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The Effect of Oxygen on Bile Resistance in *Listeria monocytogenes*

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Abstract

Listeria monocytogenes is a Gram-positive facultative anaerobe that is the causative agent of the disease listeriosis. The infectious ability of this bacterium is dependent upon resistance to stressors encountered within the gastrointestinal tract, including bile. Previous studies have indicated bile salt hydrolase activity increases under anaerobic conditions, suggesting anaerobic conditions influence stress responses. Therefore, the goal of this study was to determine if reduced oxygen availability increased bile resistance of *L. monocytogenes*. Four strains representing three serovars were evaluated for changes in viability and proteome expression following exposure to bile in aerobic or anaerobic conditions. Viability for F2365 (serovar 4b), EGD-e (serovar 1/2a), and 10403S (serovar 1/2a) increased following exposure to 10% porcine bile under anaerobic conditions ($P < 0.05$). However, HCC23 (serovar 4a) exhibited no difference ($P > 0.05$) in bile resistance between aerobic and anaerobic conditions, indicating that oxygen availability does not influence resistance in this strain. The proteomic analysis indicated F2365 and EGD-e had an increased expression of proteins associated with cell envelope and membrane bioenergetics under anaerobic conditions, including thioredoxin-disulfide reductase and cell division proteins. Interestingly, HCC23 had an increase in several dehydrogenases following exposure to bile under aerobic conditions, suggesting that the NADH:NAD+ is altered and may impact bile resistance. Variations were observed in the expression of the cell shape proteins between strains, which corresponded to morphological differences observed by scanning electron microscopy. These data indicate that oxygen availability influences bile resistance. Further research is needed to decipher how these changes in metabolism impact pathogenicity *in vivo* and also the impact that this has on susceptibility of a host to listeriosis.

Keywords: *Listeria monocytogenes;* Bile; Oxygen; Proteomic; Mass spectrometry; Scanning electron microscopy

Abbreviations: ACN: Acetonitrile; BHI: Bran Heart Infusion; FA: Formic Acid; PBS: Phosphate Buffered Saline; SEM: Scanning Electron Microscope

Introduction

Listeria monocytogenes is a Gram-positive facultative anaerobe and the causative agent of listeriosis [1]. With a nearly 20% mortality rate in the United States, *L. monocytogenes* typically manifests disease in the young, elderly, pregnant women and the immunocompromised through the consumption of contaminated foods [2,3]. Upon consumption *L. monocytogenes* must resist the multiple stressors encountered within the gastrointestinal tract, including bile, variations in pH, and oxygen availability [4,5]. Bile is specific to the gastrointestinal tract and consists of multiple components such as ions, cholesterol, proteins, bile salts, and pigments [6]. Of these, bile salts have been shown to possess antimicrobial activity through the induction of DNA damage and degradation of viral and bacterial membranes [7,8]. *Listeria monocytogenes* is able to grow in the gall bladder [4], indicating this bacterium is resistant to the highest concentration of bile salts encountered within the body.

Bile resistance mechanisms have been extensively studied, including the bile salt hydrolase *bsh* [9,10], the general stress response sigma factor *sigB* [11,12], the bile exclusion system *bilE* [13], and virulence regulator *prfA* [9]. However, information is lacking in regards to the response of *L. monocytogenes* to bile under physiologically relevant anaerobic and microaerophilic conditions. The expression of genes needed for survival under acidic conditions was found to increase under anaerobic conditions [14,15] and an increase in branch-chain fatty acids in the cell membrane was observed when *L. monocytogenes* was cultured under elevated carbon dioxide and anaerobic conditions [15]. Additionally, the activity of the bile salt hydrolase has been found to increase under anaerobic conditions [9]. Oxygen restriction also enhances growth at lower temperatures (~ 19°C). Together, these data suggest that oxygen availability influences resistance to stressors, which could potentially impact the virulence capability of *L. monocytogenes*.

Although much research has been conducted to identify and characterize proliferation and pathogenesis, few studies have analyzed the effect of oxygen availability on the bile resistance properties of *L. monocytogenes* including the influence of oxygen on regulation of the proteome expressed in response to bile. Therefore, this study focused on comparing the influence that reduced oxygen has on bile resistance in strains representing three serovars of *L. monocytogenes*. Two of these serovars (1/2a and 4b) represent nearly 90% of all listeriosis cases, whereas serovar 4a is rarely associated with listeriosis [16]. Here, we

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report that anaerobiosis increased bile resistance with the serovar 1/2a and 4b strains tested and the resistance may be linked to differential expression patterns of metabolic and membrane bioenergetic proteins.

Materials and Methods

Bacterial strains and culture conditions

Strains used in this study were F2365 (serotype 4b), EGD-e (serotype 1/2a), 10403S (serotype 1/2a), and HCC23 (serotype 4a) [17-19]. All strains of *L. monocytogenes* were cultured in Brain Heart Infusion (BHI) broth and incubated in a shaker incubator at 37°C at 250 rpm.

Survival assays

Anaerobic conditions: Overnight cultures were diluted 1:100 into 10 mL of fresh BHI in an anaerobic chamber (Coy Laboratories) with a gas mix of 95% N_2, 5% H_2 in Wheaton serum vials capped with rubber stoppers and sealed with aluminum; the redox indicator resazurin (5 μM) was added in order to visually monitor anaerobiosis. Inoculated vials were grown to mid-logarithmic phase (OD_{600} ~ 0.4) at 37°C with agitation, at which time cells were divided into 4 separate 2 mL aliquots, pelleted immediately at 8,000 × g for 5 min, then resuspended in 2 mL of BHI supplemented with 0%, 1% (0.02 g), 5% (0.1 g), or 10% (0.2 g) porcine bile extract (Sigma B8631, Sigma Aldrich) and 0.1% methanol (for solubility of bile) in the anaerobic chamber. Samples (100 μL) were collected using a syringe needle at 0, 1, 2, 3, 4, 5 and 6 h post exposure to porcine bile extract. Samples were serially diluted in phosphate buffered saline (PBS) and plated on BHI agar. Plates were incubated under anaerobic conditions using an AnaeroPack System (Mitsubishi Gas Chemical) at 37°C for 18 h prior to viable plate count analysis.

Aerobic conditions: Bacterial cultivation was performed as described for the anaerobic cultivation assay, but without the use of sealed vials or the addition of resazurin. All cultures were incubated under normal atmospheric conditions at 37°C. A minimum of three independent experiments was performed for each strain under each condition tested.

Protein sample preparation

Cultures were grown to mid-log in either aerobic or anaerobic conditions or subsequently exposed to either 0% or 5% porcine bile for 1 h as described for the survival assays. At 1 h post exposure, 10 mL were collected and immediately centrifuged at 8,000 × g for 10 min at 10°C. Cell pellets were resuspended in 700 μL NP-40 lysis buffer (150 mM NaCl, 1.0% IGEPAL, 50 mM Tris, pH 8.0) supplemented with protease inhibitors (Sigma P2714, Sigma Aldrich) and lysed for 60 sec through sonication using Covaris model S220. Lysate was plated to ensure complete lysis. Lysates were centrifuged at 20,000 × g for 15 min to remove cell debris. A Fluka Protein Quantification Kit was used to determine protein concentration using the manufacturer's protocol (Sigma Aldrich). Proteins (100 μg - 200 μg) were then purified by chloroform-methanol precipitation in order to remove any residual detergent, which would have interfered with mass spectrometry analysis. Briefly, samples were mixed with 600 μL of methanol, 150 μL of chloroform, and 450 μL of sterile deionized water, vortexed, and centrifuged at 13,000 × *g* for one min. Supernatant was removed, 450 μL of methanol was added to the interface layer, and centrifuged again at 13,000 × *g* for two min. Protein pellets were dried in a vacuum centrifuge at room temperature, denatured and alkylated, and digested with 2 μg of trypsin overnight as previously described [20] and desalted using a macrotrap (Michrome Bioresources). Cleaned samples were resuspended in 20 μL of 0.1% formic acid (FA) and 5% acetonitrile (ACN) for proteomic analysis.

Proteomic analysis by mass spectrometry

Peptides were analyzed using a Dionex UltiMate 3000 (Thermo Scientific) high performance liquid chromatography machine (HPLC) coupled with an LTQ-OrbiTrap Velos (Thermo Scientific) tandem mass spectrometer. The linear trap of the LTQ was used for precursor and fragment scans, as the resolution of the OrbiTrap was not required for these analyses [21]. The HPLC was configured for reverse phase chromatography using a C18 Acclaim PepMap RSLC column (Thermo Scientific) with a flow rate of 300 nL/min. Peptides were separated for mass spectrometry analysis using an acetonitrile gradient starting at 2% ACN, 0.1% FA and reaching 50% ACN, 0.1% FA in 120 min, followed by a 15 min wash of 95% ACN, 0.1% FA. Column equilibration was handled automatically using the Dionex UltiMate 3000. The eluate from the HPLC was fed directly to the LTQ for nanospray ionization followed by MS / MS analysis of detected peptides by Collision-induced dissociation (CID). The LTQ was configured to perform 1 ms scan followed by 17 MS / MS scans of the 17 most intense peaks repeatedly over the 135 min duration of each HPLC run. Dynamic exclusion was enabled with duration of 3 min, repeat count of 3, and a list length of 500. For fragment mass analysis, activation time was 40 ms and normalized collision energy was 35. Ion trap mass spectrometer (IT-MS) was used for both full mass analysis, as well as fragment mass analysis. Raw files from the LTQ were converted to mgf format using the MSConvert GUI software from the ProteoWizard toolset [21,22]. *L. monocytogenes* strain-specific protein FASTA databases for were downloaded from the National Center for Biotechnology Information reference sequences (NCBI RefSeq 10403S gi|386042347; EGD-e gi|16802048; F2365 gi|85700163; and HCC23 gi|217963303). The X!tandem [PMID: 14976030] and OMSSA algorithms [PMID: 15473683] were used to match ms/ms spectra to the FASTA databases. Precursor and fragment mass tolerances were set to 1 Da and 0.5 Da respectively for X!tandem. Both precursor and fragment mass tolerances were set to 1 Da for OMSSA. Tryptic cleavage rules were used when calculating *in-silico* peptide precursor masses; b and y ions were used for fragment m/z matching. Amino acid modifications that were included in the database searches were single and double oxidation of methionine and both carboxymethylation and carbamidomethylation of cysteine, phosphorylation of serine, threonine and tyrosine, as well as water loss from serine and threonine. Randomized versions of each protein FASTA were concatenated to the originals as a means to calculate false discovery rates (FDR). Peptide-spectrum matches with e-values < 0.05 were accepted for down-stream analysis. Peptide matches were organized by protein using Perl, at which time non-unique peptide sequences and proteins identified by a single peptide sequence were removed. The maximum acceptable FDR was 1%; if the FDR was above 1%, the peptide e-value cutoff was lowered incrementally by 0.005 until the FDR fell below 1%. After each iteration, proteins reduced to a single peptide sequence were removed from the results. The mass spectrometry proteomics data has been deposited to the ProteomeXchange Consortium [23] via the PRIDE partner repository with the dataset identifier PXD002243 and 10.6019/PXD002243.

Differential expression of proteins between porcine bile treated and non-treated samples as well as between aerobic and anaerobic conditions upon bile treatment was performed pairwise using peptide elution profiles. Precursor mass spectra were extracted from the raw data in MS1 format using the MSConvert GUI software from the

ProteoWizard toolset [21,22]. Peptide precursor m/z values were extracted from the previously compiled protein identifications using Perl. Elution profiles for peptide-spectrum matches were calculated by parsing each corresponding MS1 file and summing the ion current for that match's m/z value within a 0.25 Da tolerance, effectively integrating the elution profiles. Each trace started at the scan number of the peptide-spectrum match and preceded both forward and backward until the chromatogram noise level, or a distance of 250 scans, was reached. Multiple peptide-spectrum matches with the same precursor m/z were only counted once, ensuring the same integral was not included multiple times. Once all peptide-spectrum matches were processed, intensities were summed for each protein on a per-replicate basis. Proteins not identified in a replicate were represented with the average noise level of the replicate's chromatogram for further calculations. The reasoning behind this is two-fold: 1) peptides not identified in a replicate could be present at levels at or below the noise level of the chromatogram, causing the mass spectrometer to ignore them and 2) for calculating expression ratios between lines, zero cannot be in the denominator. Data were normalized using a mode-based technique. First, the mode of the protein intensities for each replicate was calculated, representing the most commonly occurring protein intensity. Next, for each identified protein, the intensity per replicate was divided by the mode of the same replicate. This ensures that normalization is not affected by the minimum and maximum intensities, which can vary tremendously between replicates. A permutation analysis was performed for each protein by evaluating the difference in means of the replicates of both conditions. From this permutation, a p-value was calculated to indicate the significance of the difference in means. Two additional permutations were performed for each protein, comparing both conditions to their own baselines. These baseline permutations provided a mechanism to further reduce false positives introduced by differences in chromatogram ion current as electron multiplier performance deceases. Proteins were considered to be differentially expressed if the difference in means between conditions resulted in a $P < 0.05$ and the difference in means between one of the conditions and its baseline was $P < 0.05$.

Pathway analysis

Protein datasets identified as significantly differentially expressed were subjected to KOBAS 2.0 analysis (http://kobas.cbi.pku.edu.cn/home.do). KOBAS 2.0 was applied to first annotate all of the entries with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and to then identify significantly enriched pathways with $P < 0.05$.

Gene expression assays

All strains were cultured in 5 mL BHI under aerobic or anaerobic conditions and exposed to either 0% or 5% porcine bile extract for 1 h. Cells were pelleted by centrifugation at 10,000 × *g* for 2 min, washed in ice cold PBS, then treated with RNAprotect Bacteria Reagent (Qiagen) according to the manufacturer's protocol. RNA was isolated using the RNeasy mini kit (Qiagen) following an altered manufacturer's protocol as followed. Briefly, 400 µL of RLT buffer with 2-β-mercaptoethanol was added to cell pellets, followed by lysis using a bead beater for two 2 min intervals. Ethanol was then directly added to lysed cells at a 60% final concentration to the samples prior to being homogenized using a QIAshredder (Qiagen). Samples were treated with RNase free DNase. RNA and DNA quantitation was determined using Qubit RNA BR assay and DNA HS assay, respectively, and analyzed using a Qubit 2.0 fluorometer (Life Technologies) following manufacturer's protocol. RNA was normalized to 100ng and converted to cDNA using an Applied Biosystems High Capacity cDNA Reverse Transcriptase Kit following manufacturer's protocol. The RT-PCR protocol was as follows: 25°C for 10 min, 37°C for 120 min and 85°C for 5 sec. Concentrations were determined using a Nanodrop ND-100.

Expression of *bsh* was determined in relation to expression of 16S rRNA gene using an Applied Biosystems Step One Plus System. For the *bsh* target, the forward primer was 5'-CCTGTTGGCGTGTTAACAAATAA-3', the reverse primer was 5'- CCATCCCACGACTATAAGCATC -3', and the probe was 5'-FAM-TCGCGTTCT/ZEN/TTCGAGTGAAACTCCA-IowaBlackFQ. For the 16S target, the forward primer was 5'-CTTGTCCCTTGACGGTATCTAAC-3' the reverse primer was 5'-GCGCTTTACGCCCAATAAATC-3' and the probe was 5'-FAM/CGGTAATAC/ZEN/GTAGGTGGCAAGCGT-IowaBlackFQ. Standard curves were generated to verify primer efficiency. Each reaction received 10 µL of 2x Taqman Gene Expression Master Mix (Life Technologies), 2 µL of 10x PrimeTime qPCR assay mix designed for either *bsh* or 16S rRNA (Integrated DNA Technologies) and 5 µL of 1:100 diluted cDNA template, with volume adjusted to 20 µL with RNAse free water. The qPCR reaction followed two stages; first stage: 50°C for 2 min and 95°C for 10min; second stage: 95°C for 15 s and 60°C for 1 min, for 40 cycles. Fold changes in expression of *bsh* were calculated based on expression levels of 16S rRNA from three independent experiments as previously described [22].

Scanning electron microscopy

F2365, EGD-e, 10403S, and HCC23 were cultured aerobically or anaerobically in 5 mL BHI to mid-log phase as described above, at which time cultures were split to two 2 mL aliquots and treated with either 0% or 5% bile for 1 h at 37°C. Cells were then processed for scanning electron microscopy (SEM) as previously described [24]. Briefly, cells were pelleted by centrifugation at 8,000 × g for 5 min, fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer, washed in 0.1 M cacodylate buffer, post-fixed in 2% osmium tetraoxide in 0.1 M cacodylate buffer, dehydrated in an ethanol series, and dried in hexamethyldisilazane series. Samples were sputter coated with platinum prior to observation on a JOEL 6500F SEM. Cell size was determined using the ImageJ software.

Statistical analysis

Survival assays were analyzed using the Proc Glimmix procedure of SAS (v. 9.4, SAS Institute, Cary, NC). Fixed effects included bacterial strain, treatment, oxygen availability, time, and interactions between these parameters. All concentrations were converted to Log_{10} to achieve normality. Means were separated using LSMeans with an alpha of 0.05 using Tukey-Kramer adjustment. For SEM analyses, the length and width of both control and treatment samples under either aerobic or anaerobic conditions were analyzed using a T-tailed type II T-test, with $P < 0.05$ deemed as significant.

Results

Oxygen availability influences the survival of *L. monocytogenes* in bile

The aim of this study was to determine if oxygen availability influenced bile resistance among strains of *L. monocytogenes*. The viability of strains 10403S (serovar 1/2a), EGD-e (serovar 1/2a), F2365 (serovar 4b) and HCC23 (serovar 4a) following exposure to porcine bile extract was first assessed under both aerobic and anaerobic conditions (Figure 1). Exposure to 1% or 5% bile under aerobic conditions did

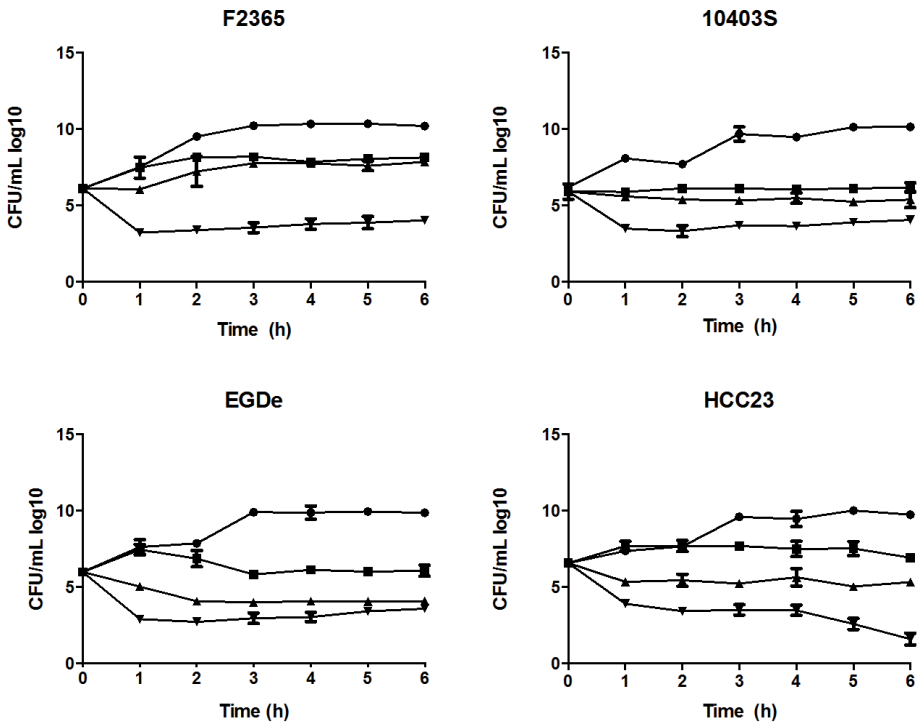

Figure 1: Viability of *Listeria monocytogenes* following exposure to bile under aerobic conditions.

Viability of strains 10403S, EGD-e, F2365, and HCC23 was determined by viable plate counts following exposure to 0% (●), 1% (■), 5% (▲) or 10% (▼) porcine bile extract under aerobic conditions. Graphs represent an average of three independent experiments; error bars represent standard deviation.

not impact viability of 10403S. The 10% concentration resulted in a 2.4 Log_{10} decrease in viability within the first hour post exposure (P = 0.001). EGD-e decreased in viability following exposure to 5% and 10% bile, with a nearly 1 Log_{10} and 3 Log_{10} reductions respectively (P < 0.01 and P < 0.001, respectively). F2365 was able to continue replicating in media supplemented with either 1% or 5% porcine bile extract, but not 10%. Exposure to 10% bile resulted in a nearly 3 Log_{10} decrease in viability within the first hour post exposure (P < 0.001), which was similar to what was observed in the other strains analyzed (P < 0.05). Interestingly, the avirulent strain HCC23 had a slight increase in viability immediately following exposure to 1% bile, but then remained static for the remainder of the times analyzed. Exposure to 5% and 10% resulted in a decrease of 1.2 Log_{10} and 2.7 Log_{10} within the first hour, respectively (P < 0.001).

The virulent strain F2365 was able to survive and continue to replicate following exposure to all concentrations of bile tested under anaerobic conditions (Figure 2). A slight increase of 0.2 Log_{10} was observed within 1 h exposure to 1% bile (P = 0.03). Also, an increase of 0.2 Log_{10} after 1 h exposure to 5% and 10% bile was observed in F2365 under anaerobic conditions.

An increase of approximately 1 Log_{10} was observed after 1 h exposure to 1% bile in both 10403S and EGD-e (P = 0.01). The viability of 10403S increased until 3 h, at which point no changes in viability were noted (Figure 2). In 10% bile, EGD-e decreased by approximately 1 Log_{10} within 1 h post exposure (P = 0.02) and recovered at 2 h. Following an initial decrease in viable colonies, the avirulent strain HCC23 had relatively no change in viability in all bile concentrations analyzed, except for a significant decrease in viability following 6 h of exposure to 10% bile HCC23 (4.69 Log_{10}, P < 0.001).

Statistical comparisons of the viability of *L. monocytogenes* exposed to bile under aerobic versus anaerobic conditions revealed a difference in F2365 in 10% bile for each time point tested (P < 0.001; Table 1); exposure to 5% bile only showed significance at 1 h (P = 0.017). 10403S also had an increase in survival under anaerobic conditions in 10% bile for up to 4 h post exposure (P < 0.05). Interestingly, no significant change was observed in HCC23 upon exposure to bile in relation to oxygen availability except for 1% at 1 h (P = 0.011) and 2 h (P = 0.002).

Bile salt hydrolase activity varies between strains under aerobic and anaerobic conditions

The expression of *bsh* has been shown to increase under anaerobic conditions [9]. To determine whether the differences in the bile survival between the four strains tested was due to variations in the expression of *bsh,* real-time PCR was utilized to quantify the expression following a 1 h exposure to bile under aerobic or anaerobic conditions (Table 2). Expression of *bsh* increased for 10403S, EGD-e, and HCC23 under anaerobic conditions (P < 0.001). The expression also increased slightly for F2365 under anaerobic conditions, but the change was not significant (P > 0.05). This suggests that the expression of *bsh* alone does not impact the increased resistance observed under anaerobic conditions.

Proteomes vary between strains in response to bile

Since variations were observed in the viability of the four strains

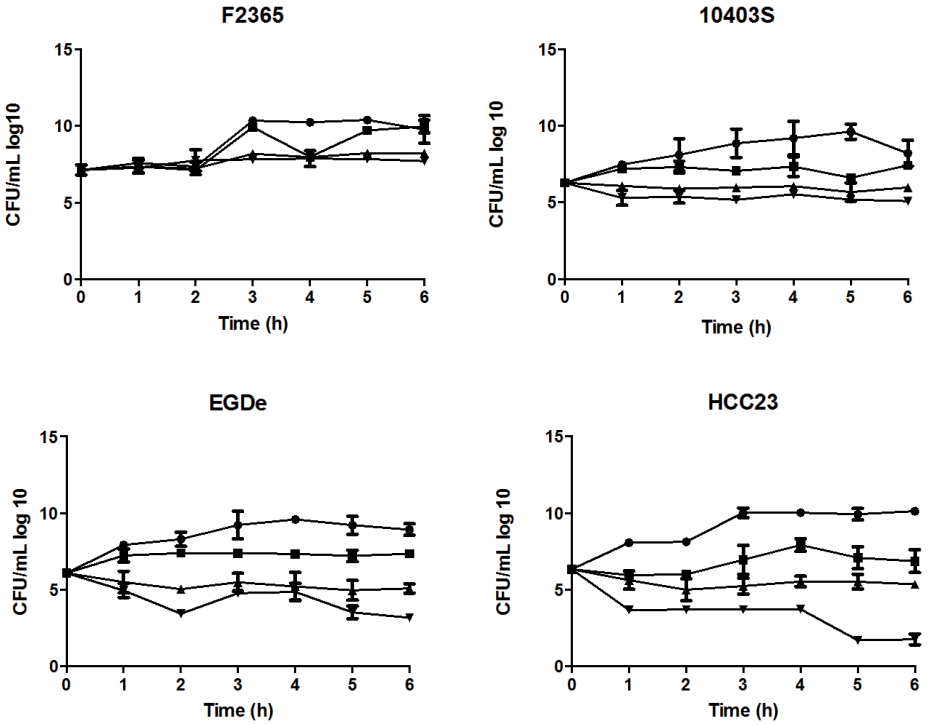

Figure 2: Viability of *Listeria monocytogenes* following exposure to bile under anaerobic conditions.

Viability of strains 10403S, EGD-e, F2365 and HCC23 was determined by viable plate counts following exposure to 0% (●), 1% (■), 5% (▲), or 10% (▼) porcine bile extract under anaerobic conditions. Graphs represent an average of three independent experiments; error bars represent standard deviation.

	0% Bile				1% Bile				5% Bile				10% Bile			
Time	F2365	10403S	EGDe	HCC23	F2365	10403S	EGDe	HCC23	F2365	10403S	EGDe	HCC23	F2365	10403S	EGDe	HCC23
1h	1.000	0.319	1.000	0.950	1.000	0.116	1.000	**0.001**	**0.017**	1.000	0.999	1.000	**< 0.001**	**0.002**	**< 0.001**	1.000
2h	**< 0.001**	0.998	1.000	1.000	0.155	0.245	0.998	**0.001**	1.000	1.000	0.402	1.000	**< 0.001**	**< 0.001**	0.914	1.000
3h	1.000	0.725	0.971	1.000	**< 0.001**	0.690	**0.002**	0.947	0.999	0.995	**0.006**	1.000	**< 0.001**	**0.034**	**< 0.001**	1.000
4h	1.000	1.000	1.000	0.998	1.000	0.126	0.068	1.000	1.000	0.999	0.129	1.000	**< 0.001**	**< 0.001**	**< 0.001**	1.000
5h	1.000	1.000	0.917	1.000	**< 0.001**	1.000	0.080	1.000	0.959	1.000	0.527	0.999	**< 0.001**	0.127	1.000	0.706
6h	1.000	**< 0.001**	0.514	1.000	**< 0.001**	0.172	**0.042**	1.000	0.998	0.999	0.311	1.000	**< 0.001**	0.522	1.000	1.000

Comparisons between aerobic and anaerobic conditions for each strain at each time point revealed significant survival difference between both conditions under various concentrations of bile salt stress

Table 1: Statistical analysis between growth in aerobic and anaerobic conditions.

	Aerobic Fold Change *bsh* (± StDev)	**Anaerobic Fold Change *bsh* (± StDev)**	***P*-value (aerobic v. anaerobic)**
10403S	0.57 (0.05)	4.19 (0.42)	< 0.001
EGD-e	1.50 (0.02)	4.38 (0.39)	< 0.001
F2365	1.22 (0.38)	1.62 (0.15)	0.175
HCC23	1.78 (0.39)	2.65 (0.08)	< 0.001

Table 2: Fold changes in expression of *bsh* in aerobic and anaerobic conditions.

tested that could not be solely attributed to the variations in *bsh* expression, the proteomes of F2365, 10403S, EGD-e, and HCC23 were analyzed following exposure to bile in either aerobic or anaerobic conditions. Strains were treated for 1 h with either 0% or 5% porcine bile extract in aerobic or anaerobic conditions and proteins were isolated from whole cell lysates. Table 3 represents a summary of the number of proteins identified as significantly differentially expressed. Proteomics data quality was assessed by including a randomized version of the protein FASTA database when performing spectrum matching. The maximum FDR reported from these analyses was 0.993%, with a mean FDR of 0.375%.

Proteins identified were analyzed by KOBAS to identify whether trends were evident in expression of specific pathways. Table 4 represents a summary of the pathways that were significantly differentially expressed following exposure to bile under either aerobic or anaerobic pathways. Alterations in purine metabolism were identified in all four strains analyzed. 10403S, F2365, and HCC23 all had an alteration in pyruvate metabolism under anaerobic conditions in the presence of bile; EGD-e only had an alteration in this pathway under aerobic conditions.

Proteins associated with cell envelope and cellular processes are differentially expressed under aerobic and anaerobic conditions upon exposure to bile

Proteomic analyses revealed a change in the expression of proteins

	Aerobic proteins			Anaerobic proteins		
Strain	**Total identified**	**1 h sig different**	**1 h bile sig different**	**Total identified**	**1 h sig different**	**1 h bile sig different**
10403S	637	179	234	623	123	341
EGD-e	628	117	313	587	126	309
F2365	620	127	323	612	136	363
HCC23	634	112	268	651	213	367

Table 3: Summary of proteins identified by mass spectrometry.

KEGG pathway name	Background number	Aerobic				Anaerobic			
		1 h sig different		1 h bile sig different		1 h sig different		1 h bile sig different	
		n[a]	*P* value	n[a]	*P* value	n[a]	*P* value	n[a]	*P* value
10403S									
Aminoacyl-tRNA biosynthesis	25	8	0.004	10	0.002	-	-	23	<0.001
D-Alanine metabolism	5	-	-	3	0.041	-	-	-	-
Nucleotide excision repair	7	3	0.043	-	-	-	-	-	-
Peptidoglycan biosynthesis	15	-	-	5	0.048	-	-	-	-
Purine metabolism	54	-	-	-	-	-	-	23	0.012
Pyrimidine metabolism	45	-	-	-	-	-	-	22	0.004
Pyruvate metabolism	33	-	-	-	-	-	-	14	0.046
Ribosome	57	-	-	13	0.025	-	-	24	0.012
RNA polymerase	5	-	-	-	-	-	-	5	0.026
Streptomycin biosynthesis	7	3	0.043	4	0.021	-	-	6	0.023
EGD-e									
Aminoacyl-tRNA biosynthesis	25	-	-	24	0.001	6	0.006	18	< 0.001
Citrate cycle (TCA cycle)	11	-	-	10	0.031	-	-	-	-
DNA replication	16	-	-	-	-	4	0.021	-	-
Homologous recombination	20	-	-	-	-	4	0.040	-	-
Mismatch repair	19	-	-	-	-	4	0.035	-	-
Purine metabolism	53	-	-	30	0.033	-	-	20	0.004
Pyrimidine metabolism	44	7	0.018	26	0.032	-	-	18	0.003
Pyruvate metabolism	33	-	-	22	0.020	-	-	-	-
Ribosome	57	-	-	49	<0.001	-	-	19	0.013
RNA degradation	14	-	-	11	0.044	4	0.015	-	-
RNA polymerase	5	-	-	-	-	-	-	4	0.033
Streptomycin biosynthesis	7	-	-	-	-	-	-	5	0.023
F2365									
Aminoacyl-tRNA biosynthesis	25	-	-	22	< 0.001	-	-	20	< 0.001
Butanoate metabolism	15	4	0.042	-	-	-	-	-	-
D-Alanine metabolism	5	3	0.015	-	-	3	0.010	5	0.035
Fatty acid biosynthesis	14	-	-	-	-	-	-	9	0.030
Fatty acid metabolism	15	-	-	-	-	-	-	10	0.019
Glycine, serine and threonine metabolism	31	6	0.045	-	-	6	0.022	-	-
Lysine biosynthesis	16	-	-	-	-	4	0.029	-	-
Purine metabolism	53	-	-	23	0.019	-	-	23	0.026
Pyrimidine metabolism	44	-	-	20	0.020	-	-	22	0.009
Pyruvate metabolism	33	7	0.021	-	-	-	-	17	0.017
Ribosome	57	-	-	23	0.035	-	-	23	0.045
RNA polymerase	5	-	-	5	0.032	-	-	5	0.035
HCC23									
Aminoacyl-tRNA biosynthesis	25	-	-	12	< 0.001	-	-	21	< 0.001
D-Alanine metabolism	5	-	-	3	0.036	-	-	4	0.031
Fatty acid metabolism	15	-	-	-	-	-	-	7	0.033
Glyoxylate and dicarboxylate metabolism	10	-	-	4	0.041	-	-	-	-
Lysine biosynthesis	16	-	-	-	-	5	0.021	-	-
Nucleotide excision repair	7	-	-	5	0.004	-	-	-	-
One carbon pool by folate	10	-	-	-	-	4	0.020	6	0.021
Purine metabolism	53	-	-	-	-	-	-	18	0.012
Pyruvate metabolism	33	-	-	-	-	7	0.034	11	0.049
Ribosome	54	-	-	-	-	-	-	16	0.043
RNA polymerase	5	-	-	-	-	-	-	4	0.031
Valine, leucine and isoleucine degradation	10	-	-	4	0.041	4	0.020	-	-

[a]Number of proteins in the input file associated with each pathway

Table 4: Significant pathways identified using KOBAS in significantly differentially expressed protein datasets.

involved in redox reactions, invasion, and cell division following bile exposure (Table 5). The MreB protein, which is involved in determining cell shape, decreased upon bile treatment under aerobic and anaerobic conditions for virulent strains 10403S, EGD-e, and F2365 (Table 5). Expression of MreC only decreased following bile exposure under anaerobic conditions; expression increased under aerobic conditions. A decrease in several cell division proteins was detected under both aerobic and anaerobic conditions following a 1h exposure to bile. FtsZ, FtsH, and FtsA decreased in all strains tested following exposure to bile under both aerobic and anaerobic conditions.

Membrane bioenergetics proteins were also differentially expressed. Thioredoxin reductase decreased following exposure to bile under both aerobic and anaerobic conditions for all strains tested (Table 5). NADH dehydrogenase decreased following exposure to bile in both aerobic and anaerobic conditions for 10403S and HCC23. Several proteins related to invasion were differentially expressed following bile treatment under aerobic and anaerobic conditions. Flavocytochrome c, which is involved in intracellular survival [25], increased after exposure to bile in HCC23 under aerobic conditions.

Additional cell envelope associated proteins detected that are worth noting include several flagellar associated proteins and cell wall anchor proteins. Expression of the flagellar motor protein and flagellin increased in HCC23 under aerobic conditions following exposure to bile. Flagellin also increased in F2365 under aerobic conditions following bile treatment. A cell wall anchor protein increased in expression under aerobic conditions only in F2365. Lipoproteins were increased in expression in HCC23 following exposure to bile under both aerobic and anaerobic conditions. However, the lipoprotein adhesin B increased following exposure to bile only under aerobic conditions in HCC23.

Morphological changes occur following exposure to bile under aerobic, but not anaerobic conditions

Proteomic analysis revealed changes in expression of cell shape determining proteins and proteins involved in cell division, including FtsZ. Therefore, scanning electron microscopy (SEM) was utilized to observe the morphological changes that occurred between aerobic and anaerobic bile treatment conditions (Figure 3). Under aerobic conditions, an increase in cell length and width was observed in 10403S, EGD-e, and F2355 ($P < 0.001$) following bile exposure. An increase in elongation of HCC23 was seen under aerobic conditions as well ($P = 0.04$), which correlated with the increase in MreC that was observed by the proteomic analysis. In contrast, under anaerobic conditions, the only change in length was observed in EGD-e ($P = 0.003$). Changes in width were only observed in 10403S and HCC23 ($P < 0.001$).

Proteins associated with metabolism are differentially expressed under aerobic and anaerobic conditions upon exposure to bile

		Aerobic		Anaerobic		Aerobic	Anaerobic
Protein	**ListiList**	**0%**	**5%**	**0%**	**5%**	**0% vs. 5%**	**0% vs. 5%**
10403S							
MreB	1.1	3.203	0.199	0.582	0.001	Down	Down
Peptide/nickel transport, ATP binding	1.2	0.001	0.976	6.666	3.459	Up	Down
PTS mannose-specific, factor IIAB	1.2	1.589	0.168	0.904	0.001	Down	Down
NADH dehydrogenase	1.4	2.980	1.001	1.652	0.001	Down	Down
Thioredoxin	1.4	3.689	2.177	1.899	0.001	Down	Down
Cell division protein FtsZ	1.7	1.558	0.293	3.718	0.259	Down	Down
EGD-e							
MreB	1.1	3.247	0.464	3.015	0.479	Down	Down
ATP synthase, epsilon chain	1.4	0.001	0.585	0.840	0.001	Up	Down
Thioredoxin reductase	1.4	1.939	0.001	2.045	0.001	Down	Down
NADPH dehydrogenase	1.4	0.574	0.001	-	-	Down	-
Cell division protein FtsZ	1.7	2.090	0.001	2.980	0.001	Down	Down
F2365							
MreB	1.1	3.497	0.001	3.528	0.001	Down	Down
PTS mannose-specific, factor IIAB	1.2	1.798	0.001	2.011	0.001	Down	Down
Lipoprotein	1.2	0.410	0.001	0.698	0.001	Down	Down
Thioredoxin reductase	1.4	2.1799	0.001	1.240	0.001	Down	Down
Flagellin	1.5	2.577	5.677	-	-	Up	-
FtsZ	1.7	2.114	0.001	2.540	0.001	Down	Down
Cell wall surface anchor family protein	1.8	0.003	1.802	0.161	0.001	Up	Down
HCC23							
MreC	1.1	0.109	0.593	0.511	0.001	Up	Down
Manganese ABC transporter protein	1.2	0.001	1.550	1.659	0.673	Up	Down
PTS mannose-specific, factor IIAB	1.2	0.769	0.328	2.446	0.001	Down	Down
Lipoprotein	1.2	0.001	0.380	0.001	0.482	Up	Up
PEP phosphotransferase	1.2	5.996	8.088	6.850	4.999	Up	Down
Thioredoxin reductase	1.4	1.929	0.001	1.755	0.001	Down	Down
ATP synthase epsilon chain	1.4	0.001	0.594	-	-	Up	-
NADH dehydrogenase	1.4	3.306	1.380	2.933	0.001	Down	Down
Flagellin	1.5	11.901	19.518	-	-	Up	-
FtsZ	1.7	1.768	0.001	2.877	0.001	Down	Down

Table 5: Select proteins associated with the cell envelope with a significant change in expression following bile exposure under aerobic and anaerobic conditions.

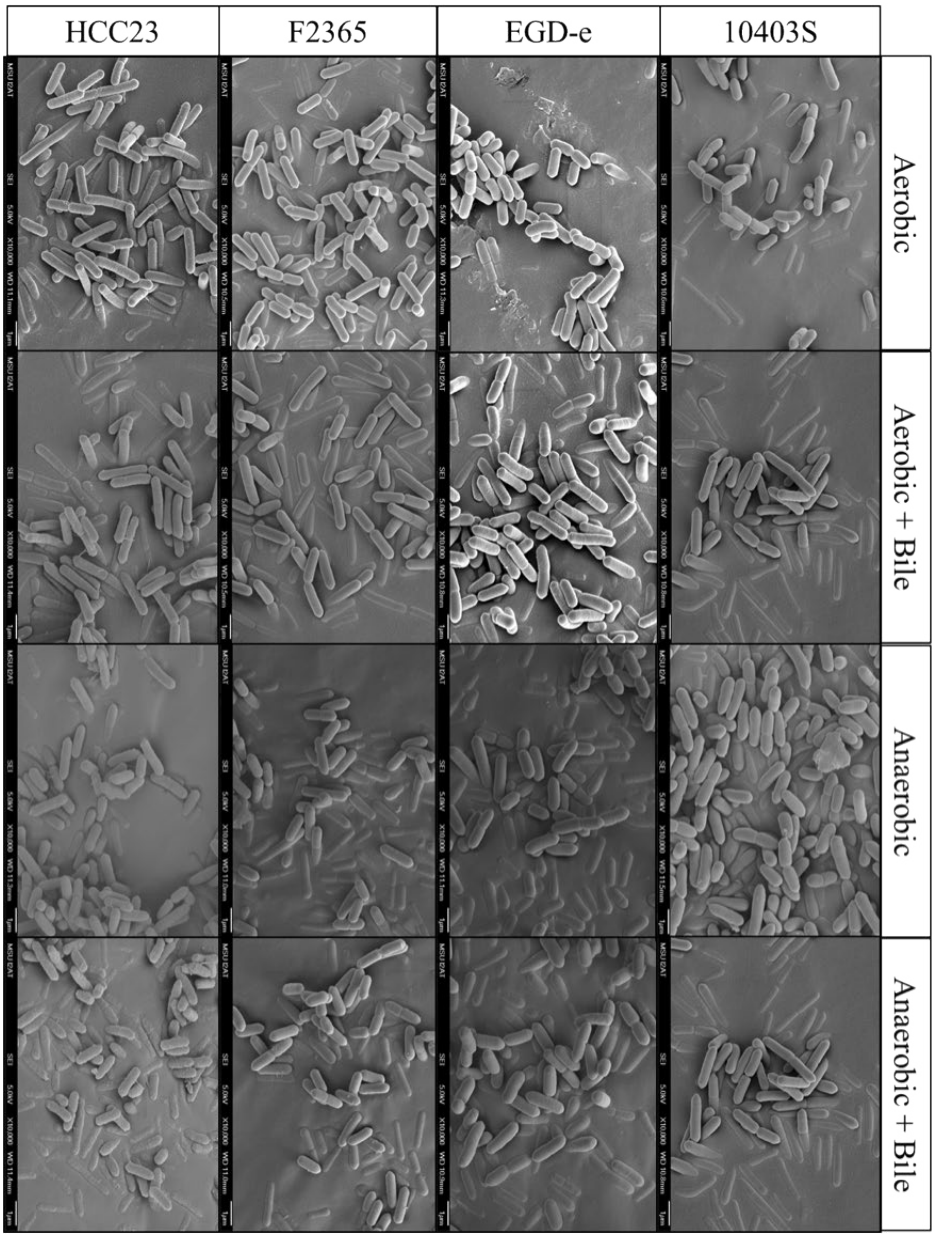

Figure 3A: Morphological changes following exposure to bile.

10403S, EGD-e, F2365 and HCC23 treated with 5% porcine bile extract for 1 h under aerobic or anaerobic conditions were analyzed using a JOEL field emission scanning electron microscope. (A) Images represent the predominant morphology of the representative strain under the condition tested (n = 30).

Many proteins involved in metabolism were differentially expressed following bile treatment under aerobic and anaerobic conditions (Table 6). Enzymes involved in the entrance into the TCA cycle were differentially expressed between aerobic and anaerobic conditions. For instance, pyruvate dehydrogenase, which is involved in the decarboxylation of pyruvate into acetyl CoA, decreased following bile exposure in both 10403S and F2365. However, expression of pyruvate dehydrogenase increased in HCC23 following bile exposure in aerobic conditions. The expression of pyruvate carboxylase, which converts pyruvate to oxaloacetate, decreased following bile exposure in aerobic conditions for HCC23, 10403S, and F2365. Tagatose-diphosphate aldolase, which is involved in galactose metabolism, decreased in expression for 10403S, F2365, and HCC23 under aerobic and anaerobic conditions. Several dehydrogenases were increased in expression in HCC23 following exposure to bile under aerobic conditions, including aspartate dehydrogenase, alanine dehydrogenase, glutamate dehydrogenase, and naloxone dehydrogenase.

The osmotic stress response protein alanine dehydrogenase had altered expression following bile exposure in either aerobic or anaerobic conditions. Expression increased in HCC23 and 10403S following exposure to bile under aerobic conditions. Interestingly, expression decreased in F2365 and EGD-e following bile exposure under aerobic

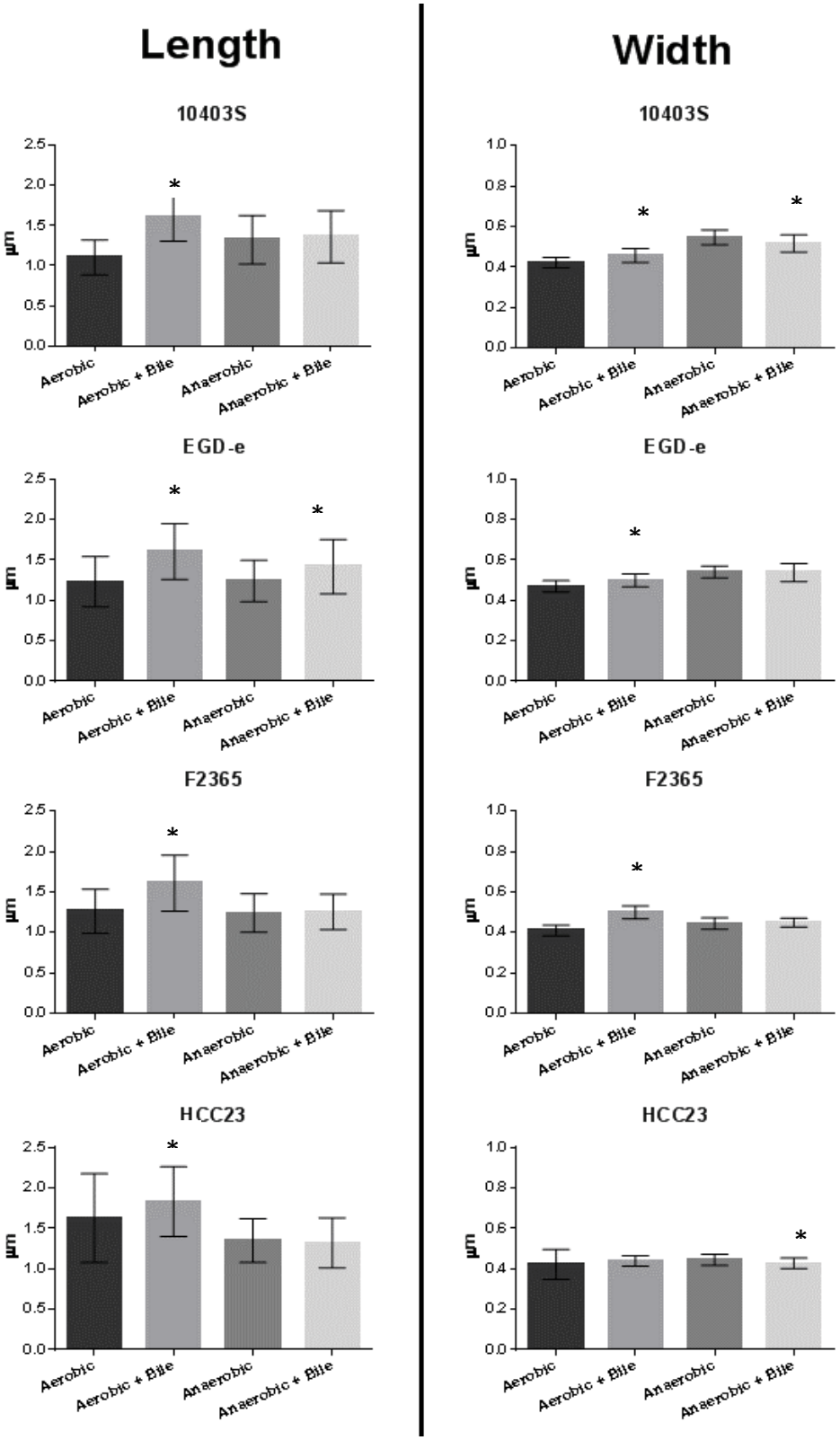

Figure 3B: Morphological changes following exposure to bile.

(B) Following exposure to bile under aerobic conditions, elongation was observed in all strains; this was not observed anaerobically. Error bars indicate standard deviation. * Indicates P-value < 0.05.

		Aerobic		Anaerobic		Aerobic	Anaerobic
Protein	**ListiList**	**0%**	**5%**	**0%**	**5%**	**0% vs. 5%**	**0% vs. 5%**
10403S							
Pyruvate carboxylase	2.1	1.430	0.001	2.444	1.086	Down	Down
Ribulose P 3- epimerase	2.1	0.456	0.992	2.617	0.001	Up	Down
Transketolase	2.1	6.016	1.904	-	-	Down	-
Tagatose 1,6-P aldolase	2.1	2.264	0.805	1.719	0.001	Down	Down
Alanine dehydrogenase	2.2	0.624	2.214	1.521	0.301	Up	Down
Adenine phosphoribosyltransferase	2.3	0.768	2.639	0.285	0.001	Up	Down
Purine nucleoside phosphorylase	2.3	1.866	2.915	2.199	0.459	Up	Down
EGD-e							
Tagatose-6-P kinase	2.1	-	-	2.079	0.001	-	Down
Pyruvate carboxylase	2.1	2.616	0.001	3.757	0.001	Down	Down
Transketolase	2.1	5.709	0.479	2.838	0.001	Down	Down
Adenine phosphoribosyltransferase	2.3	0.001	1.132	-	-	Up	-
Ferrochelatase	2.5	0.805	0.001	0.974	0.001	Down	Down
F2365							
Tagatose 1,6-P aldolase	2.1	2.186	0.001	2.419	0.001	Down	Down
Pyruvate carboxylase	2.1	4.147	0.001	5.992	0.001	Down	Down
Ribulose P-3-epimerase	2.1	0.606	0.001	0.591	0.001	Down	Down
Transketolase	2.1	4.656	0.001	1.719	0.001	Down	Down
Alanine dehydrogenase	2.2	1.525	0.001	1.604	0.001	Down	Down
Aspartate dehydrogenase	2.2	0.001	0.303	0.478	0.001	Up	Down
Dihydroxy-acid dehydratase	2.2	-	-	0.001	0.151	-	Up
Propanediol utilization: diol dehydratase	2.2	0.001	0.652	-	-	Up	-
Purine nucleoside phosphorylase	2.3	0.987	0.134	1.106	0.001	Down	Down
HCC23							
Aldehyde-alcohol dehydrogenase	2.1	11.443	3.889	20.873	5.317	Down	Down
Tagatose 1,6-P aldolase	2.1	3.001	0.475	3.588	0.001	Down	Down
Aspartate dehydrogenase	2.2	0.001	0.579	-	-	Up	-
Alanine dehydrogenase	2.2	0.183	1.807	1.320	0.001	Up	Down
Propanol dehydrogenase	2.2	-	-	0.236	0.001	-	Down
Adenylate kinase	2.3	1.317	2.236	-	-	Up	-
Ferrochelatase	2.5	0.284	0.001	1.036	0.001	Down	Down
Purine nucleoside phosphorylase	2.3	1.904	2.534	2.271	1.248	Up	Down

Table 6: Select proteins associated with intermediary metabolism with a significant change in expression following bile exposure under aerobic and anaerobic conditions.

conditions. Metabolism protein GuaB, an inosine-monophosphate dehydrogenase associated with protection from stress through protein folding, decreased in all strains tested under both aerobic and anaerobic conditions.

Proteins associated with information pathways are differentially expressed under aerobic and anaerobic conditions upon exposure to bile

There were several significant differences in the expression of proteins related to stress responses and repair mechanisms between aerobic and anaerobic conditions (Table 7). Expression of the chaperone proteins DnaK and DnaJ decreased in HCC23, EGD-e, F2365, and 10403S following bile exposure in both aerobic and anaerobic conditions. Multiple proteins involved in the Clp operon, which is transcribed under stress conditions, were found to decrease in expression under both aerobic and anaerobic conditions following bile exposure in EGD-e, 10403S, and F2365. Interestingly, ClpP, which is involved in intracellular replication, increased in expression in HCC23 following bile exposure in aerobic conditions [23].

The nucleotide excision repair protein UvrA decreased following bile exposure in EGD-e, 10403S, and F2365. Expression of RecA decreased following bile treatment in EGD-e, F2365, and HCC23 under both aerobic and anaerobic conditions. Expression of the recombination repair protein RecA increased in 10403S following bile exposure under aerobic conditions. The SigB negative regulator RsbW decreased in all strains tested following bile exposure in aerobic conditions.

OsmC, which is involved in the osmotic stress response, increased in HCC23 and 10403S under aerobic conditions following bile treatment, but not anaerobic conditions. Expression of the pore-forming toxin listeriolysin O decreased following exposure bile salts under anaerobic conditions for F2365 and EGD-e. Catalase, which is involved in degradation of hydrogen peroxide, was detected in all strains. However, expression in catalase decreased following exposure to bile under both aerobic and anaerobic conditions for all strains tested.

Discussion

Bile salts, the bactericidal component of bile, induce oxidative damage in the DNA and membrane [8]. Therefore, the ability of enteric pathogens to colonize the GI tract is dependent on their ability to survive in the presence of bile salts. Though many studies have elucidated the bile salt stress response of Listeria, information is lacking in regards to this response under physiologically relevant anaerobic conditions. A previous study conducted by our group analyzed the anaerobic bile response, but no information is available as to the comparative analysis between bile survival under aerobic and anaerobic conditions [20].

		Aerobic		Anaerobic		Aerobic	Anaerobic
Protein	**ListiList**	**0%**	**5%**	**0%**	**5%**	**0% vs. 5%**	**0% vs. 5%**
10403S							
UvrA	3.2	-	-	1.804	0.001	-	Down
RecA	3.3	1.218	2.417	-	-	Up	-
GTP-sensing repressor CodY	3.5	1.718	0.508	-	-	Down	-
Chaperone DnaK	3.9	24.927	10.697	0.669	0.001	Down	Down
RsbW	4.1	1.992	0.001	-	-	Down	-
Catalase	4.2	3.382	1.008	0.471	0.001	Down	Down
EGD-e							
UvrA	3.2	0.281	0.001	2.731	0.001	Down	Down
RecA	3.3	-	-	2.69	0.001	-	Down
GTP-sensing repressor CodY	3.5	1.882	0.704	2.204	0.001	Down	Down
Chaperone DnaK	3.9	23.248	10.482	15.975	4.779	Down	Down
Chaperone DnaJ	4.1	1.143	0.452	0.835	0.001	Down	Down
RsbW	4.1	1.035	0.001	0.922	0.001	Down	Down
Catalase	4.2	4.856	0.547	4.813	0.537	Down	Down
F2365							
UvrA	3.2	0.653	0.001	1.486	0.001	Down	Down
RecA	3.3	1.506	0.001	1.992	0.001	Down	Down
GTP-sensing repressor CodY	3.5	2.077	0.001	1.951	0.001	Down	Down
Chaperone DnaK	3.9	19.512	6.407	12.607	0.993	Down	Down
Chaperone DnaJ	4.1	0.982	0.001	0.768	0.001	Down	Down
RsbW	4.1	1.920	0.001	2.578	0.001	Down	Down
Glyoxalase	4.2	0.147	3.192	-	-	Up	-
Catalase	4.2	12.697	2.023	6.162	0.001	Down	Down
HCC23							
UvrA	3.2	1.136	0.001	1.503	0.001	Down	Down
GTP-sensing repressor CodY	3.5	1.486	0.547	1.979	0.001	Down	Down
Chaperone DnaK	3.9	22.873	8.949	21.888	6.368	Down	Down
Chaperone DnaJ	4.1	1.638	0.821	1.085	0.821	Down	Down
RsbW	4.1	1.550	0.201	1.146	0.001	Down	Down
Clp protease	4.1	1.701	0.268	0.861	0.001	Down	Down
Catalase	4.2	1.688	0.497	2.857	0.001	Down	Down

Table 7: Select proteins associated with information pathways with a significant change in expression following bile exposure under aerobic and anaerobic conditions.

Therefore, the purpose of this study was to determine if reduced oxygen affects the bile survival of *L. monocytogenes*.

In this study resistance of *L. monocytogenes* to various concentrations of bile salts was shown to be strain specific, as well as influenced by oxygen availability. A significant increase in bile resistance was observed for strains F2365, 10403S, and EGD-e under anaerobic conditions. Interestingly, HCC23 was the only strain tested that had an increase in bile resistance under aerobic conditions. This suggests that the mechanism by which *L. monocytogenes* responds to bile will differ depending upon oxygen availability in a strain dependent manner. This is of particular interest as the gastrointestinal tract is an anaerobic / microaerophilic environment.

Listeria monocytogenes possesses many proteins that assist in invasion and dispersal throughout the body [26]. Even though these bacteria have the ability to evade stressors and the immune system by invading phagocytic and non-phagocytic cells, *L. monocytogenes* has been found to remain extracellular within the gallbladder, where the concentration of bile salts is the greatest [12]. In the current study, lipoproteins involved in the invasion of cells increased upon exposure to porcine bile extract in HCC23, but not other strains tested. As lipoproteins are involved in cell invasion and intracellular survival [27], the differences in expression between strains suggests that the mechanism by which *L. monocytogenes* replicates within the lumen of the gallbladder is mediated by bile exposure [4]. Additionally, exposure to bile has been found to increase biofilm formation [8]. This finding supports previous data reported by our group that indicated variations in biofilm production occurs following exposure to bile [20] and provides additional support that oxygen availability may contribute to the ability of *L. monocytogenes* to invade.

Following bile salt stress, changes in morphology were observed through SEM analysis. Significant elongation and width of *L. monocytogenes* cells were seen following treatment. However, bile treatment under anaerobic conditions resulted in very little morphological changes in comparison to controls. This suggests that the mechanism utilized by *L. monocytogenes* to adequately respond to bile differs based on oxygen availability.

Previous studies have suggested a link between the phosphotransferase system (PTS) and PrfA (virulence regulator), in which PrfA-regulated genes are repressed by sugars such as mannose, glucose, and fructose that are transported through the PTS [28]. Interestingly, PrfA has been shown to interfere with glucose uptake, resulting in a decrease in expression of PTS [29,30]. Expression of PTS

associated proteins, including glucose and mannose-specific systems, decreased following bile exposure. Interestingly, HCC23 exhibited an increase in PrfA under aerobic conditions, but this was not observed in the other strains tested. This suggests that a link between carbon metabolism and PrfA expression may reflect the invasive capability of *L. monocytogenes*. Also, since proteins associated with the PTS system were detected under aerobic conditions, this could provide a way of reducing the activation of virulence genes until appropriate conditions are encountered.

Several studies have demonstrated the importance of repair mechanisms to overcome damage induced by bile salts. In *Salmonella enterica*, bile salts were found to induce oxidative DNA damage primarily in the form of transitions, specifically GC–AT [31]. However, the type of damage induced under anaerobic conditions has not been characterized. To provide an assessment of the response to bile, the stress response proteins expressed by bile resistant strains 10403S, EGD-e, and F2365 and the bile sensitive strain HCC23 were analyzed. HCC23 had several stress response proteins that were differentially expressed in response to bile in comparison to the other strains tested. For instance, OsmC, which is an enzyme involved in osmotic and oxidative stress [32], was increased under aerobic conditions for HCC23 and 10403S and decreased in expression following bile treatment under anaerobic conditions. UvrA, which is associated with DNA repair, decreased following bile exposure in all strains tested. Catalase, which is needed for detoxifying reactive oxygen species that can induce oxidative damage, was detected in all strains tested. However, the expression of this protein decreased following exposure to bile regardless of whether exposure was aerobic or anaerobic. Superoxide dismutase was also detected; the expression increased in 10403S, though never to concentrations detected under aerobic conditions. Recombinational repair protein RecA was also detected in all strains, though expression only increased following bile treatment in 10403S. Together, the increased expression of DNA repair proteins under anaerobic conditions suggests oxygen influences the expression of stress response genes required for DNA repair. However, oxidative damage may be a secondary effect of bile as the increase in expression was not observed in all strains tested. The dependency of repair proteins in bile survival is most likely a multi-faceted mechanism that relies on strain specific mechanisms.

Phosphogluconate dehydrogenase is an enzyme involved in reducing NADP+ through the Pentose Phosphate Pathway. This protein was decreased in all strains analyzed following bile exposure, suggesting that bile shunts metabolism away from this pathway. Therefore, in order to recycle NAD+, the expression of additional dehydrogenases was assessed following bile exposure. HCC23 had an increase in expression of several dehydrogenases, following bile exposure under aerobic conditions. This was interesting, as F2365 and 10403S only had an increase in expression of aspartate dehydrogenase and alanine dehydrogenase, respectively. This could indicate an imbalance of NADH:NAD+ that could impact the membrane integrity and therefore damage induced by bile. Further research is needed to characterize these aspects of *L. monocytogenes* in relation to oxygen availability.

In summary, bile resistance for all strains studied was found to be strain dependent and this resistance is influenced by a reduction in available oxygen. Several changes in expression of proteins associated with the cell morphology, DNA repair, invasion, and metabolism were identified to be involved in the resistance under this stress. Proteins expressed under anaerobic conditions, including virulence factors and the SOS response, suggest that limited oxygen is needed for regulation of these proteins to overcome the damaging affects of bile salts. Oxygen availability influences bile resistance and this may be dependent on regulation of general stress responses by available oxygen. Further research is needed to characterize the mechanisms at which oxygen is detected and how it is used to regulate genes needed for survival under stress.

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Conflict of Interest

The authors have declared no conflict of interest.

References

1. Gellin BG, Broome CV (1989) Listeriosis. JAMA 261: 1313-1320.
2. Gray MJ, Freitag NE, Boor KJ (2006) How the bacterial pathogen *Listeria monocytogenes* mediates the switch from environmental Dr. Jekyll to pathogenic Mr. Hyde. Infect Immun 74: 2505-2512.
3. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, et al. (2011) Foodborne Illness Acquired in the United States-Major Pathogens. Emerg Infect Dis 17: 7-15.
4. Hardy J, Francis KP, DeBoer M, Chu P, Gibbs K, et al. (2004) Extracellular replication of *Listeria monocytogenes* in the murine gall bladder. Science 303: 851-853.
5. O'Driscoll B, Gahan CG, Hill C (1996) Adaptive acid tolerance response in *Listeria monocytogenes*: isolation of an acid-tolerant mutant which demonstrates increased virulence. Appl Environ Microbiol 62: 1693-1698.
6. Vlahcevic ZR, Heuman DM, Hylemon PB (1996) Physiology and pathophysiology of enterohepatic circulation of bile acids. In Hepatology: A Textbook of Liver Disease. 1: 376-417.
7. Gunn JS (2000) Mechanisms of bacterial resistance and response to bile. Microbes Infect 2: 907-913.
8. Begley M, Kerr C, Hill C (2009) Exposure to bile influences biofilm formation by *Listeria monocytogenes*. Gut Pathog 1: 11.
9. Dussurget O, Cabanes D, Dehoux P, Lecuit M, Buchrieser C, et al. (2002) *Listeria monocytogenes* bile salt hydrolase is a PrfA-regulated virulence factor involved in the intestinal and hepatic phases of listeriosis. Mol Microbiol 45: 1095-1106.
10. Begley M, Gahan CG, Hill C (2005) The interaction between bacteria and bile. FEMS Microbiol Rev 29: 625-651.
11. Begley M, Sleator RD, Gahan CG, Hill C (2005) Contribution of three bile-associated loci, bsh, pva, and btlB, to gastrointestinal persistence and bile tolerance of *Listeria monocytogenes*. Infect Immun 73: 894-904.
12. Dowd GC, Joyce SA, Hill C, Gahan CG (2011) Investigation of the mechanisms by which *Listeria monocytogenes* grows in porcine gallbladder bile. Infect Immun 79: 369-379.
13. Sleator RD, Wemekamp-Kamphuis HH, Gahan CG, Abee T, Hill C (2005) A PrfA-regulated bile exclusion system (BilE) is a novel virulence factor in *Listeria monocytogenes*. Mol Microbiol 55: 1183-1195.
14. Buchanan RL, Klawitter LA (1990) Effects of temperature and oxygen on the growth of *Listeria monocytogenes* at pH 4.5. J Food Sci 55: 1754-1756.
15. Jydegaard-Axelsen AM, Hoiby PE, Holmstrom K, Russell N, Knochel S (2004) CO_2- and anaerobiosis-induced changes in physiology and gene expression of different *Listeria monocytogenes* strains. Appl Environ Microbiol 70: 4111-4117.
16. Ramaswamy V, Cresence VM, Rejitha JS, Lekshmi MU, Dharsana KS, et al. (2007) Listeria-review of epidemiology and pathogenesis. J Microbiol Immunol Infect 40: 4-13.
17. Murray EGD, Webb RA, Swann MBR (1926) A disease of rabbit characterised by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus: *Bacterium monocytogenes* (n. sp.). J Pathol Bacteriol 29: 407-439.

18. Linnan MJ, Mascola L, Lou XD, Goulet V, May S, et al. (1988) Epidemic listeriosis associated with Mexican-style cheese. N Engl J Med 319: 823-828.

19. Liu D, Lawrence ML, Ainsworth AJ, Austin FW (2005) Comparative assessment of acid, alkali and salt tolerance in *Listeria monocytogenes* virulent and avirulent strains. FEMS Microbiol Lett 243: 373-378.

20. Payne A, Schmidt TB, Nanduri B, Pendarvis K, Pittman JR, et al. (2013) Proteomic analysis of the response of *Listeria monocytogenes* to bile salts under anaerobic conditions. J Med Microbiol 62: 25-35.

21. Haas W, Faherty BK, Gerber SA, Elias JE, Beausoleil SA, et al. (2006) Optimization and use of peptide mass measurement accuracy in shotgun proteomics. Mol Cell Proteomics 5: 1326-1337.

22. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3: 1101-1108.

23. Gaillot O, Pellegrini E, Bregenholt S, Nair S, Berche P (2000) The ClpP serine protease is essential for the intracellular parasitism and virulence of *Listeria monocytogenes*. Mol Microbiol 35: 1286-1294.

24. Merritt ME, Lawrence AM, Donaldson JR (2010) Comparative Study of the Effect of Bile on the *Listeria monocytogenes* Virulent Strain EGD-e and Avirulent Strain HCC23 Arch. Clinical Micro 1: 4-9.

25. Chatterjee SS, Hossain H, Otten S, Kuenne C, Kuchmina K, et al. (2006) Intracellular gene expression profile of *Listeria monocytogenes*. Infect Immun 74: 1323-1338.

26. Gaillard JL, Berche P, Frehel C, Gouin E, Cossart P (1991) Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. Cell 65: 1127-1141.

27. Machata S, Tchatalbachev S, Mohamed W, Jänsch L, Hain T, et al. (2008) Lipoproteins of *Listeria monocytogenes* are critical for virulence and TLR2-mediated immune activation. J Immunol 181: 2028-2035.

28. Scortti M, Monzó HJ, Lacharme-Lora L, Lewis DA, Vázquez-Boland JA (2007) The PrfA virulence regulon. Microbes Infect 9: 1196-1207.

29. Marr AK, Joseph B, Mertins S, Ecke R, Müller-Altrock S, et al. (2006) Overexpression of PrfA leads to growth inhibition of *Listeria monocytogenes* in glucose-containing culture media by interfering with glucose uptake. J Bacteriol 188: 3887-3901.

30. Stoll R, Mertins S, Joseph B, Müller-Altrock S, Goebel W (2008) Modulation of PrfA activity in *Listeria monocytogenes* upon growth in different culture media. Microbiology 154: 3856-3876.

31. Prieto AI, Ramos-Morales F, Casadesús J (2006) Repair of DNA damage induced by bile salts in *Salmonella enterica*. Genetics 174: 575-584.

32. Atichartpongkul S, Loprasert S, Vattanaviboon P, Whangsuk W, Helmann JD, et al. (2001) Bacterial Ohr and OsmC paralogues define two protein families with distinct functions and patterns of expression. Microbiology 147: 1775-1782.