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Proteomic analysis reveals the temperature-dependent presence of extracytoplasmic peptidases in the biofilm exoproteome of *Listeria monocytogenes* EGD-e[§]

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The foodborne pathogen *Listeria monocytogenes* resists environmental stresses by forming biofilms. Because this pathogen transmits between the environment and the host, it must adapt to temperature as an environmental stress. In this study, we aimed to identify which proteins were present depending on the temperature in the biofilms of *L. monocytogenes* EGD-e. Proteins in the supernatants of biofilms formed at 25°C and 37°C were compared using two-dimensional gel electrophoresis and liquid chromatography with tandem mass spectrometry. The larger number of extracytoplasmic proteins associated with cell wall/membrane/envelop biogenesis was identified from the supernatant of biofilms formed at 25°C (7) than those at 37°C (0). Among the 16 extracytoplasmic proteins detected only at 25°C, three were peptidases, namely Spl, Cwh, and Lmo0186. Moreover, mRNA expression of the three peptidases was higher at 25°C than at 37°C. Interestingly, this adaptation of gene expression to temperature was present in sessile cells but not in dispersed cells. After inhibiting the activity of extracytoplasmic peptidases with a protease inhibitor, we noted that the levels of biofilm biomass increased with higher concentrations of the protease inhibitor only when *L. monocytogenes* grew biofilms at 25°C and not at 37°C. Overall, our data suggest an effect of temperature on the presence of peptidases in *L. monocytogenes* biofilms. Additionally, increasing the levels of extracytoplasmic peptidases in biofilms is likely a unique feature for sessile *L. monocytogenes* that causes a naturally occurring breakdown of biofilms and facilitates the pathogen exiting biofilms and disseminating into the environment.

***Keywords*:** cell wall metabolism, extracytoplasmic peptidases, sessile mode, biofilm dispersion

Introduction

Listeriosis is a serious disease caused by ingesting food contaminated by *Listeria monocytogenes*. This disease causes 15–20% of annual food-related deaths worldwide and is especially dangerous for the elderly, fetuses, newborns, pregnant women, and immunocompromised patients (Scallan *et al.*, 2011; European Food Safety Authority; European Centre for Disease Prevention and Control, 2016). *L. monocytogenes* can adhere to multiple abiotic surfaces (Beresford *et al.*, 2001; Silva *et al.*, 2008) and form biofilms, which leads to persistent contamination of ready-to-eat foods (Lee and Wang, 2017). During biofilm development, some *L. monocytogenes* cells within biofilms are released into the surrounding environment through dispersion. The release of bacteria with advanced resistance to sanitizers is also a great concern for food processing industries (Chavant *et al.*, 2004; van der Veen and Abee, 2010).

Biofilms are surface-associated microbial communities that exhibit resistance to antimicrobials and protection against environmental stresses. Biofilm formation is a dynamic process whereby microbes first attach to a surface, produce extracellular polymeric substances (EPS) that immobilize the biofilm structure, and finally disperse from the surface for the next cycle of biofilm formation at a new location (Hall-Stoodley and Stoodley, 2002). Bacteria undergo profound physiological changes during their transition from a planktonic life mode to a sessile, biofilm-associated life stage and then to a dispersed, free-floating life stage (O'Toole *et al.*, 2000). Sessile and dispersed cells have been reported to be highly tolerant to antimicrobial agents and to be highly virulent against immune cells compared with planktonic cells (Pan *et al.*, 2006; Uppuluri *et al.*, 2010; Chua *et al.*, 2014; Stewart *et al.*, 2015). Therefore, characteristics acquired during biofilm development make biofilm removal difficult and exacerbate cross-contamination between food processing equipment and food (Manios and Skandamis, 2014).

In addition to the intrinsic adaptations of bacteria, EPS produced by bacteria contribute substantial protection to biofilm communities. EPS are composed of polysaccharides, proteins, extracellular DNA, and lipids (Flemming and Wingender, 2010). For *Listeria*, proteins are the most abundant biomolecules within EPS (Frølund *et al.*, 1996; Combrouse *et al.*, 2013). Depending on the environment, these extracytoplasmic proteins of EPS vary in composition and quantity. *L. monocytogenes* biofilms are mainly found in environments with temperatures lower than those inside host cells, although increasing the environmental temperature to that inside host cells

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certainly accelerates biofilm formation and increases biofilm biomass of *L. monocytogenes*. This observation suggests that *L. monocytogenes* behave distinctively to shape biofilms at suboptimal growth temperatures, such as 25°C. Therefore, we hypothesized that the components of biofilm exoproteomes would change with environmental temperatures. To test that hypothesis and investigate links between extracytoplasmic proteins and biofilm development, we compared the exoproteomes of *L. monocytogenes* biofilms formed at 25°C, a temperature encountered in food processing environments, with those formed at 37°C, the temperature inside host cells.

Materials and Methods

Bacterial strain and culture conditions

L. monocytogenes strain EGD-e, a serovar 1/2a strain, was used in this study, as serovar 1/2a accounts for > 50% of *L. monocytogenes* isolates recovered from foods and the environment (Aarnisalo *et al.*, 2003; Gilbreth *et al.*, 2005). Frozen stock of this strain was kept at -80°C and subcultured in brain heart infusion (BHI) broth (Difco) for 16 h at 37°C for the following experiments.

Preparation of stainless-steel coupons

Stainless-steel chips (2 × 2 × 0.2 cm, type 304, #4 finish; Stainless Supply, Inc.) were used to mimic the materials used in food processing equipment. The chips were initially washed, air dried, and then autoclaved at 121°C for 15 min.

Biofilm formation

Overnight cultures of *L. monocytogenes* strain EGD-e were diluted to colony-forming units (CFU) of 10^7 per ml in modified Welshimer's broth (MWB; HiMedia Laboratories LLC). CFU were confirmed by serial dilution, plating, and enumeration of the colonies on the BHI agar plates after a 24 h incubation at 37°C. To establish biofilms on the stainless steel surface, 5 ml of bacterial diluent or blank MWB (control) was added to a well of 6-well sterile polystyrene microtiter plates (CELLTREAT) that were preloaded with a single stainless steel chip per well. Each bacterial and control group had three replicates in a plate. The plates were incubated at 25°C for 24 h and then either kept at 25°C or transferred to 37°C for an additional 48-h incubation. The broth was replaced by fresh broth once every 24 h during the incubation.

Separation of sessile and dispersed cells

After 3 days of incubation, the biofilm culture in a single well contained attached and non-attached cells. Three millimeters of the suspensions from the biofilm cultures were removed and centrifuged at 2,800 × *g* for 30 min. The pellets, comprising dispersed (non-attached) cells, were washed with sterile phosphate-buffered saline (PBS) and centrifuged at 5,000 × *g* for 10 min, whereas the supernatant was kept for protein extraction. After the remaining suspension was removed from the well, the chips were gently rinsed with fresh MWB once. Then, we flushed the chips thoroughly with fresh MWB to remove the sessile (attached) cells on the stainless steel surface and centrifuged them at 2,800 × *g* for 10 min. The cell pellets were kept at -80°C for subsequent RNA extraction.

Protein extraction

Proteins were extracted from the suspensions of biofilm cultures after removing the bacterial cells using the phenol extraction method described by Faurobert *et al.* (2007). Collected supernatants were loaded onto 10 kDa-cut-off concentrators (MilliporeSigma) and centrifuged at 2,800 × *g* at 4°C for 30 min. The concentrated supernatants were desalted with an equal-to-original volume of PBS. A final concentration of 200 μM phenylmethylsulfonyl fluoride (PMSF, Sigma) was added to the protein concentrates to inhibit protease activities. The protein concentrates were mixed with extraction buffer (0.5 M Tris-HCl; pH 8.8, 50 mM EDTA, 0.9 M sucrose, 0.1 M KCl, 2% β-mercaptoethanol, 1 mM PMSF) and Tris-phenol (pH 8.0) at a ratio of 1:4:1. After 10 min of shaking and 10 min of centrifugation at 7,500 × *g* at 4°C, the phenol phase was collected and re-treated with extraction buffer at a ratio of 1:4. The recovered phenol phase was then treated with a precipitation solution (0.1 M ammonium acetate in methanol) at a ratio of 1:4 and incubated overnight at -20°C. The crude proteins were pelleted and washed three times with the cooled precipitation solution and another three times with 80% acetone. The resultant pellets were dried and stored at -80°C for further analysis. Protein concentrations were determined using the Bradford Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

2D-PAGE was performed as previously described by Renier *et al.* (2013). For isoelectric focusing (IEF), 75-μg protein extracts were mixed with IEF buffer (7 M urea, 2 M thiourea, 4% [w/v] CHAPS, 1% DTT, 0.2% ampholytes with pH 3–10) to make a total volume of 200 μl. The mixtures were loaded on immobilized pH gradient (IPG) strips with a pH 4–7 linear gradient (Bio-Rad). The strips were subjected to rehydration and IEF for a total of 35,000 Vh (12 h at 50 V, 15 min at 250 V, linear gradient to 8,000 V over 2.5 h, and 8,000 V until the end) at 23°C in a re-swelling tray within a PROTEAN IEF cell (Bio-Rad). The strips were equilibrated in an equilibration solution (6 M urea, 0.375 M Tris-HCl; pH 8.8, 2% SDS, 20% glycerol) containing 2% (w/v) DTT for 15 min and in 2.5% (w/v) iodoacetamide (IAA) for another 15 min. The second-dimension electrophoresis (SDS-PAGE) was carried out with 10% acrylamide gel in a mini-Protean system (Bio-Rad). The obtained 2D gels were stained overnight with 0.5% (w/v) Coomassie Blue G250 in 45% (v/v) methanol and 10% (v/v) acetic acid and scanned using a ProteomeWorks Spot cutter (Bio-Rad).

2D-PAGE gel imaging and statistical analysis

Images of six 2D-PAGE gels (three for each experimental temperature) from three independent experiments were analyzed using PDQuest software, version 8.1 (Bio-Rad). Normalization using a local regression method was applied to calibrate all the sample data with those of the reference gel (representing exoproteomes at 25°C). A Student's t-test was

applied to determine significant changes in protein spot intensities ($P < 0.02$). A spot specific to exoproteomes formed at either 25 or 37°C was determined if the same spot was present on all three individual gel with exoproteomes from the same temperature and whether its signal intensity was significantly greater than that of the corresponding spot for the other temperature ($P < 0.05$). For each temperature, the exclusive spots were manually selected from one of the three gels for peptide identification.

Peptide identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

In-gel tryptic digestion was carried out using the In-Gel Tryptic Digestion Kit (Thermo Fisher Scientific) according to the manufacturer's instruction with minor modification. The spots of interest were cut into 1 mm × 1 mm pieces and de-stained using 200 µl de-staining solutions (100 mM NH_4HCO_3, 50% methanol, 50% water and 25 mM NH_4HCO_3, 50% acetonitrile, 50% water) at 37°C and for 30 min in each de-staining solution. We dehydrated the gels with 100% acetonitrile for 30 sec and dried them for 5 min. In-gel proteins were reduced using 50 mM Tris(2-carboxyethyl)phosphine at 60°C for 10 min and then alkylated by 100 mM IAA in the dark at room temperature for 1 h. The gel pieces were dehydrated with acetonitrile and further treated with 10 µl activated trypsin solution (10 ng/µl) for 15 min and subsequently with an extra 25 µl digestion buffer (25 mM NH_4HCO_3 in double-DW). The resultant samples were digested overnight at 30°C with 200 rpm agitation. The supernatants were cleaned up using Pierce C18 Spin Columns (Thermo Fisher Scientific) according to the manufacturer's instructions. The resulting samples were dried and stored at -20°C until use.

The peptide samples were shipped to the Arizona Proteomics Consortium and analyzed using an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) equipped with an Advion nanomate ESI source (Advion). Tandem mass spectra were processed by the SEQUEST (Xcorr) algorithm (Yates *et al.*, 1995), and results were queried in the protein database Listeria_UniprotKB_021118_Cont.fasta (98952 entries). Scaffold v4.8.4 (Proteome Software Inc.) was used to validate the MS/MS-based protein and peptide identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.9% probability and contained at least five identified peptides.

Bioinformatic analysis

Protein identification data from Scaffold were imported into the web-based platform, Listeriomics (https://listeriomics.pasteur.fr), which functionally annotates proteins and predicts the subcellular localization of proteins (Bécavin *et al.*, 2017). Among the identified proteins, proteases were categorized using the MEROPS database (https://www.ebi.ac.uk/merops/index.shtml) and assigned using MEROPS identifiers based on the clan or family to which it belonged.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

To quantify the mRNA expression of target genes, the total RNA was extracted from the bacterial pellets using acid phenol-chloroform extraction (Chomczynski and Sacchi, 2006) combined with the RNeasy Plus Universal Mini Kit (Qiagen). After the growth of biofilms and the separation of sessile and dispersed cells (as described above), the cell pellets were resuspended in lysis buffer (15 mg/ml lysozyme and 200 µg/ml Proteinase K in TE buffer) and incubated at 37°C for 10 min. The resultant samples were transferred to lysing matrix B tubes (MP Biomedicals) and vortexed for 15 sec four times using a disruptor (Scientific Industries) with a 1-min pause on ice between vortexes. The mixture of phenol/chloroform/isoamyl alcohol (25:24:1) was added into the samples and centrifuged at 16,000 × *g* at 4°C for 10 min. The aqueous phase was then treated with 100% chloroform and centrifuged at 16,000 × *g* at 4°C for 10 min. Contaminating DNA was eliminated, and total RNA was purified using RNeasy Plus Universal Mini Kit according to the manufacturer's instructions. In addition, RNase-free DNase (Promega) was applied to the samples at 37°C for 15 min after samples were loaded onto the columns. The purity and concentration of the RNA were determined using gel electrophoresis and a Nanodrop ND1000 UV-visible light spectrophotometer (Thermo Fisher Scientific). A 1-µg aliquot of RNA samples was reverse-transcribed to cDNA using the SuperScript VILO cDNA synthesis kit (Qiagen). cDNAs diluted by a factor of 10 or 100 (for *16S rRNA*) were used as the template in a 10-µl qPCR reaction with the primers for *cwh* (locus tag: lmo0582; F: GAG CCGTGGATGTTATCGTATTTAAC; R: GTAACGGACC AACTACATTTGATTGC), *spl* (locus tag: lmo2505; F: AG GCTATAAGGTTTTCCTAGTTGTG; R: TAGTTCGGAT ACCTCTACACCAAG), *lmo0186* (locus tag: lmo0186; F: AACACCAGTTTCTAACGTATCCACTTC; R: GGATCA ACCGCAATTACTTTTAGTCC), and *16S rRNA* (locus tag: lmor01; F: GAGGGTCATTGGAAACTGGAAGAC; R: C CTAACACTTAGCACTCATCGTTTACG). NCBI Primer-BLAST was used to design the primers. qPCR was performed using the SYBR Green Master Kit (Applied Biosystems) and the 7500 Fast Real-Time PCR system (Applied Biosystems) under the following conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. We used 16S rRNA as an internal control. Relative gene expression values were normalized with respect to the internal control (16S rRNA) and presented as the fold change compared to the control group (sessile cells at 25°C).

Protease inhibitor assay

Overnight cultures of *L. monocytogenes* strain EGD-e were diluted to 10^7 CFU in MWB containing 0, 5, 50, 500, or 1,000 µM protease inhibitor 4-(2-Aminoethyl)-benzenesulfonyl-fluoride hydrochloride (AEBSF; Gold Biotechnology). Bacteria treated with 500 ng/ml TPCK-treated trypsin (Thermo Fisher Scientific) in the presence or absence of 500 µM AEBSF served as positive controls. We added 250 µl bacterial diluents or blank MWB (negative control) to 96-well sterile polystyrene microtiter plates and incubated them at 25 or 37°C for 24 h.

Crystal violet staining

Crystal violet staining was used to quantify the biofilm biomass in protease inhibitor assays as described by Lourenço *et al.* (2012) with minor modifications. In brief, the biofilms that formed in wells as described above were dried for 30 min after removing the liquid suspension from the well. The wells were then stained with 100 µl 0.1% crystal violet solution with 20% ethanol for 30 min at room temperature. The unbound dye in the well was removed by rinsing the wells three times with 100 µl sterile double-DW. Crystal violet was then solubilized in 100 µl 95% ethanol for 30 min at room temperature with 100 rpm agitation. The optical density at 595 nm (OD_{595}) was measured using a Synergy HT microplate reader (BioTek).

Statistical analysis

Data are from three independent experiments that were performed in triplicate. The significance of differences was assessed by one-way analysis of variance (ANOVA) using SigmaPlot (Systat Software). Pairwise comparisons were analyzed using Tukey's test. The strength and direction of the association between the concentration of protease inhibitor and the measured biofilm biomass was performed with Spearman correlation. For all tests, $P < 0.05$ was considered significant.

Results

Extracytoplasmic peptidases were detected in biofilm exoproteomes formed at 25°C

2D gel images show the exoproteomes of *L. monocytogenes* biofilms formed at 25°C and 37°C (Fig. 1). Circled protein spots in the images represent proteins that appeared only in the supernatant of biofilms formed at either 25 or 37°C. A total of 64 distinct proteins was identified from these circled protein spots: 51 at 25°C and 13 at 37°C. Fifty-one identified proteins present at 25°C were generally distributed into three major functional categories: cell envelope and cellular processes (17 [number of proteins in the indicated category];

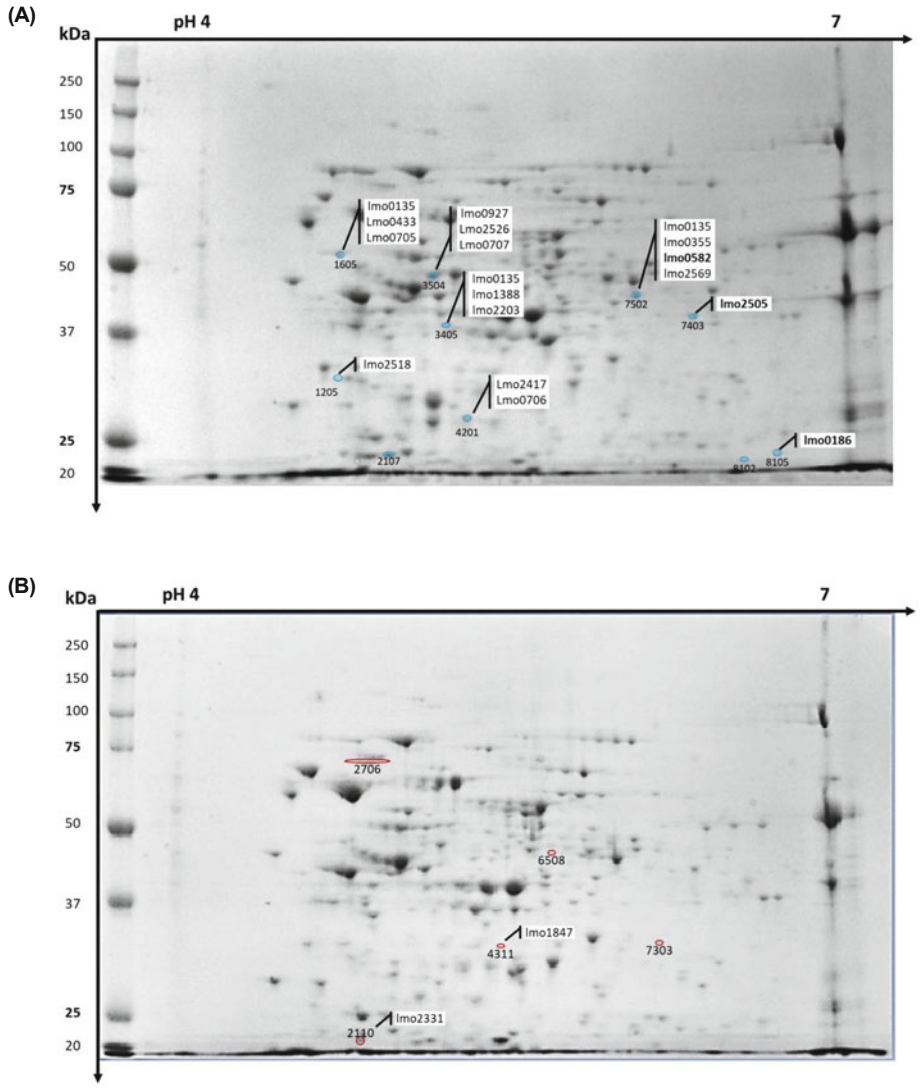

Fig. 1. Two dimensional-PAGE gels of biofilm exoproteomes formed at 25 and 37°C. Gels from two temperatures, 25°C (A) and 37°C (B), were compared to determine significant changes in protein spot intensities ($P < 0.05$). Hollow circles indicate spots only present at one of two temperatures. The proteins identified from these exclusive spots are labeled with their locus tags only if they were predicted to be localized extracellularly and on the cell surface.

Table 1. Extracytoplasmic proteins obtained from the supernatants of biofilm cultures at 25°C and 37°C

Spot ID	Locus tag	Gene name	Protein accession ID	Subcellular localization[a]	Functional category[b]	Function in cell wall/membrane/ envelop biogenesis[c]	Description	Theoretical[d]		Experimental		Unique peptide count	Percentage sequence coverage
								pI	MW (kDa)	pI	MW (kDa)		
25°C													
7403	**lmo2505**	*spl*	Q7AP49	EM	1.1	Y	Secreted protein with lytic activity/P45 as a D-glutamyl-L-m-Dpm peptidase, C40/M23[e]	8.56	42.7	6–6.5	35–40	9	22.70%
4201	lmo2417		Q8Y4M0	EM/CS	1.2	Y	ABC transporter, substrate-binding protein	5.27	30.7	5–5.5	25	5	17.00%
3405	lmo1388	*tcsA*	Q48754	EM/CS	1.2		CD4+ T-cell-stimulating antigen	5.02	38.4	5–5.5	35–40	9	28.60%
3405 7502	lmo0135	*ctaP*	Q8YAJ0	EM/CS	1.2		ABC transporter, oligopeptide-binding protein	4.95	58.3	5.8–6	40	11	27.10%
7502	lmo0355	*frdA*	Q8YA11	EM/CS	1.4		Flavocytochrome c	5.71	46.8	5.8–6	40	6	13.20%
3504	lmo2526	*murA1*	Q8Y4C4	CS	1.1	Y	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	4.96	46	5–5.2	45–50	6	16.00%
7502	**lmo0582**	*cwh*	P21171	CS	1.1	Y	Cell-wall hydrolase/P60 belonging to peptidase C40[e]	9.22	50.6	5.8–6	40	13	35.70%
7502	lmo2569		Q8Y486	CS	1.2		ABC transporter, dipeptide-binding protein	5.25	61.9	5.8–6	40	7	28.60%
1605	lmo0705	*flgK*	Q8Y936	CS	1.5		Flagellar hook-associated protein 1	4.65	54.2	4.5–5	50	25	46.40%
3504	lmo0707	*fliD*	Q8Y934	CS	1.5		Flagellar hook-associated protein 2	4.97	45.5	5–5.2	45–50	5	54.00%
4201	lmo0706	*flgL*	Q8Y935	CS	1.5		Flagellar hook-associated protein 3	5.03	31.7	5–5.5	25	1	60.80%
1605	lmo0433	*inlA*	P0DJM0	CS	1.8	Y	Internalin A	4.93	86.5	4.5–5	50	5	8.87%
3405	lmo2203		Q8Y572	CS	1.8	Y	N-acetylmuramoyl-L-alanine amidase	5	41.8	5–5.5	35–40	4	23.20%
1205	lmo2518		Q8Y4D2	CS	3.5.2		Transcriptional regulator belonging to LytR family	5.57	39.1	4.5–5	30	5	15.90%
3504	lmo0927	*ltaS*	Q8Y8H6	CS	5.2	Y	Putative phosphatidyl-membrane phosphoglyceroltransferase; lipoteichoic acid (LTA) synthase	6.01	74.7	5–5.2	45–50	12	17.00%
8105	**lmo0186**		Q8YAE4	CS	5.2		Membrane-bound lytic murein transglycosylase with 3D, G5, and DUF348 domains, which belongs to peptidase M23[e]	9	44.3	6.5–7	20–25	3	15.70%
37°C													
2110	lmo2331		Q8Y4U9	EM/CS	4.3		Uncharacterized protein	4.89	25.4	4.5–5	20	5	30.10%
4311	lmo1847	mntA	Q8Y653	CS	1.2		Manganese-binding lipoprotein	5.43	34.4	5–6	30–35	21	52.60%

[a] EM, Extracellular milieu; CS, Cell surface.
[b] Numbers indicate the functional groups to which the identified proteins belong. More details about the names of the groups are in Supplementary data Table S2.
[c] Y, yes; the protein is predicted to be involved in the cell/membrane/envelope biogenesis.
[d] Theoretical average isoelectric point (pI) and molecular weight (MW) are given for the predicted mature protein without signal peptide.
[e] The MEROPS identifier of the identified peptidase proteins.

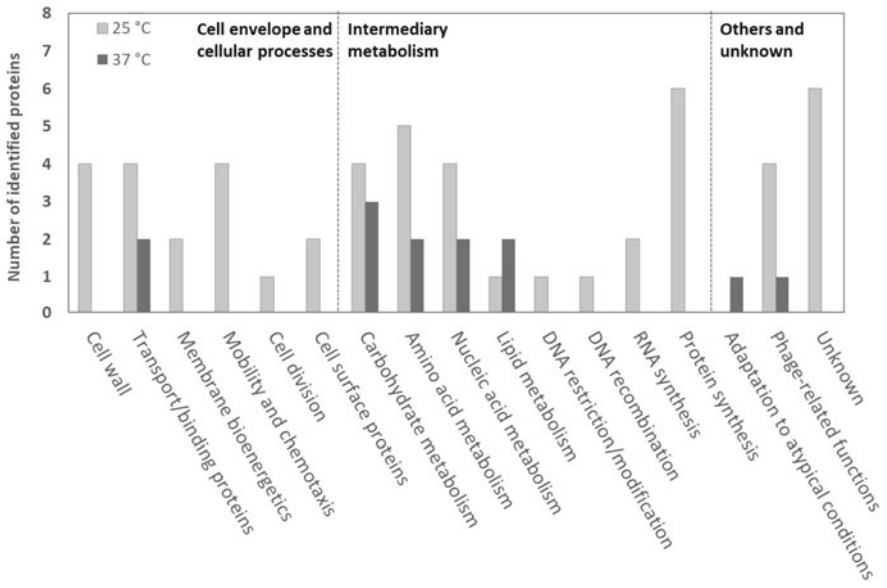

Fig. 2. Functional distributions of the proteins produced during *L. monocytogenes* biofilm formation at 25 and 37°C. The 51 and 13 identified proteins present exclusively in the biofilm exoproteomes formed at 25 and 37°C, respectively, were categorized into a total of 50 functional groups. These functional groups can be generally classified into three major categories: cell envelope and cellular processes (left), intermediary metabolism (middle), and others and unknown functions (right). Only the groups (of the first hierarchy of categories) with identified proteins are shown here. More details about every functional group and its assigned proteins are listed in Supplementary data Table S1.

33% [proportion of proteins to the total identified proteins]), intermediary metabolism (24; 47%), and others with unknown functions (10; 20%). However, over half the proteins (9; 69%) present at 37°C were related to intermediary metabolism (Fig. 2 and Supplementary data Table S1).

Based on the biology of protein transportation and localization, there were 16 out of 51 proteins present at 25°C being predicted as localized extracellularly (EM) and on the cell surface (CS) (Table 1). Of those 16 extracytoplasmic proteins, 13 were annotated with functions related to cell envelope and cellular processes (functional group 1.1 to 1.8 in Table 1). More specifically, seven are predicted to be involved in cell wall/membrane/envelop biogenesis (marked with a 'Y' in Table 1) and recognized as essentially associated with biofilm formation. Additionally, 3 out of 16 extracytoplasmic proteins are peptidases cleaving the amide bonds between amino acids in peptidoglycans, namely secreted protein with lytic activity (Spl/Lmo2505), a cell wall hydrolase (Cwh/Lmo0582), and Lmo0186. On the other hand, 35 out of 51 proteins present at 25°C were predicted to be localized intracellularly (CP). One of these (MurE/Lmo2038) is involved in cell wall/membrane/envelop biogenesis, although it is not a peptidase but a ligase (Supplementary data Table S2).

When biofilms formed at 37°C, only 2 (Lmo2331 and MntA/Lmo1847) of 13 identified proteins were predicted to be extracytoplasmic proteins. Of those, only MntA was annotated as functioning in the cell envelope and cellular processes and neither of the two were peptidases (Table 1). On the other hand, 1 (Lmo0096) of the other 11 intracellular proteins present at 37°C is predicted to be involved in cell envelope and cellular processes, specifically in carbohydrate transport and metabolism. Overall, more extracytoplasmic peptidases (4) were present in the supernatant of biofilms formed at 25°C compared to none at 37°C.

Gene expression of peptidase Spl, Cwh, and Lmo0186 was higher in sessile cells at 25°C

Given that a mature biofilm community generally consists of attached (sessile) and free-living (dispersed) cells (see Materials and Methods for the separation of these two cell populations), we investigated how the size (number of cells) of the two populations (sessile and dispersed cells) in biofilms was influenced by environmental temperatures (Fig. 3). At the end of the 3-day biofilm incubation, an average of 1×10^9 CFU/ml sessile bacteria were living in the biofilm formed at 37°C, and the number was significantly greater than that at 25°C (7.5×10^8 CFU/ml). In contrast, a 10-fold greater number (5×10^9 CFU/ml) of viable dispersed bacteria were observed in the supernatant of the biofilm formed at 25°C compared to the number at 37°C. Comparing the numbers of two populations at a single temperature, the size of the dispersed population was greater than the size of the sessile population at 25°C but smaller than that at 37°C (number of dispersed cells < number of sessile cells).

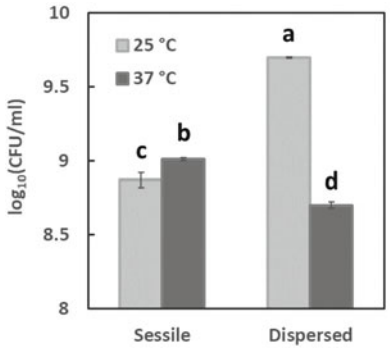

Fig. 3. The numbers of sessile and dispersed *L. monocytogenes* in the biofilms formed at 25 or 37°C. The numbers of sessile and dispersed *L. monocytogenes* at 25 and 37°C are shown here in units of $\log_{10}$ CFU/ml. Data are shown as means ± standard errors ($n \geq 3$). Different lowercase letters indicate statistically significant differences, $P < 0.05$.

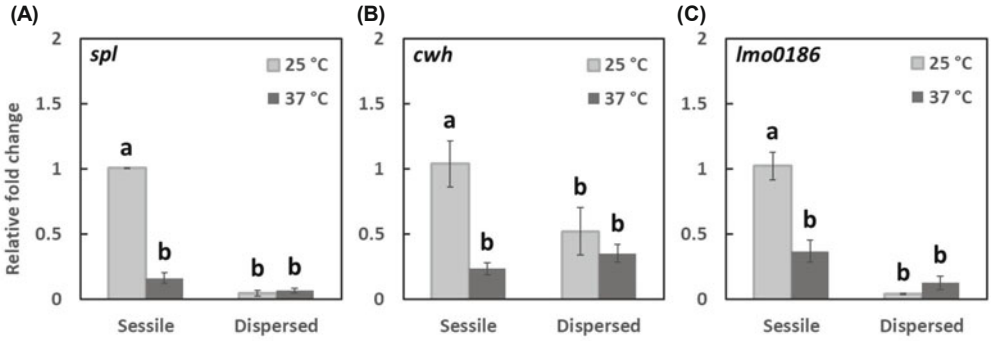

Fig. 4. Gene expression of identified extracytoplasmic peptidases in sessile and dispersed *L. monocytogenes* cells. Relative fold changes in expression of peptidase genes *spl* (A), *cwh* (B), and *lmo0186* (C) in two cell types and at two temperatures were calculated by setting a value of 1 for the group of sessile cells at 25°C. The light gray bars represent the results for 25°C-treated conditions, whereas the dark gray bars are for 37°C. Data are shown as means ± standard errors (n ≥ 3). Different lowercase letters indicate statistically significant differences, $P < 0.05$.

Because the size of the sessile and dispersed populations in the biofilm was affected by environmental temperatures, we explored the contribution of temperature and bacterial life stage, *i.e.* sessile or dispersed, to the gene expression of extracytoplasmic peptidases. The expression of *spl*, *cwh*, and *lmo0186* in sessile cells was significantly higher at 25°C than at 37°C (Fig. 4A, B, and C). Unlike in sessile cells, the expression of these three genes in dispersed cells did not change with incubation temperatures. When we compared the outputs of sessile cells and dispersed cells at a single temperature, we found that the expression of three extracytoplasmic peptidase genes was higher in sessile cells at 25°C but not at 37°C. Overall, for extracytoplasmic peptidases Spl, Cwh, and Lmo0186, the patterns of their gene expression and protein production changed in the same way in response to environmental temperature changes.

Inhibition of extracytoplasmic peptidase activity led to enhanced biofilm biomass at 25°C

A link between extracytoplasmic peptidases and biofilm formation was shown with protease inhibitor (AEBSF) treatment and the crystal violet assay. The results from the protease inhibitor AEBSF and trypsin combination treatment revealed that AEBSF restored trypsin-reduced biofilm biomass at both 25°C and 37°C (Fig. 5A). When *L. monocytogenes* were treated with AEBSF in gradually increasing concentrations at 25°C, their biofilm biomass increased as well (Fig. 5B). The correlation coefficient of 0.633 ($P = 0.011$) indicates a positive correlation between the two variables, AEBSF concentration, and biofilm biomass. However, the effect of AEBSF on biofilm biomass was not observed at 37°C, as the correlation coefficient of the two variables was -0.074 ($P = 0.811$).

Discussion

Extracytoplasmic proteins are primary determinants of environmental adaption because they mediate the direct interactions between the bacterium and surrounding environments such as food facilities (Lourenço *et al.*, 2013) or the bodies of hosts (Desvaux *et al.*, 2010; Soni *et al.*, 2011). Most previous studies of host infection and intracellular escape have focused on the role of extracytoplasmic proteins as virulence factors. Interestingly, one of the secreted virulence factors,

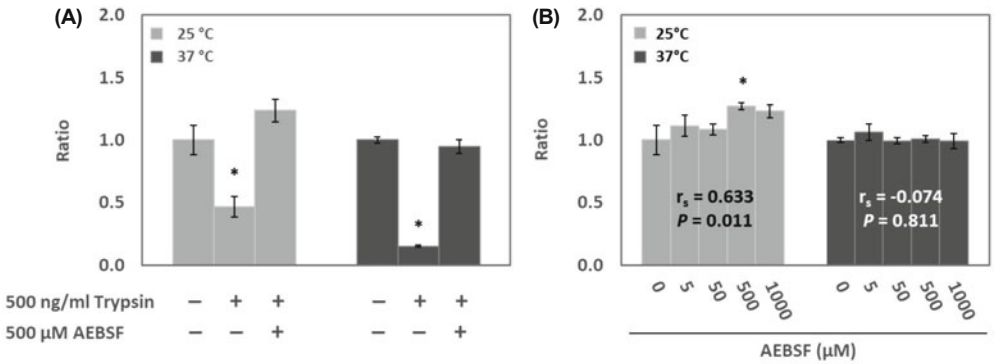

Fig. 5. The effect of protease inhibitor AEBSF on biofilm formation at 25°C and 37°C. Biofilm biomass was quantified by crystal violet staining and measuring OD_{595}. (A) The effects of the protease trypsin, the protease inhibitor AEBSF, or both on biofilm biomass of *L. monocytogenes* at 25 and 37°C. (B) The effect of AEBSF at different concentrations on biofilm biomass at 25 and 37°C. Data are shown as the ratios of OD values from indicated treatment groups to the OD value from the control group in the same set. * indicates statistically significant differences relative to the control group in the same set ($P < 0.05$). r_s denotes the Spearman correlation coefficient, and P denotes the P-value.

actin assembly-inducing protein (ActA), has been validated for its role in a non-infectious context by supporting *L. monocytogenes* biofilm formation in the environment (Tiensuu *et al.*, 2013). This finding suggests that extracytoplasmic proteins have a global role in modulating different life stages of *L. monocytogenes*. For the food industry, the ability to form biofilms on varied surfaces presents a considerable challenge, as biofilms make it harder to eradicate pathogens (Martínez-Suárez *et al.*, 2016). Given that extracytoplasmic proteins are key elements for the bacterial ability to form biofilms (Alcorlo *et al.*, 2017), understanding the role of these proteins in the *L. monocytogenes* sessile stage of growth is imperative for more effective strategies to prevent biofilm formation or eliminate established biofilms. In this study, we have demonstrated (1) an increased abundance of three extracytoplasmic peptidases, Spl, Cwh, and Lmo0186, in biofilm exoproteomes formed at 25°C, (2) unequal expression of the same extracytoplasmic peptidase gene when comparing sessile and dispersed *L. monocytogenes*, and (3) a positive correlation between the increased levels of biofilm biomass and the inhibited extracytoplasmic peptidase activity at 25°C. Thus, our results suggest that temperature-dependent presence of extracytoplasmic peptidases is a property of sessile *L. monocytogenes* that leads to the natural breakdown of biofilms and the release of the pathogen to the environment.

By comparing the biofilm exoproteomes of *L. monocytogenes* at 25 and 37°C, a greater number of extracytoplasmic proteins involved in cell wall/membrane/envelop biogenesis were identified in biofilms formed at 25°C (Table 1). Similarly, proteomic analysis of the marine extracytoplasmic proteins of the bacterium *Sphingopyxis alaskensis* at 10°C revealed increased abundance of several proteins related to cell wall structure, exopolysaccharide biosynthesis, and envelope biogenesis functions (Ting *et al.*, 2010). The upregulation of genes and proteins associated with peptidoglycan (a cell wall component) biogenesis has also been shown in proteomic and transcriptomic studies of *L. monocytogenes* growing at low temperatures, such as 4, 11, and 20°C (Cabrita *et al.*, 2013; Durack *et al.*, 2013). Altogether, these studies and ours highlight the relevance of proteins for cell wall metabolism in coping with relatively low environmental temperatures, ranging from 4°C to 25°C. Most of these proteins are demonstrated to act in cold stress adaptation (Chan and Wiedmann, 2009). However, our results suggest that extracytoplasmic proteins that promote cell wall metabolism are also required for *L. monocytogenes* biofilm formation at the relatively low environmental temperatures.

Differences in the presence of three specific extracytoplasmic peptidases, namely Spl, Cwh, and Lmo0186, between two growth temperatures, 25 and 37°C, were observed in this study (Fig. 1 and Table 1). In addition to the temperature-dependent presence of extracytoplasmic peptidase Spl, Cwh, and Lmo0186, gene expression of these peptidases was higher in sessile cells growing at 25°C than at 37°C (Fig. 4). The clear distinctions between bacteria at different life stages allowed for our analysis of gene expression and finding a specific contribution of sessile cells to the expression of extracytoplasmic peptidases. In fact, previous studies have illustrated that the expression of *cwh*, *spl*, and *lmo0186* was not different between planktonic cells and sessile cells at 37°C, nor was the expression in planktonic cells altered by changes in environmental temperatures (Luo *et al.*, 2013; Kaspar *et al.*, 2014). However, these two studies demonstrated that the mRNA and protein levels of Spl were higher in sessile cells than in planktonic cells at 25°C (Lourenço *et al.*, 2013; Tiong and Muriana, 2016). Altogether, previous studies and our data suggest that sessile cells are more susceptible to environmental temperatures than the other two bacterial forms, planktonic and dispersed cells, in terms of the relative expression of peptidase genes. In other words, the temperature-dependent expression and production of extracytoplasmic peptidases could be a benefit conferred by the sessile mode of growth to *L. monocytogenes* in the environment. Further experiments using peptidase overexpression and deletion strains (Loh *et al.*, 2009; Klinkert *et al.*, 2012) can determine if any temperature-sensing regulators, such as non-coding RNAs, interact with the extracytoplasmic peptidases and thus shed light on the molecular basis for temperature-dependent regulation on extracytoplasmic peptidases.

Temperature is also a determining factor for mammalian pathogens, including *L. monocytogenes*, in controlling the production of virulence factors (Lam *et al.*, 2014). Among 16 extracytoplasmic proteins identified in the biofilm exoproteomes formed at 25°C, internalin A (InlA/Lmo0433) was initially unexpected because its transcription has been clearly demonstrated to be upregulated by the *L. monocytogenes* positive regulatory factor A (PrfA), a transcriptional regulator of virulence genes, at a 37°C host temperature (Johansson *et al.*, 2002). However, several lines of evidence have revealed that virulence factors, such as InlA and ActA, have functions other than the colonization and invasion of host cells. In *L. monocytogenes*, these virulence factors can keep a pathogen alive outside the host. Biofilm formation is one of these nonclassical functions (Franciosa *et al.*, 2009; Tiensuu *et al.*, 2013). Moreover, the involvement of virulence factors, including ActA and PrfA, in biofilm formation is regulated by a mechanism involving sigma factor B (σ^B) activation below the host body temperature (Lemon *et al.*, 2010; Tiensuu *et al.*, 2013). Along with these findings, it is conceivable that the level of InlA is determined in a life stage- and a temperature-dependent manner. Investigating how the contribution of σ^B to InlA-involved biofilm formation is affected by environmental temperatures will be a future interest to further understand the molecular basis underlying the non-classical functions of *L. monocytogenes* virulence factors.

In this study, we also confirmed the correlation between the extracytoplasmic peptidases and biofilm formation, as well as an effect of temperature on this correlation, with our finding that biofilm formation was affected by the modulation of activity of extracytoplasmic peptidases at 25°C, but not 37°C (Fig. 5). The cell wall peptidases, including Spl, Cwh, and Lmo0186, examined in this study, had a specific role in breaking the amide bonds between amino acids within the peptidoglycan chain (Vermassen *et al.*, 2019). The degree of peptidase activity can alter the global charge of the cell surface, affect ion binding, and/or modify the exposure of adhesin, favoring aggregation/adhesion or biofilm formation of bacteria (Mercier *et al.*, 2002). One piece of supporting evidence is that a *L. monocytogenes* rough isolate with reduced secretion of the peptidase Cwh has an enhanced ability to

form biofilms (Monk *et al.*, 2004). A similar scenario was demonstrated in other Gram-positive organisms as well. For example, the σ^B cascade in *Staphylococcus aureus* decreases *agr* RNAIII levels (a quorum sensing system) and reduces the activity of extracytoplasmic peptidases, resulting in incremental biofilm formation (Lauderdale *et al.*, 2009). For *Bacillus subtilis*, increased production of extracytoplasmic peptidases is observed when the bacteria formed mature biofilms (Marlow *et al.*, 2014). Despite a lack of direct evidence, our data are in line with previous findings about temperature-oriented roles for virulence factors mentioned above, and consistently suggest that the involvement of *L. monocytogenes* extracytoplasmic peptidases in biofilm formation is dependent on environmental temperatures. Additionally, it is very likely that the global stress regulator σ^B participates in coordinating the presence of peptidases and temperature signals for biofilm formation, given that the activity of σ^B and transcript levels of σ^B-dependent genes are reported as being regulated in a temperature-dependent manner (McGann *et al.*, 2007; Lee *et al.*, 2014; NicAogáin and O'Byrne, 2016). Overall, the extracytoplasmic peptidases produced by sessile cells might make biofilms vulnerable to physical disruption from the surrounding environment. Upon collapse of biofilms, the bacteria detaching from the biofilm and dispersing into the surrounding environment may contaminate environments where human food is present (Colagiorgi *et al.*, 2017). This peptidase-involved biofilm dispersion is also a potential explanation for the observation of a larger dispersed population at 25°C (Fig. 3). It appears that *L. monocytogenes* can modulate the production of the extracytoplasmic peptidases to control a dynamic process of biofilm development and bacterial spreading can benefit from biofilm dispersion. Though we have revealed that certain extracytoplasmic peptidases can be part of the biofilm remodeling apparatus for bacteria, several studies have validated the effect of exogenously supplied peptidases on the prevention and removal of biofilms (Longhi *et al.*, 2008; Nguyen and Burrows, 2014). However, the application of peptidases or other biofilm-dispersal agents onto the EPS of biofilms can be problematic if these treatments do not completely kill the bacteria. In this case, unsuccessful treatment can trigger the dissemination of bacteria from the original site into the environment (Verderosa *et al.*, 2019). Hence, a combination treatment in which the biofilm dispersal agent is administered concurrently with an antimicrobial agent is a better approach to killing bacteria embedded in the EPS (Marvasi *et al.*, 2014; Yu *et al.*, 2015). In conclusion, extracytoplasmic proteins, especially peptidases, are critical for bacteria to remodel their biofilm structures. Despite the potential to disperse biofilms, peptidase treatment in combination with antimicrobial agents as a strategy to eradicate biofilm still requires rigorous evaluation, including studies to determine the effective concentrations and appropriate window of time for administration.

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Conflict of Interest

The authors declare no conflict of interest.

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