Journal of Food Protection, Vol. 77, No. 2, 2014, Pages 254–261
doi:10.4315/0362-028X.JFP-13-182

Gene Transcription Patterns of pH- and Salt-Stressed *Listeria monocytogenes* Cells in Simulated Gastric and Pancreatic Conditions

MARIOS MATARAGAS,[1,2] ANNA GREPPI,[1] KALLIOPI RANTSIOU,[1] AND LUCA COCOLIN[1]*

[1]*Università di Torino, Dipartimento di Scienze Agrarie, Forestali e Alimentari, Via Leonardo da Vinci 44, Grugliasco, 10095 Turin, Italy; and* [2]*Agricultural University of Athens, Department of Food Science and Technology, Laboratory of Food Quality Control and Hygiene, Iera Odos 75, 11855 Athens, Greece*

MS 13-182: Received 4 May 2013/Accepted 19 September 2013

ABSTRACT

A *Listeria monocytogenes* subgenomic array, targeting 54 genes involved in the adhesion, adaptation, intracellular life cycle, invasion, and regulation of the infection cycle was used to investigate the gene expression patterns of acid- and salt-stressed *Listeria* cells after exposure to conditions similar to those in gastric and pancreatic fluids. Three *L. monocytogenes* strains, one laboratory reference strain (EGDe) and two food isolates (wild strain 12 isolated from milk and wild strain 3 isolated from fermented sausage), were used during the studies. Differences in the expressed genes were observed between the gastric and pancreatic treatments and also between the serotypes. Increased transcripts were observed of the genes belonging to the adaptation and regulation group for serotype 4b (strain 12) and to the invasion and regulation group for serotype 1/2a (strain EGDe). Interestingly, no significantly differentially expressed genes were found for serotype 3c (strain 3) in most cases. The genes related to adaptation (serotype 1/2a) and to intracellular life cycle and invasion (serotype 4b) were down-regulated in order to cope with the hostile environment of the gastric and pancreatic fluids. These findings may provide experimental evidence for the dominance of serotypes 1/2a and 4b in clinical cases of listeriosis and for the sporadic occurrence of serotype 3c.

The physiological properties of *Listeria monocytogenes,* including its capability to survive on food processing equipment, are well known, as well as the disease that the pathogen causes, symptoms of the disease, and the food commodities most often implicated in disease outbreaks *(12, 35)*. The microorganism, however, still remains a concern because the numbers of cases of listeriosis have not decreased over the years, and it continues to show a high mortality rate *(14, 37)*. More studies are therefore necessary to understand its pathogenicity. Over the last few decades, much has been learned, by means of molecular methods, about the genes involved in the infection cycle *(9)*. However, the elucidation of the mechanism of *L. monocytogenes* pathogenicity still remains a challenge, and tools such as microarrays and reverse transcriptional quantitative polymerase chain reaction (RT-qPCR) can be used for such purposes *(26)*.

L. monocytogenes serotypes 1/2a, 1/2b, and 4b cause a majority (>95%) of clinical cases of listeriosis *(32)*. Most studies done to determine the genes involved in the pathogenicity mechanism of *L. monocytogenes* have used strains belonging to serotypes 1/2a (EGDe and 10403S) or 1/2c (LO28) and, therefore, may not adequately address the virulence-related features of other serotypes, such as 1/2b or 4b, which are also of great importance *(17)*. Although all serotypes include the known key virulence factors, it is possible that the regulation of their expression is different from serotype to serotype *(17)*. Comparative genomics has, in fact, revealed differences between 4b and 1/2a *(23)*.

Acids and salts are often used to preserve food, especially fermented food *(19)*. Low pH and a high salt content may cause changes in gene expression as a result of the adaptation of *L. monocytogenes* to these stresses *(1, 13, 24, 36)*. pH values of 5.5 and a salt content of 4.5% are often found in fermented food, such as in fermented sausages, and these conditions (acid and salt stress) have therefore been used in the current microarray experiment to investigate the gene regulation of *L. monocytogenes*. To understand better the molecular mechanism by which *L. monocytogenes* copes with hostile environments, gene expression was studied after treatment with synthetic gastric and pancreatic fluids.

The objective was, therefore, to compare gene expression patterns of three different *L. monocytogenes* strains, in terms of their origin and serotype, treated or not treated with synthetic gastric and pancreatic fluids, in order to identify the genes that allow this pathogen to persist in the gastrointestinal tract. A subgenomic array, targeting 54 genes, was used for this purpose. Finally, the effect of food-related stress (acidity and salinity) on gene expression of *L. monocytogenes* was simulated in vitro.

* Author for correspondence. Tel: +39-011-6708553; Fax: +39-011-6708549; E-mail: lucasimone.cocolin@unito.it.

MATERIALS AND METHODS

***Listeria monocytogenes* strains.** The following *L. monocytogenes* strains were used: strain EGDe (serotype 1/2a, sequenced laboratory strain), strain 12 (serotype 4b, wild strain isolated from milk), and strain 3 (serotype 3c, wild strain isolated from fermented sausage) *(25)*. All the bacterial cultures were kept frozen at −80°C in brain heart infusion (BHI) broth (Oxoid, Milan, Italy) and were supplemented with 30% glycerol (Sigma, Milan, Italy). Before each experiment, the cultures were resuscitated twice in BHI broth (1% inoculum) at 37°C for 24 h.

Synthetic gastric and pancreatic fluids and experimental procedure. The synthetic gastric and pancreatic fluids were prepared as described in Corcoran et al. *(6)* and Bautista-Gallego et al. *(2)*. The resuscitated *Listeria* cultures (strains EGDe, 12, and 3) were used to inoculate the following experimental conditions (5 ml in total; 4.5 ml of the respective substrate and experimental condition plus 0.5 ml of the resuscitated *Listeria* culture): BHI broth (unstressed cells), BHI broth adjusted to pH 5.5 (pH-stressed cells), and BHI broth containing 4.5% (wt/vol) NaCl (salt-stressed cells). Prestress was applied to simulate pH and salt conditions commonly found in foods. *Listeria* cells were subjected to a sequence of challenges, i.e., unstressed, pH- or salt-stressed cells plus gastric plus pancreatic stresses; note that the treated cells were exposed sequentially to pH or salt, gastric, and pancreatic juices and not to each stress alone. For each strain, the unstressed, pH-, and salt-stressed *Listeria* cells were exposed to the subsequent simulated gastrointestinal stresses, in parallel.

The samples (27 total; 3 experimental conditions × 3 strains × 3 repetitions performed on different days) were incubated at 37°C for 24 h. After incubation, the bacterial cells were harvested (each experimental condition of 5 ml split into two Eppendorf tubes of 2 ml each), using an Eppendorf 5417R refrigerated centrifuge (Eppendorf, Milan, Italy) at 13,400 × *g* for 1 min at 4°C, and were washed with Ringer's solution. After washing, the pellets were suspended in 1 ml of synthetic gastric juice (final pH 3.0; buffer solution: 976.7 μl, pH 2.0; 2.05 g/liter NaCl, 0.60 g/liter KH_2PO_4, 0.11 g/liter $CaCl_2$, 0.37 g/liter KCl [all from Sigma]; pepsin [13.3 μl; 1 mg/ml; Sigma]; lysozyme [10 μl; 1 mg/ml; Sigma]) and were incubated at 37°C for 3 h under continuous shaking (300 rpm) using a Thermomixer (Eppendorf). After incubation, the bacterial cells were harvested and washed as before. The resulting pellets were suspended in 1 ml of synthetic pancreatic juice (0.2 M phosphate buffered saline [PBS] buffer solution adjusted to pH 8.0 [860 μl; Sigma], bile solution [30 μl; 100 mg/ml; Oxoid], pancreatin [100 μl; 1 mg/ml; Sigma], NaH_2CO_3 [10 μl; 100 mg/ml; Sigma]) and were incubated under the same conditions, i.e., 37°C for 3 h under continuous shaking (300 rpm). Next, the cultures were centrifuged and washed, as before, and the resulting pellets were used for RNA extraction, as described in the next paragraph. In order to avoid changes in the transcriptome of *L. monocytogenes*, the duration of the centrifugations was very short (1 min) and RNA*later* (Ambion, Applied Biosystems, Milan, Italy) was immediately added to the pellets (see below). *L. monocytogenes* was enumerated on BHI agar plates at each washing step, that is, after the stress, gastric, and pancreatic treatments. Two plates from three dilutions were spread with the prepared culture suspensions and were incubated at 37°C for 48 h.

RNA extraction. The procedure described by Rantsiou et al. *(25)* was followed, with slight modifications. RNA was extracted from the bacterial cells at each level of stress, i.e., after pH and salt (first level), gastric (second level), and pancreatic (third level) treatments, and at each repetition. After the bacterial cells were washed and the supernatant was removed, the pellet was suspended in RNA*later* (50 μl; Ambion) and the cell suspension was treated with lysozyme (50 μl; 50 mg/ml solution; Sigma) and proteinase K (50 μl; 25 mg/ml solution; Sigma) for 20 min at 37°C for cell lysis. The RNA was extracted using the MasterPure Complete DNA and RNA Purification kit (Epicentre, Madison, WI) according to the manufacturer's instructions. At the end of the procedure, Turbo DNase (Ambion) was used to eliminate the DNA through enzymatic digestion. Integrity of the extracted RNA was checked using agarose gel (1.2%) electrophoresis, and its quantity was determined using a Nanodrop ND-1000 spectrophotometer (Celbio, Milan, Italy).

cDNA synthesis. Reverse transcription (RT) was performed as in Rantsiou et al. *(25)*, with slight modifications. Two RT reactions, each with ca. 4.5 μg of RNA, were performed for each sample. The reactions also contained random hexamer primers (Promega, Milan, Italy); 150 μM biotin-11-dUTP (Fermentas, M-Medical, Milan, Italy); and a mixed solution consisting of 5× RT buffer (Promega), a mix of dNTPs (150 μM dTTP and 0.7 mM each dATP, dCTP, and dGTP; Promega), an M-MLV reverse transcriptase enzyme (Promega), and an RNase ribonuclease inhibitor (Promega), according to the manufacturer's instructions. The RT reaction was performed in a DNA Engine Peltier Thermal Cycler (Bio-Rad, Hercules, CA) at 25°C for 10 min and 42°C for 50 min, and it was stopped when the enzyme became inactive. The two RT reactions were combined as one, and cDNA precipitation was carried out, as described in Sambrook et al. *(27)*. The pellet was suspended with 350 μl of the microarray hybridization buffer QHyb (QInstruments, Jena, Germany).

Microarray hybridization. The SureArray for *L. monocytogenes* (Congen, Berlin, Germany) was used, as in Rantsiou et al. *(25)*. This is a subgenomic array that relies on the complete genome sequences of the *L. monocytogenes* EGDe and F2365 strains and that comprises 54 gene spots (Table 1). Positive, negative, and hybridization control spots are also present on the slides. Each slide has two hybridization areas, to load two different samples; the gene and control spots were repeated four times. The Silverquant Detection kit (Eppendorf) was used for the microarrays, according to the manufacturer's instructions. Hybridization was carried out in a Thermomixer comfort (Eppendorf) at 30°C for 24 h. The slides were scanned using the Silverquant scanner (Eppendorf) after staining with silver. The images were analyzed using Silverquant analysis software (Eppendorf).

Statistical analysis. The spot intensities obtained at each condition (reference: unstressed cells receiving no treatment with synthetic gastric or pancreatic fluid, and unstressed cells treated with synthetic gastric or pancreatic fluid; test: pH- or salt-stressed cells receiving no treatment with synthetic gastric or pancreatic fluid, and pH- or salt-stressed cells treated with synthetic gastric or pancreatic fluid) were preprocessed, filtered, and transformed to $\log_2$(ratio), where ratio = test/reference; then they were normalized (lowess normalization) using a microarray data analyzer *(11)*. Missing values were imputed using the K-nearest-neighbor method *(33)* embedded in J-Express Pro 2012 *(10)*. The imputed lowess normalized $\log_2$(ratio) values were used further. Differentially expressed genes between two groups, i.e., the control (the aforementioned pH- or salt-stressed cells receiving no treatment with synthetic gastric or pancreatic fluid) and treated (the aforementioned pH- or salt-stressed cells treated with synthetic gastric or pancreatic fluid) samples, were identified using the Significance Analysis of Microarrays (SAM) implemented in J-Express Pro 2012 *(34)*. Those genes whose false discovery rate

TABLE 1. Listeria monocytogenes *subgenomic array with the 54 genes used to study the gene expression of pH- and salt-stressed cells in the synthetic gastric and pancreatic fluids*

Gene	Symbol	Description	Locus	Group assigned[a]
1	*actA*	Actin-assembly inducing protein precursor	lmo0204	Adhesion/invasion/ intracellular life cycle
2	*ami*	Autolysin, amidase	lmo2558	Adhesion
3	*arpJ*	Similar to an amino acid ABC transporter, permease	lmo2250	Adaptation (general stress)
4	*aut*	Similar to autolysin	lmo1076	Invasion
5	*betL*	Glycine betaine transporter	lmo2092	Adaptation (osmotic stress)
6	*bsh*	Similar to conjugated bile acid hydrolase	lmo2067	Adaptation (bile salts stress)
7	*bvrA*	Transcription antiterminator	lmo2788	Regulation
8	*bvrB*	Beta-glucoside–specific phosphotransferase enzyme II ABC component	lmo2787	Regulation
9	*bvrC*	Putative ADP-ribosylglycohydrolase	lmo2786	Regulation
10	*clpC*	Endopeptidase Clp ATP-binding chain C	lmo0232	Adaptation (general stress)
11	*clpE*	ATP-dependent protease	lmo0997	Adaptation (general stress)
12	*clpP*	ATP-dependent Clp protease proteolytic subunit	lmo2468	Adaptation (general stress)
13	*ctsR*	Very similar to the transcription repressor of class III stress genes	lmo0229	Adaptation/regulation
14	*cysK*	Very similar to cysteine synthase	lmo0223	Stress (general stress)
15	*dal*	Similar to alanine racemase	lmo0898	Intracellular life cycle
16	*dat*	D-Amino acid aminotransferase	lmo1619	Intracellular life cycle
17	*fbpA*	Similar to fibronectin-binding proteins	lmo1829	Adhesion
18	*flaA*	Flagellin	lmo0690	Adhesion/invasion
19	*fri*	Nonheme iron-binding ferritin	lmo0943	Intracellular life cycle
20	*gadA*	Similar to glutamate decarboxylase	lmo0447	Adaptation (acid stress)
21	*gadB*	Similar to glutamate decarboxylase	lmo2363	Adaptation (acid stress)
22	*gadC*	Similar to amino acid antiporter (acid resistance)	lmo2362	Adaptation (acid stress)
23	*gadE*	Similar to amino acid antiporter	lmo0448	Adaptation (acid stress)
24	*gbuA*	Very similar to glycine betaine ABC transporter (ATP-binding protein)	lmo1014	Adaptation (osmotic stress)
25	*gbuB*	Very similar to glycine betaine ABC transporters (permease)	lmo1015	Adaptation (osmotic stress)
26	*gbuC*	Very similar to glycine betaine ABC transporters (glycine betaine–binding protein)	lmo1016	Adaptation (osmotic stress)
27	*hly*	Listeriolysin O precursor	lmo0202	Invasion/intracellular life cycle
28	*iap*	Invasion-associated secreted endopeptidase	lmo0582	Invasion/intracellular life cycle
29	*iap(2)*	Invasion-associated secreted endopeptidase	lmo0582	Invasion/intracellular life cycle
30	*inlA*	Internalin A	lmo0433	Invasion
31	*inlB*	Internalin B	lmo0434	Invasion
32	*inlC*	Internalin C	lmo1786	Adhesion
33	*inlD*	Internalin D	LMOf2365_0282	Invasion
34	*inlE*	Internalin E	lmo0264	Adhesion
35	*inlF*	Internalin F	LMOf2365_0429	Adhesion
36	*inlG*	Internalin G	lmo0262	Adhesion
37	*inlH*	Internalin H	lmo0263	Adhesion
38	*lisK*	Two-component sensor histidine kinase	lmo1378	Adaptation/regulation
39		Similar to heat-shock protein htrA serine protease	lmo0292	Adaptation (heat stress)
40		Similar to oxidoreductase	lmo0669	Adaptation (general stress)
41	*mpl*	Zinc metalloproteinase precursor	lmo0203	Intracellular life cycle
42	*opuCA*	Similar to glycine betaine/carnitine/choline ABC transporter (ATP-binding protein)	lmo1428	Adaptation (osmotic stress)
43	*plcA*	Phosphatidylinositol-specific phospholipase C	lmo0201	Intracellular life cycle
44	*plcB*	Phospholipase C	lmo0205	Intracellular life cycle
45	*prfA*	Listeriolysin positive regulatory protein	lmo0200	Adhesion/invasion/intracellular life cycle/regulation
46	*proA*	Gamma-glutamyl phosphate reductase	lmo1259	Adaptation (osmotic stress)
47	*proB*	Gamma-glutamyl kinase	lmo1260	Adaptation (osmotic stress)
48	*proC*	Similar to 1-pyrroline-5-carboxylate reductase	lmo0396	Adaptation (osmotic stress)
49	*relA*	Similar to (p)ppGpp synthetase	lmo1523	Intracellular life cycle/ adaptation (osmotic stress)
50	*rpoB*	DNA-directed RNA polymerase subunit beta	lmo0258	Regulation
51	*sigB*	RNA polymerase sigma factor SigB	lmo0895	Invasion/adaptation/regulation

TABLE 1. *Continued*

Gene	Symbol	Description	Locus	Group assigned[a]
52	*sod*	Superoxide dismutase	lmo1439	Adaptation (oxidative stress)
53	*srtA*	Similar to sortase	lmo0929	Invasion
54	*svpA*	Hypothetical protein	lmo2185	Invasion

[a] Genes assigned to general groups, on the basis of their functions in the Gene Set Enrichment Analysis (GSEA).

was equal to or less than 25% and whose fold change was at least 2.0 were considered statistically significant.

Gene set enrichment analysis (GSEA) was performed to identify the differentially expressed genes that shared some characteristics *(29)*. For this analysis, general groups of genes were organized, in advance, on the basis of their direct or indirect involvement in the infection cycle *(9)*, i.e., adhesion, invasion, intracellular life cycle, adaptation, and regulation. The gene members of these groups are shown in Table 1. Those gene sets between two groups, i.e., the control and treated samples, that had a false discovery rate equal to or less than 25% GSEA were considered differentially expressed using GSEA software v2.0 *(21, 29)*. This procedure differs from SAM in that sets of genes rather than individual genes are analyzed. Finding significant changes by just looking at each gene separately may be difficult, especially when the change in genes is low or moderate. Smaller changes can instead be detected by means of GSEA, because a whole set of related genes is affected, even when the change in genes is low or moderate. Some genes within the gene set contribute more to the enrichment score, and this reflects the degree to which a gene set is up- or down-regulated. This subset is called "leading edge," and it contains the genes that contribute the most to the up- or down-regulation of a specific gene set. A heat-map graph was drawn up of the lowess normalized $\log_2$(ratio) of the control and treated samples using Genesis software *(28)*. Survival of each strain after exposure to the synthetic gastric and pancreatic fluids was compared by one-way analysis of variance using SPSS v15.0 (SPSS, Inc., Chicago, IL).

RESULTS

***L. monocytogenes* survival after gastric and pancreatic treatment.** Table 2 shows that all the tested strains became sensitive after their exposure to the synthetic gastric and pancreatic fluids. After 3 h of exposure to the synthetic gastric juice, adjusted to pH 3.0, a significant ($P < 0.05$) reduction in survival was observed (20 to 25%). Subsequent exposure of the cells to harsher conditions, i.e., 3 h in the synthetic pancreatic fluid, resulted in an additional population loss of about 40% ($P < 0.05$).

***L. monocytogenes* gene transcription patterns after gastric and pancreatic treatment.** Figure 1 provides a general view of the gene expression of the three strains for all of the experimental conditions tested. Heterogeneity in gene expression among the serotypes and experimental conditions can be observed. The relatively low intensities of the expression values of several genes were also found. Transcription data for each *L. monocytogenes* strain were statistically analyzed using SAM to discover any differences in the gene expression between the gastric and pancreatic treatments and the control. This was performed for each physiological state of the investigated *Listeria* cell, i.e., pH- or salt-stressed cells. In most cases no significant genes were found (Table 3) owing to low transcription levels of the genes, making the interpretation of the results challenging. Subsequently, GSEA was also employed on the genes that were organized in general groups (Table 1). Note that leading edges (Table 4) included genes that were not marked as being significantly differentially expressed genes by SAM. GSEA revealed a clearer trend in gene expression for each serotype, i.e., up- or down-regulation of

TABLE 2. *Survival of the unstressed, pH-, and salt-stressed* Listeria *cells subjected to a sequence of challenges*[a]

Conditions	Survival (%)		
	EGDe	12	3
Unstressed cells			
After 24 h in BHI	98.1 ± 1.7 A	98.7 ± 1.2 A	98.2 ± 1.2 A
After 3 h in the gastric fluid	79.5 ± 7.6 B	74.4 ± 13.8 B	79.2 ± 10.2 B
After 3 h in the pancreatic fluid	43.7 ± 1.1 C	35.8 ± 5.7 C	42.4 ± 1.6 C
pH-stressed cells			
After 24 h in BHI adjusted to pH 5.5	96.1 ± 1.8 A	95.5 ± 0.8 A	96.4 ± 1.2 A
After 3 h in the gastric fluid	77.8 ± 2.0 B	72.4 ± 13.5 B	76.6 ± 11.2 B
After 3 h in the pancreatic fluid	41.0 ± 1.8 C	35.4 ± 5.8 C	40.9 ± 3.5 C
Salt-stressed cells			
After 24 h in BHI containing 4.5% (wt/vol) NaCl	95.7 ± 2.2 A	98.2 ± 1.5 A	96.9 ± 0.8 A
After 3 h in the gastric fluid	78.8 ± 5.7 B	79.6 ± 8.5 B	76.1 ± 8.6 B
After 3 h in the pancreatic fluid	40.9 ± 4.2 C	41.2 ± 3.6 C	39.1 ± 5.3 C

[a] Survival (average ± standard deviation) was calculated from three replicates ($n = 3$). Survival (%) = (final *L. monocytogenes* concentration × 100)/(initial *L. monocytogenes* concentration). *L. monocytogenes* concentration in log CFU per milliliter. Values followed by different letters are significantly different ($P < 0.05$).

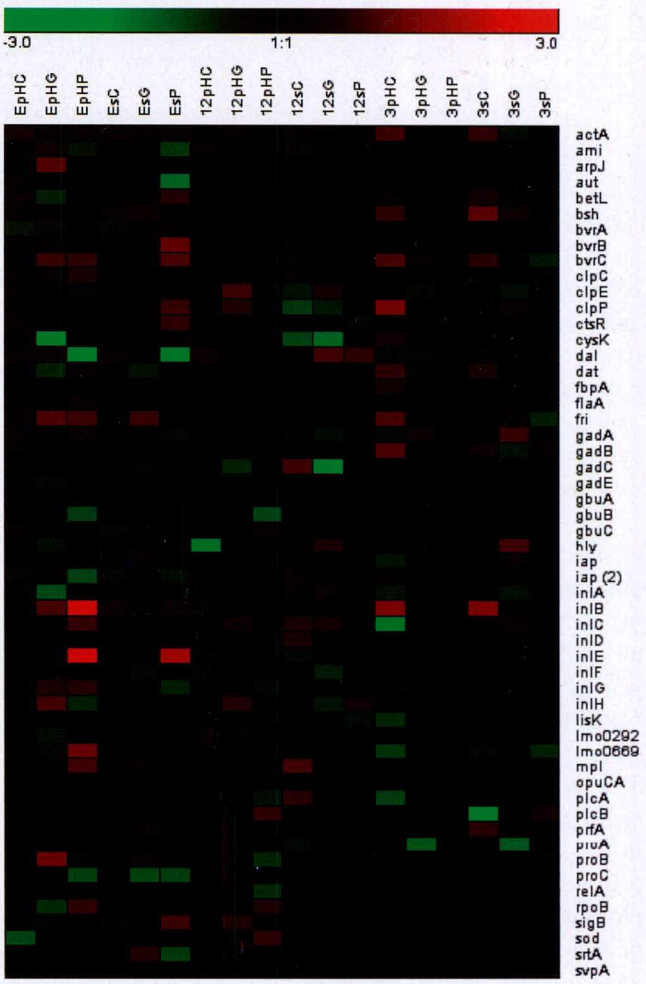

FIGURE 1. *Heat map showing the gene lowess normalized mean log_2(ratio) (n = 3) of the pH- and salt-stressed cells treated with the synthetic gastric and pancreatic fluids relative to their respective control conditions for the three* L. monocytogenes *strains. All log_2(ratio) values have been determined based on the test/reference ratio, i.e., prestressed cultures relative to cultures without prestress. E, strain EGDe; 12, strain 12; 3, strain 3; pH, pH-stressed cells; s, salt-stressed cells; C, control; G, synthetic gastric fluid; P, synthetic pancreatic fluid; the down-regulated genes are in green; the upregulated genes are in red.*

certain gene sets. Furthermore, differences between the gastric and pancreatic treatments were also revealed, i.e., which genes were expressed in each condition.

Gene transcription patterns of acid- and salt-stressed *Listeria* cells after gastric and pancreatic treatment. After exposure of pH- or salt-stressed *Listeria* cells to synthetic gastric fluid, the invasion (in strain EGDe), adaptation and regulation (in strain 12), and intracellular life cycle (in strain 3, only in salt-stressed cells) groups were upregulated, whereas some genes belonging to the adaptation and intracellular life cycle groups were down-regulated (in strains EGDe and 12, respectively). The overrepresented upregulated genes of the invasion group in strain EGDe included the *srtA, inlB, iap, hly, prfA,* and *sigB* members. Down-regulation of some adaptation-related genes, such as *lmo0669, clpE, cysK, sigB, clpC, gadE, lmo0292,* and *betL,* was observed. The upregulated adaptation- and regulation-related genes for strain 12 were *clpE, gadB, proC, sigB, bvrA,* and *bvrC*. The genes involved in the intracellular life cycle, e.g., *mpl, iap, dat, dal, plcA, relA, fri,* and *prfA,* were down-regulated. On the contrary, upregulation of the genes *mpl, iap, plcA, plcB,* and *hly,* belonging to the intracellular life cycle group, was only observed after exposure of the salt-stressed cells of strain 3 to the synthetic gastric fluid.

TABLE 3. *Differentially expressed genes found with Significance Analysis of Microarrays at false discovery rate ≤25% and fold change ≥2.0 for the pH- and salt-stressed* L. monocytogenes *cells treated with the synthetic gastric and pancreatic fluids, relative to their respective control conditions*

Conditions[a]	Gene (fold change)[b]
E/pH/G	*inlB* (2.23), *betL* (−2.38)
E/s/G	—
E/pH/P	—
E/s/P	—
12/pH/G	—
12/s/G	*clpE* (2.07)
12/pH/P	—
12/s/P	—
3/pH/G	—
3/s/G	*gadA* (2.14), *gadB* (−2.05), *actA* (−2.13)
3/pH/P	—
3/s/P	—

[a] E, strain EGDe; 12, strain 12; 3, strain 3; pH, pH-stressed cells; s, salt-stressed cells; G, synthetic gastric fluid; P, synthetic pancreatic fluid.

[b] A negative number indicates down-regulation of the gene, and a positive number indicates upregulation of the respective gene; —, no gene was found with false discovery rate ≤25% and fold change ≥2.0.

Strain EGDe showed upregulation of the *rpoB, ctsR, bvrA, prfA, bvrC,* and *sigB* genes (regulation group) after exposure of both pH- and salt-stressed cells to the synthetic pancreatic fluid. Acid- and salt-stressed *Listeria* cells of strain 12 showed upregulation of the adaptation group (*clpE, cysK, clpC, clpP,* and *proA*) and down-regulation of the intracellular life cycle group (*iap, fri, dat, plcA, dal, relA, prfA, mpl,* and *actA*), as previously observed for the synthetic gastric fluid. In addition, down-regulation of the main invasion-related genes, such *as aut, inlD, prfA, actA, iap,* and *inlA,* was observed for the salt-stressed cells. Finally, no significantly differentially expressed genes were found for strain 3 after exposure to the synthetic pancreatic fluid.

DISCUSSION

The tested *L. monocytogenes* strains proved to be sensitive after exposure to the synthetic gastric and pancreatic fluids. However, a small number of populations survived at the end of the treatment. Other authors have also found a reduction in the *L. monocytogenes* population after exposure to gastrointestinal tract conditions *(15)*. A subgenomic array was used to study the gene expression of the pH- and salt-stressed *L. monocytogenes* cells; the suitability of this array has been validated elsewhere *(25)*.

TABLE 4. *Gene set enrichment analysis results obtained for the pH- and salt-stressed cells treated with the synthetic gastric and pancreatic fluids relative to their respective control conditions*[a]

Conditions	Enriched groups	ES	NES	Leading edge
E/pH/G	Invasion	0.66	1.53	*srtA, inlB, iap(2)*
	Adaptation	−0.38	−1.21	*lmo0669, clpE, cysK, sigB, clpC, gadE, lmo0292, betL*
E/s/G	Invasion	0.67	1.51	*prfA, iap, srtA, sigB, iap(2), hly*
E/pH/P	Regulation	0.71	1.75	*rpoB, ctsR, bvrA, prfA, bvrC*
E/s/P	Regulation	0.75	1.55	*prfA, ctsR, sigB, bvrC*
12/pH/G	Intracellular life cycle	−0.60	−1.43	*mpl, iap(2), iap, dat, dal, plcA, relA, fri, prfA*
12/s/G	Adaptation	0.54	1.45	*clpE, gadB, proC, sigB*
	Regulation	0.61	1.32	*sigB, bvrA, bvrC*
12/pH/P	Intracellular life cycle	−0.59	−1.69	*iap, fri, dat, plcA, dal, relA, prfA*
12/s/P	Adaptation	0.62	1.58	*clpE, cysK, clpC, clpP, proA*
	Intracellular life cycle	−0.60	−1.36	*dat, mpl, prfA, actA, iap(2), plcA*
	Invasion	−0.57	−1.38	*aut, inlD, prfA, actA, iap(2), inlA*
3/pH/G	None	—	—	No significant genes were found
3/s/G	Intracellular life cycle	0.51	1.33	*plcA, hly, plcB, mpl, iap*
3/pH/P	None	—	—	No significant genes were found
3/s/P	None	—	—	No significant genes were found

[a] The table shows the groups overrepresented at false discovery rate ≤25%, the enrichment score (ES), the normalized enrichment score (NES), and the gene members of each group that contributed most to the enrichment score (leading edge). NES accounts for differences in gene set size and correlations between gene sets and the expression data set. E, strain EGDe; 12, strain 12; 3, strain 3; pH, pH-stressed cells; s, salt-stressed cells; G, synthetic gastric fluid; P, synthetic pancreatic fluid. A negative number indicates down-regulation of the gene, and a positive number indicates upregulation of the respective gene.

The genetic study revealed that heterogeneity in gene expression exists among serotypes. Some differences between the gastric and pancreatic treatments were also observed, in terms of what genes were expressed. It can be speculated that *L. monocytogenes* strains responded differently to environmental conditions so as to be able to survive in such harsh conditions. Rantsiou et al. *(25)* also found that the same strains (EGDe, 12, and 3) responded quickly and individually to a new environment when they were inoculated into minced meat and fermented sausage juices for 30 min and 48 h at 4°C. Another feature that has been observed in the present study concerns the weak expression of several genes. This observation confirms the finding that virulence gene expression in *L. monocytogenes* at 37°C in bacteriological culture media is weaker than the intracellular levels *(8)*. The use of GSEA has allowed some general conclusions to be drawn on the effect of the simulated gastrointestinal conditions on the gene expression patterns of acid- and salt-stressed *Listeria* cells.

Serotype 1/2a (strain EGDe) displayed increased invasiveness and potential stress responsiveness through the upregulation of the invasion-related genes (*iap, inlB, srtA,* and *hly*) and of the major gene regulators involved in the regulation of other genes related to virulence and stress (*prfA* and *sigB*). PrfA is the master regulator of virulence gene expression in *L. monocytogenes (8)*. Sigma B regulon contains several stress response and virulence genes and gene regulators *(18, 20, 22, 30, 31)*. In the present study, the *sigB* gene was specifically expressed in the salt-stressed *Listeria* cells. In *L. monocytogenes,* expression of the σ^B factor is strongly induced by salt stress *(3)*. Walecka-Zacharska et al. *(36)* concluded that *L. monocytogenes* enhanced invasiveness in response to salt stress. Overexpression of the gene regulators *ctsR, bvrA,* and *bvrC* was observed, especially after the treatment of acid- and salt-stressed cells with synthetic pancreatic fluid. The gene *ctsR* negatively regulates the expression of the *clp* genes (*clpB, clpP, clpE,* and *clpC*) required for stress survival and intracellular growth *(16)*. The *bvr* locus encodes a specific beta-glucoside sensor that mediates virulence gene repression *(4)*. The speculation that stress-adapted *Listeria* cells may promote invasiveness by regulating (repressing) other nonvirulence- and/or virulence-related genes could be supported by the fact that acid-stressed cells, after the synthetic gastric passage, down-regulated several genes belonging to the adaptation group, for instance, the genes *lmo0669, clpE, cysK,* and *clpC,* which are related to general stress; the general stress regulator *sigB*; the gene *gadE,* which is related to acid stress; or the genes *lmo0292* (heat stress) and *betL* (osmotic stress). The short- and long-term adaptation of *L. monocytogenes* to acidic and osmotic stress has been shown to increase the capability of the pathogen to invade Caco-2 cells *(5, 13, 24)*. Serotype 4b (strain 12) showed upregulation of the adaptation- and regulation-related genes. The *gadB* gene is part of the glutamate decarboxylase acid resistance system of *L. monocytogenes (7)*. This system is essential for survival in the stomach after ingestion *(9)*. The stress-related genes *cysK, clpC, clpE,* and *clpP* were upregulated, as well as the osmotic-stress–related genes *proA* and *proC*. The *clpC, clpE,* and *clpP* genes have been shown to increase their transcription after exposure of *L. monocytogenes* to conditions similar to those in the gastrointestinal tract *(15)*. Down-regulation of the major genes involved in the intracellular life cycle and in invasion, such as *mpl, fri, dal, dat, plcA, relA, prfA, actA, aut, inlA, inlD,* and *iap,* occur in serotype 4b. This could be part of a strategy of *L. monocytogenes* to enhance the expression of stress-related genes and repress the transcription of non–stress-related genes in order to survive in hostile environments. Jiang et al. *(15)*

found that *L. monocytogenes* strains, after exposure to the conditions that prevail in the gastrointestinal tract, enhanced the expression of stress-related genes (*clpC, clpE,* and *clpP*) and decreased the transcription of an adhesion-related gene (*ami*) in order to survive in the diverse microenvironments. Interestingly, for serotype 3c (strain 3), no genes were found to be overrepresented (up- or down-regulated) in most of the cases.

In conclusion, *L. monocytogenes* strains exposed to conditions simulating those found in foods and hosts responded differently to environmental stresses. Note that serotype 1/2a showed upregulation of the master regulator genes (*prfA* and *sigB*) and the genes involved in the invasion process; serotype 4b displayed enhanced expression in genes related to adaptation and regulation by increasing the expression of stress-responsive genes such as *sigB*; whereas, in general, serotype 3c did not show significantly differentially expressed genes after the treatments or the upregulated genes were not found to be associated with invasion and/or adaptation. These interesting results provide experimental (gene-expression) evidence for the potential dominance of serotypes 1/2a and 4b over serotype 3c in clinical cases. *L. monocytogenes* serotypes 1/2a, 1/2b, and 4b are responsible for 95% of human infections, and the majority of outbreaks are caused by these serotypes, especially by serotype 4b *(32)*.

Studies of *L. monocytogenes* gene expression in the conditions usually encountered during gastric and pancreatic digestion may provide critical information on the biology of this pathogen and may lead to improved understanding of the strategies that *L. monocytogenes* pathogens use to facilitate survival and transmission in such environments. To the authors' knowledge, this is the first report of the effects of acid- and salt-adapted *Listeria* cells on the expression of the genes involved in the pathogenicity mechanism of *L. monocytogenes* after treatment with synthetic gastric and pancreatic fluids using microarrays. Most studies have focused on the investigation and measurement of the expression of a specific number of genes using RT-qPCR *(15, 24, 30, 31)*. Although definitive conclusions cannot be made, due to common problems that occur in experiments conducted in vitro (weak expression of genes), a general trend in the behavior of pH- and salt-stressed *L. monocytogenes* cells after a gastrointestinal passage can be observed.

ACKNOWLEDGMENT

This work was supported by the FP7-People-2011-CIG (LisGenOmics) project through a Marie Curie scholarship (Marie Curie—Career Integration Grant) to M. Mataragas (grant no. PCIG09-GA-2011-293406).

REFERENCES

1. Bae, D., C. Liu, T. Zhang, M. Jones, S. N. Peterson, and C. Wang. 2012. Global gene expression of *Listeria monocytogenes* to salt stress. *J. Food Prot.* 75:906–912.
2. Bautista-Gallego, J., F. N. Arroyo-Lopez, K. Rantsiou, R. Jimenez-Diaz, A. Garrido-Fernandez, and L. Cocolin. 2013. Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. *Food Res. Int.* 50:135–142.
3. Becker, L. A., M. S. Cetin, R. W. Hutkins, and A. K. Benson. 1998. Identification of the gene encoding the alternative sigma factor σ^B from *Listeria monocytogenes* and its role in osmotolerance. *J. Bacteriol.* 180:4547–4554.
4. Brehm, K., M.-T. Ripio, J. Kreft, and J.-A. Vazquez-Boland. 1999. The *bvr* locus of *Listeria monocytogenes* mediates virulence gene repression by β-glucosides. *J. Bacteriol.* 181:5024–5032.
5. Conte, M. P., G. Petrone, A. M. di Biase, M. G. Ammendolia, F. Superti, and L. Seganti. 2000. Acid tolerance in *Listeria monocytogenes* influences invasiveness of enterocyte-like cells and macrophage-like cells. *Microb. Pathog.* 29:137–144.
6. Corcoran, B. M., C. Stanton, G. F. Fitzgerald, and R. P. Ross. 2007. Growth of probiotic lactobacilli in the presence of oleic acid enhances subsequent survival in gastric juice. *Microbiology* 153:291–299.
7. Cotter, P. D., C. G. Gahan, and C. Hill. 2001. A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. *Mol. Microbiol.* 40:465–475.
8. de las Heras, A., R. J. Cain, M. K. Bielecka, and J. A. Vazquez-Boland. 2011. Regulation of *Listeria* virulence: PrfA master and commander. *Curr. Opin. Microbiol.* 14:118–127.
9. Dussurget, O. 2008. New insights into determinants of *Listeria monocytogenes* virulence. *Int. Rev. Cell Mol. Biol.* 270:1–38.
10. Dysvik, B., and I. Jonassen. 2001. J-Express: exploring gene expression data using Java. *Bioinformatics* 17:369–370.
11. Ellrott, A., C. Wurdemann, J. Peplies, and F. O. Glockner. 2007. MADA—a software tool for the highly transparent initial processing of microarray raw data based on Microsoft Excel. Max Planck Institute for Marine Microbiology, Microbial Genomics Group. Available at: http://www.megx.net/mada. Accessed 5 June 2012.
12. Farber, J. M., and P. I. Peterkin. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Rev.* 55:476–511.
13. Garner, M. R., K. E. James, M. C. Callahan, M. Wiedmann, and K. J. Boor. 2006. Exposure to salt and organic acids increases the ability of *Listeria monocytogenes* to invade Caco-2 cells but decreases its ability to survive gastric stress. *Appl. Environ. Microbiol.* 72:5384–5395.
14. Goulet, V., C. Hedberg, A. Le Monnier, and H. de Valk. 2008. Increasing incidence of listeriosis in France and other European countries. *Emerg. Infect. Dis.* 14:734–740.
15. Jiang, L., I. Olesen, T. Andersen, W. Fang, and L. Jespersen. 2010. Survival of *Listeria monocytogenes* in simulated gastrointestinal system and transcriptional profiling of stress- and adhesion-related genes. *Foodborne Pathog. Dis.* 7:1–8.
16. Karatzas, K. A. G., J. A. Wouters, C. G. M. Gahan, C. Hill, T. Abee, and M. H. J. Bennik. 2003. The CtsR regulator of *Listeria monocytogenes* contains a variant glycine repeat region that affects piezotolerance, stress resistance, motility and virulence. *Mol. Microbiol.* 49:1227–1238.
17. Kathariou, S. 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *J. Food Prot.* 65:1811–1829.
18. Kazmierczak, M. J., S. C. Mithoe, K. J. Booe, and M. Wiedmann. 2003. *Listeria monocytogenes* sigma B regulates stress response and virulence functions. *J. Bacteriol.* 185:5722–5734.
19. Lucke, F.-K. 2000. Utilization of microbes to process and preserve meat. *Meat Sci.* 56:105–115.
20. McGann, P., M. Wiedmann, and K. J. Boor. 2007. The alternative sigma factor sigma B and the virulence gene regulator PrfA both regulate transcription of *Listeria monocytogenes* internalins. *Appl. Environ. Microbiol.* 73:2919–2930.
21. Mootha, V. K., C. M. Lindgren, K.-F. Eriksson, A. Subramanian, S. Sihag, J. Lehar, P. Puigserver, E. Carlsson, M. Ridderstrale, E. Laurila, N. Houstis, M. J. Daly, N. Patterson, J. P. Mesirov, T. R. Golub, P. Tamayo, B. Spiegelman, E. S. Lander, J. N. Hirschhorn, D. Altshuler, and L. C. Groop. 2003. PGC-1α-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat. Genet.* 34:267–273.
22. Nadon, C. A., B. M. Bowen, M. Wiedmann, and K. J. Boor. 2002. Sigma B contributes to PrfA-mediated virulence in *Listeria monocytogenes*. *Infect. Immun.* 70:3948–3952.
23. Nelson, K. E., D. E. Fouts, E. F. Mongodin, J. Ravel, R. T. DeBoy, J. F. Kolonay, D. A. Rasko, S. V. Angiuoli, S. R. Gill, I. T. Paulsen,

J. Peterson, O. White, W. C. Nelson, W. Nierman, M. J. Beanan, L. M. Brinkac, S. C. Daugherty, R. J. Dodson, A. S. Durkin, R. Madupu, D. H. Haft, J. Selengut, S. Van Aken, H. Khouri, N. Fedorova, H. Forberger, B. Tran, S. Kathariou, L. D. Wonderling, G. A. Uhlich, D. O. Bayles, J. B. Luchansky, and C. M. Fraser. 2004. Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. *Nucleic Acids Res.* 32: 2386–2395.

24. Olesen, I., F. K. Vogensen, and L. Jespersen. 2009. Gene transcription and virulence potential of *Listeria monocytogenes* strains after exposure to acidic and NaCl stress. *Foodborne Pathog. Dis.* 6:669–680.
25. Rantsiou, K., A. Greppi, M. Garosi, A. Acquadro, M. Mataragas, and L. Cocolin. 2012. Strain dependent expression of stress response and virulence genes of *Listeria monocytogenes* in meat juices as determined by microarray. *Int. J. Food Microbiol.* 152:116–122.
26. Rantsiou, K., M. Mataragas, L. Jespersen, and L. Cocolin. 2011. Understanding the behavior of foodborne pathogens in the food chain: new information for risk assessment analysis. *Trends Food Sci. Technol.* 22:S21–S29.
27. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Springs Harbor Laboratory Press, Cold Springs Harbor, NY.
28. Sturn, A., J. Quackenbush, and Z. Trajanoski. 2002. Genesis: cluster analysis of microarray data. *Bioinformatics* 18:207–208.
29. Subramanian, A., P. Tamayo, V. K. Mootha, S. Mukherjee, B. L. Ebert, M. A. Gillette, A. Paulovich, S. L. Pomeroy, T. R. Golub, E. S. Lander, and J. P. Mesirov. 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. USA* 102:15545–15550.
30. Sue, D., K. J. Boor, and M. Wiedmann. 2003. Sigma(B)-dependent expression patterns of compatible solute transporter genes *opuCA* and lmo1421 and the conjugated bile salt hydrolase gene *bsh* in *Listeria monocytogenes*. *Microbiology* 149:3247–3256.
31. Sue, D., D. Fink, M. Wiedmann, and K. J. Boor. 2004. σ^B-dependent gene induction and expression in *Listeria monocytogenes* during osmotic and acid stress conditions simulating the intestinal environment. *Microbiology* 150:3843–3855.
32. Swaminathan, B., and P. Gerner-Smidt. 2007. The epidemiology of human listeriosis. *Microbes Infect.* 9:1236–1243.
33. Troyanskaya, O., M. Cantor, O. Alter, G. Sherlock, P. Brown, D. Botstein, R. Tibshirani, T. Hastie, and R. Altman. 2001. Missing value estimation methods for DNA microarrays. *Bioinformatics* 17: 520–525.
34. Tusher, V. G., R. Tibshirani, and G. Chu. 2001. Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. USA* 98:5116–5121.
35. Vazquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland, and J. Kreft. 2001. *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.* 14:584–640.
36. Walecka-Zacharska, E., K. Kosek-Paszkowska, J. Bania, R. Karpiskova, and T. Stefaniak. 2013. Salt stress-induced invasiveness of major *Listeria monocytogenes* serotypes. *Lett. Appl. Microbiol.* 56: 216–221.
37. Watson, R. 2009. Listeriosis remains a cause for concern in Europe. *BMJ* 338:b319.