.
309 Ouyang et al. (22) verified the importance of a PBP for biofilm formation, as an insertion mutant of
310 this gene presented 86 % reduction in biofilm forming ability, compared to the wild type.
311 The actin-assembly inducing protein, ActA, is a virulence factor, as it allows assembly of actin from
312 the host cytoskeleton. The actin polymerization in one pole of the bacterial cell, forming the comet tail,
313 allows the propulsion of the bacteria towards the host cell's outer membrane (26). Travier et al. (27)
314 have pointed out a new role for ActA, showing the importance of PrfA and ActA for cell aggregation,
315 as the mutants for these genes displayed very low aggregation levels. Also, these authors found
316 evidence that the deletion of *actA* caused a reduction in biofilm formation, similar to the one caused
317 by the deletion of *prfA*. These authors further observed, by confocal microscopy, a thin and
318 homogenous layer biofilm for the *L. monocytogenes* Δ*actA* opposed to a mushroom-shaped and
319 dense biofilm from the wild type strain. The results presented here reinforce a possible extracellular
320 function of this protein as higher amount of ActA was detected in the biofilm exoproteomes from both
321 strains.

A predicted protein, coded by a gene homologous to *L. monocytogenes* EGD-e *lmo2504*, (Lmo2504) was secreted by both strains in greater amounts in the biofilm state, especially for strain 3119 (Table 2). This protein, similar to cell wall binding proteins, is predicted to be secreted according to Secretome 2.0 Server (28) with a signal peptide starting at position 1 and ending at position 25. Eight conserved domains are identified for this protein, including a domain belonging to the peptidase family M23, a group of zinc metallopeptidases (pfam 01551) (29). This group of enzymes may have various functions involving murein hydrolytic activity, cell growth, cell wall turnover, peptidoglycan maturation, cell division and separation, formation of flagella, sporulation, chemotaxis and biofilm formation (30). An endopeptidase from this same family, along with other proteins with mureinic activity from *Staphylococcus aureus*, has been shown to have influence on biofilm forming ability, as the induction of a regulator for those genes increased their expression levels and an increase in biofilm forming ability was verified (31).

Lmo2504 may also have a role in a structural maintenance of chromosomes (SMC). Like P45, Lmo2504 has a SMC-related N-terminal domain and therefore may play a role in peptidoglycan remodeling during segregation of the nucleoid (32).

Since Lmo2504 was the second most abundant protein detected in the supernatants of both strains when grown in biofilm state compared to planktonic state and since the most abundant one (P*lcA)* had already been shown not to have influence on biofilm formation (19), a deletion mutant was created on the gene coding for this protein and therefore access its influence in biofilm formation. The deletion mutant strain 3119Δ*lmo2504* constructed in the present study allowed confirming the importance of this protein in biofilm formation both by the crystal violet assay that mainly targets cells, and by the ruthenium red assay that targets exopolysaccharides (Fig. 2). Observations using SEM (Fig. 3) corroborate these results, as it was possible to observe larger and more numerous microcolonies for the wild type strain and fewer and more dispersed for 3119Δ*lmo2504* indicating lower attachment ability and a less structured biofilm of the mutant.

The role of proteins in biofilm development has been a subject of intense study in the recent years and some progress has been made in several species. The role of biofilm associated proteins (BAP's) has been established for Gram positive species such as *S. aureus*, *S. epidermidis* and *Enterococcus faecalis.* These proteins present common characteristics in common such as high molecular weight, the presence of tandem repeats, and the role in bacteria's infectious processes (reviewed by Lasa and Penades 33). In the present work, the exoproteins more intensely detected in the biofilm than in planktonic state are of lower molecular weight than those reported for BAPs. Nevertheless, three of them are virulence factors (PlcA, ActA and InlC), two (Lmo2504 and Lmo0927) have tandem repeats of small length and the protein ActA has a tandem repeat with potential biological meaning according to T-REKS algorithme (34).

In this work we have explicitated differences in the exoproteomes of *L. monocytogenes* biofilms *versus* its planktonic counterparts opening new avenues for future investigation. Furthermore, we provide evidence for the importance of a cell wall binding protein in biofilm formation. These results will assist in work to identify compounds capable of inhibiting the expression of proteins required for biofilm production.

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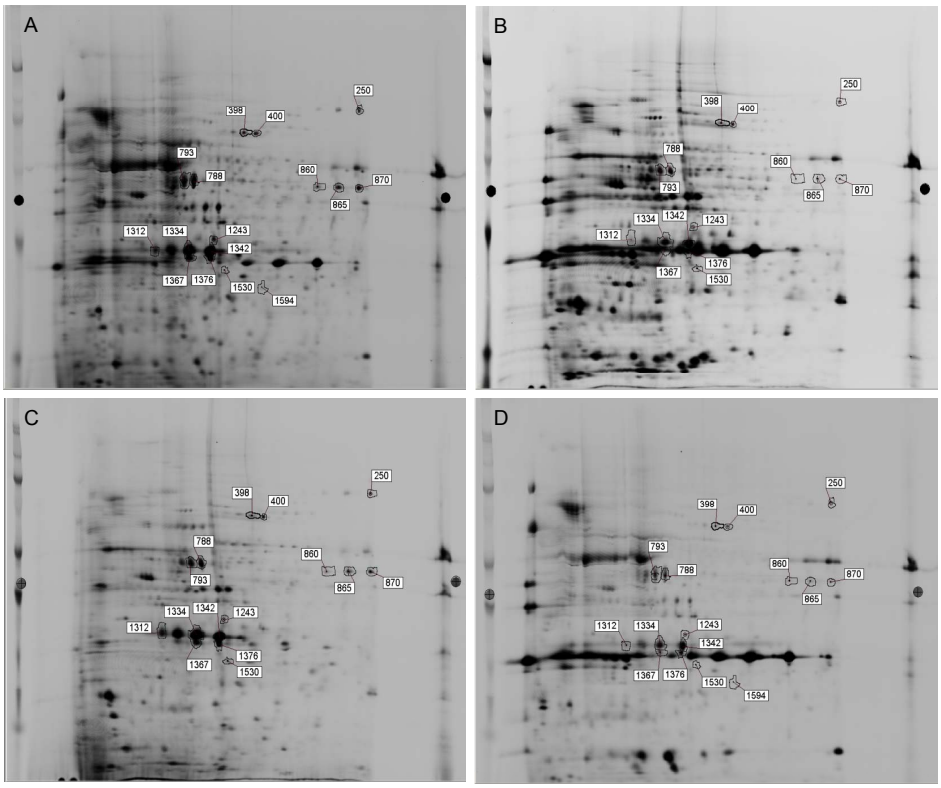

Figure 1- 2D-gel electrophoresis of the exoproteins of *L. monocytogenes* strains 3119 and J311 from biofilm (A and C) and from planktonic cells (B and D), respectively, on pH 3–7 IPG strips.
Spots are numbered according to table 2.

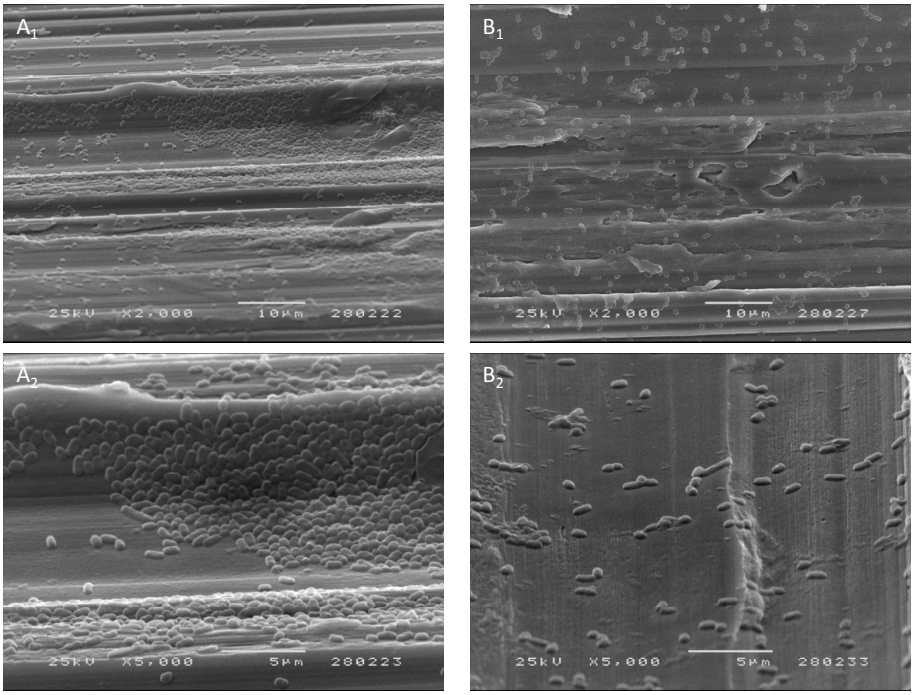

Figure 2 – Scanning electron microscope (SEM) images of strain 3119 (A_1 and A_2) and strain 3119Δ*lmo2504* (B_1 and B_2) on stainless steel coupons. Biofilms grown in MWB for 48 h at 25 °C followed by media renewal and subsequent incubation of another 48 hours.

Table 1 – Primers used for construction and confirmation of strain 3119Δ*lmo2504*

Primer	5’ 3’
P1	CG**GGATCC**ACAGCTTTACGTGAA
P2	TGGATCGACTGGAATTAGTGGCGCGGAAAT
P3	ATTTCCGCGCCACTAATTCCAGTCGATCCA
P4	G**GAATTC**CACCCATTGCATCATA
P5	CATGAATATGAAGCACAACA
P6	ACCAACATCTCCATCGATTA
P7	TGGTTATTTCCGCGCCACTA
P8	GGTGCTGGATCGACTGGAAT

Restriction sites for *Bam* HI and *Eco* RI are marked in bold in primers P1 and P4 sequences, respectively.

Table 2 – Exoproteins present in the supernatant fluids obtained from biofilms and planktonic cells, after six hours of secretion in MWB* at 25 ºC.

Master Gel Spot Number	Biofilm/Planktonic						Identification **	Accession Number **	Gene **	unique pep	% cov.
	Joint analysis		3119		J311						
	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test					
1342	6.5	0.000026	9.54	0.0046	4.79	0.0047	PlcA	gi|16802247	lmo0201	7	21
1334	4.55	0.000026	6.1	0.0046	3.56	0.0081	PlcA	gi|16802247	lmo0201	6	18
250	4.53	0.001	7.17	0.0046	3.55	0.015	Lmo2504	gi|16411992	lmo2504	5	19
870	4.03	0.0034	7.73	0.0046	2.98	0.045	Lmo2504	gi|16411992	lmo2504	3	15
1243	3.34	0.000067	3.37	0.0074	3.31	0.013	actA actin-assembly inducing protein precursor	gi|16802250	lmo0204	7	11
1312	3.3	0.00057	4.99	0.0055	-	-	flagellin	gi|17433724	lmo0609	11	38
1376	2.75	0.00024	2.63	0.021	2.88	0.039	internalin C	gi|16803826	lmo1786	11	39
865	2.63	0.014	4.94	0.0046	1.93	0.042	putative cell wall binding protein	gi|225877514	lmo2504	5	19
400	2.39	0.00015	2.55	0.021	2.25	0.0073	putative penicillin-binding protein	gi|16410867	lmo1438	4	25
1594	2.24	0.0003	2.76	0.0053	-	-	upp	gi|16412026	lmo2530	6	34
788	2.17	0.0014	3	0.0095	1.61	0.037	hypothetical protein Lmo0927	gi|16802967	lmo0297	3	6.9
1367	2.11	0.000048	2	0.0081	2.25	0.0047	internalin C	gi|16803826	lmo1786	14	49
1530	1.74	0.042	2.85	0.014	-	-	indole-3-glycerol phosphate synthase	gi|21363054	lmo1630	7	27
398	1.7	0.002	1.87	0.044	1.56	0.0094	putative penicillin-binding protein	gi|16410867	lmo1438	20	36
860	1.7	0.05	2.85	0.0076	-	-	Lmo2504	gi|16411992	lmo2504	2	11
793	1.53	0.023	2.21	0.0091	-	-	hypothetical protein Lmo0927	gi|16802967	lmo0927	9	16
397	-	-	1.55	0.026	-	-	putative penicillin-binding protein	gi|16410867	lmo1438	6	12
773	-	-	-	-	1.84	0.02	glucose-6-phosphate isomerase	gi|16804405	lmo2367	9	22
936	-	-	-	-	1.71	0.0073	enolase	gi|16411943	lmo2455	18	49
1000	-	-	2.31	0.014	-	-	peptidoglycan lytic protein P45	gi|16804543	lmo2505	5	19
1023	-	-	-	-	1.64	0.028	glyceraldehyde-3-phosphate dehydrogenase	gi|16804497	lmo2459	12	52
1134	-	-	-1.77	0.0063	-	-	translation elongation factor Ts	gi|16803697	lmo1657	6	22
1255	-	-	2.71	0.017	-	-	6-phosphofructokinase	gi|16803611	lmo1571	8	29
1283	-	-	2.08	0.03	-	-	putative AA3-600 quinol oxidase subunit II	gi|16802061	lmo0013	3	12
1414	-	-	-	-	-6.76	0.028	internalin C	gi|16803826	lmo1786	5	24
1425	-	-	-	-	-6.65	0.014	internalin C	gi|16803826	lmo1786	10	32

Table 2 – *(continued)*

Master Gel Spot Number	Biofilm/Planktonic						Identification **	Accession Number **	Gene **	unique pep	% cov.
	Joint analysis		3119		J311						
	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test					
1433	-	-	-3.97	0.013	-11.22	0.0047	internalin C	gi|16803826	lmo1786	15	64
1436	-	-	-2.63	0.016	-7.55	0.014	internalin C	gi|16803826	lmo1786	15	63
1615	-	-	-2.45	0.013	-	-	hypothetical protein Lmo0125	gi|16802173	lmo0125	10	53
1618	-	-	-2.37	0.0065	-	-	hypothetical protein Lmo0125	gi|16802173	lmo0125	11	51
1702	-	-	-1.54	0.014	-	-	Antigen A	gi|16802166	lmo0118	5	34
1736	-	-	-1.98	0.013	-	-	Antigen A	gi|16802166	lmo0118	5	27
1800	-	-	4.56	0.0064	-	-	Antigen A	gi|16802166	lmo0118	4	30
1886	-	-	-2.13	0.018	-	-	antigen B	gi|16802165	lmo0117	4	26
1894	-	-	7.6	0.0046	2.87	0.016	listeriolysin O precursor	gi|16802248	lmo0202	8	15
1962	-	-	-6.1	0.046	-	-	actA actin-assembly inducing protein precursor	gi|16802250	lmo0204	4	6.6
1987	-	-	-2.01	0.014	-4.97	0.0085	listeriolysin O precursor	gi|16802248	lmo0202	2	4.5
2007	-	-	-2.09	0.014	-10.4	0.0077	P60 extracellular protein	gi|1171970	lmo0582	4	9.9
2015	-	-	-8.46	0.0055	-	-	listeriolysin O precursor	gi|16802248	lmo0202	2	4.5
2034	-	-	-9.02	0.0046	-	-	listeriolysin O precursor	gi|16802248	lmo0202	2	4.5
2058	-	-	-9.29	0.0053	-	-	30S ribosomal protein S10	gi|16804671	lmo2633	2	23
2097	-	-	-2.1	0.0065	-	-	listeriolysin O precursor	gi|16802248	lmo0202	2	4.5
2118	-	-	-	-	-1.95	0.034	listeriolysin O precursor	gi|16802248	lmo0202	2	4.5
2276	-	-	-9.65	0.014	-6.58	0.034	internalin C	gi|16803826	lmo1786	14	64
2279	-	-	-	-	-2.1	0.016	listeriolysin O precursor	gi|16802248	lmo0202	15	38
2280	-	-	-2.49	0.0074	-	-	Lmo0127	gi|16409486	lmo0127	10	58
2281	-	-	-	-	-3.91	0.0077	P60 extracellular protein	gi|1171970	lmo0582	4	11
2282	-	-	-	-	1.93	0.014	glyceraldehyde-3-phosphate dehydrogenase	gi|16804497	lmo2459	10	42
2283	-	-	-	-	2.9	0.021	listeriolysin O precursor	gi|16802248	lmo0202	2	4.5
2284	-	-	-12.35	0.0091	-11.01	0.013	internalin C	gi|16803826	lmo1786	18	53
2287	-	-	-9.43	0.0053	-9.99	0.0047	internalin C	gi|16803826	lmo1786	16	47

Table 2 – *(continued)*

Master Gel Spot Number	Biofilm/Planktonic						Identification **	Accession Number **	Gene **	unique pep	% cov.
	Joint analysis		3119		J311						
	Av. Ratio	T-test	Av. Ratio	T-test	Av. Ratio	T-test					
2288	-	-	6.22	0.0063	2.8	0.038	Lmo2504	gi|16411992	lmo2504	3	8.9
685	-2.2	0.04	-1.75	0.03	-2.45	0.0047	listeriolysin O precursor	gi|16802248	lmo0202	4	10
678	-2.44	0.018	-1.91	0.012	-2.78	0.0077	listeriolysin O precursor	gi|16802248	lmo0202	16	33
1379	-6.26	0.00018	-6.23	0.011	-6.32	0.013	internalin C	gi|16803826	lmo1786	15	56
878	-7.68	0.00018	-7.76	0.0064	-7.63	0.0047	actA actin-assembly inducing protein precursor	gi|16802250	lmo0204	8	13
1078	-8.14	0.00037	-8.81	0.0063	-7.84	0.0055	actA actin-assembly inducing protein precursor	gi|16802250	lmo0204	15	26
1510	-8.27	0.0000068	-7.12	0.0053	-9.27	0.0055	Lmo0129	gi|16409488	lmo0129	9	41
1712	-9.32	0.004	-5.25	0.019	-11.42	0.014	antigen A	gi|16802166	lmo0118	3	20
1410	-11.52	0.0044	-14.91	0.013	-10.78	0.0047	internalin C	gi|16803826	lmo1786	4	18
1435	-13.54	0.0003	-15.32	0.0055	-12.89	0.0062	actA actin-assembly inducing protein precursor	gi|16802250	lmo0204	11	18
1382	-14.34	0.00017	-16.95	0.0074	-	-	internalin C	gi|16803826	lmo1786	2	12

Positive ratio values indicate higher abundance in the biofilm state. Negative ratios indicate higher abundance in the planktonic state.
The spots ratios marked with a dash did not comply with the established cutoff parameters for considering a valid difference of protein abundance between biofilm and planktonic states (absolute value of ratio > 1.5 with $p < 0.05$).
*MWB – Modified Welshimer's broth
** According to EGDe strain

Table 3 - Biofilm forming ability of strains 3119 and 3119Δ*lmo2504* using the crystal violet assay (Abs_{600nm}) and the ruthenium red assay (Abs_{450nm}) after growth for 24 or 48 h in MWB at 25 ºC.

Strain	Crystal violet		Ruthenium red	
	24 hours	48 hours	24 hours	48 hours
3119	0.1412 ± 0.0044	0.2561 ± 0.0164	0.3546 ± 0.0865	0.3440 ± 0.0134
3119Δ*lmo2504*	0.1331 ± 0.0048	0.2101 ± 0.0096	0.1320 ± 0.0527	0.2105 ± 0.0774
Comparison p value*	0.0158	0.0064	0.0041	0.0149

Average values ± standard deviation are indicated.
*p values obtained using ANOVA *post hoc* LSD comparison test. For both assays and both times, the level of significance considered was $p < 0.05$ and therefore differences between wild type and mutant were significant.