Transcriptome Analysis of Alkali Shock and Alkali Adaptation in *Listeria monocytogenes* 10403S

Efstathios S. Giotis,^{1,2} Arunachalam Muthaiyan,² Senthil Natesan,³ Brian J. Wilkinson,² Ian S. Blair,¹ and David A. McDowell¹

Abstract

Alkali stress is an important means of inactivating undesirable pathogens in a wide range of situations. Unfortunately, *Listeria monocytogenes* can launch an alkaline tolerance response, significantly increasing persistence of the pathogen in such environments. This study compared transcriptome patterns of alkali and nonalkali-stressed *L. monocytogenes* 10403S cells, to elucidate the mechanisms by which *Listeria* adapts and/or grows during short- or long-term alkali stress. Transcription profiles associated with alkali shock (AS) were obtained by DNA microarray analysis of midexponential cells suspended in pH 9 media for 15, 30, or 60 min. Transcription profiles associated with alkali adaptation (AA) were obtained similarly from cells grown to midexponential phase at pH 9. Comparison of AS and AA transcription profiles with control cell profiles identified a high number of differentially regulated open-reading frames in all tested conditions. Rapid (15 min) changes in expression included upregulation of genes encoding for multiple metabolic pathways (including those associated with Na⁺/H⁺ antiporters), ATP-binding cassette transporters of functional compatible solutes, motility, and virulence-associated genes as well as the σ^B controlled stress resistance network. Slower (30 min and more) responses to AS and adaptation during growth in alkaline conditions (AA) involved a different pattern of changes in mRNA concentrations, and genes involved in proton export.

Introduction

LISTERIA MONOCYTOGENES, a causative agent of both sporadic and epidemic foodborne illness with a high mortality rate (30%), emerged as an important human pathogen in the 1980s (Kathariou, 2002). This organism is resistant to a range of adverse environmental conditions (Farber and Peterkin, 1991) and has been reported to survive and adequately grow at pH values as high as 9 (Cheroutre-Vialette *et al.*, 1998; Vasseur *et al.*, 2001), although it grows best at pH 6–8 (Farber and Peterkin, 1991).

The alkali resistance of *Listeria* is of concern in the food industry where mild alkali treatments are frequently applied in disinfection of food contact surfaces and processing equipment (Vasseur *et al.*, 2001; Taormina and Beuchat, 2002b). Concerns are increased by the ability of *Listeria* to become stress hardened by sublethal concentrations of alkaline detergents, making the pathogen capable of resisting previously lethal alkali-, ethanol-, osmotic-, or thermal-based cleaning procedures (Taormina and Beuchat, 2002a, 2002b; Giotis *et al.*, 2008a), and contributing to reported failures to eradicate *Listeria* from food-

processing environments (Sharma *et al.*, 2003). The ability to resist mild alkaline pH values may also contribute to the pathogenicity of *Listeria* by increasing its survival in alkali conditions in phagolysosomes (Segal *et al.*, 1981) and pancreatic secretions (Maurer *et al.*, 2005).

Scanning electron microscopy studies revealed that exposure of *L. monocytogenes* to sublethal alkaline stress induced statistically significant changes in mean cell length, radius and volume, and single filamentous or elongated chain forms that may be associated with increased survival of *Listeria* in adverse environments and may also contribute to qualitative and quantitative underestimation of this important pathogen in food-processing environments, with potential implications in public health (Giotis *et al.*, 2007a, 2009). Also, in alkaline conditions, *L. monocytogenes* cells develop higher proportions of branched-chain fatty acids, including more *anteiso* forms that are important in adaptation to adverse pH (Giotis *et al.*, 2007b).

Bacteria have developed many sophisticated strategies to resist alkali damage and maintain cytoplasmic pH at optimal values. These include four broad categories (Padan *et al.*,

¹Food Microbiology Research Group, School of Health Sciences, University of Ulster, Northern Ireland, United Kingdom.

²Microbiology Group, Department of Biological Sciences, Illinois State University, Normal, Illinois.

³Department of Pharmaceutical Sciences, Albany College of Pharmacy and Health Sciences, Colchester, Vermont.

2005): (1) increased metabolic production of acids through deamination of amino acids and fermentation of sugars, (2) increased activity of ATP synthase, which couples H^+ entry to ATP generation, (3) changes in cell surface properties, and (4) increased expression and activity of the monovalent cation/proton antiporters. A recent study has shown strong evidence that *Listeria* cells stressed for 1 h at pH 9.5 resulted to gene expression changes related to a minimization of excess alkalization and energy expenditures and a simultaneous recruitment of available carbon sources (Giotis *et al.*, 2008b). However, relatively little is known about the exact mechanisms by which *Listeria* survives levels of alkaline pH stress that inactivate other bacteria. Even less is known about the details of the timescales and processes used in dealing with short-term, abrupt changes to high pH conditions (alkali shock [AS]) and the timescales/processes involved in longer-term acclimatization to high pH conditions (alkali adaptation [AA]). This study applied global transcriptional profiling using DNA microarrays to identify genes and protein systems involved in the alkali tolerance responses of *Listeria* during (1) AS of mid exponential cells recovered from neutral medium (pH 7) and resuspended into pH 9 media or (2) AA of cells grown to midexponential phase in pH 9 media.

Materials and Methods

Culture media

Tris-buffers pH 7 and 9 were prepared by mixing appropriate amounts of 0.1 M solutions of Trizma-hydrochloride and Trizma-base (Sigma). Brain heart infusion broth base (BHI; Difco Laboratories) was prepared and sterilized as per the manufacturer's instructions, substituting distilled water with Tris-buffer, to form a suitably pH stable complex culture medium (Butler *et al.*, 2001). The resultant modified BHI (MBHI) preparations were confirmed as pH 7 or 9 before use. Preliminary investigations established this medium as maintaining the pH ± 0.3 pH units, during the course of the study (data not shown).

Bacterial strains and growth conditions

Overnight cultures of *Listeria* 10403S (serotype 1/2a) (Edman *et al.*, 1968) were inoculated (1:100) into 20 mL of BHI (pH 7) in a 50 mL Erlenmeyer flask, and incubated at 30°C with shaking at 200 rpm. Growth was monitored by measuring OD₆₀₀, using a Beckman DU-65 spectrophotometer.

Cells to be subjected to AS were harvested by centrifugation (8000 g, 10 min) from 20 mL of this culture when the optical density at 600 nm reached 0.4. Recovered cells were washed, and immediately resuspended in 20 mL prewarmed (30°C) pH 9 MBHI (test) or neutral MBHI (pH 7 control) for 15, 30, or 60 min.

Cells to be examined in terms of AA were produced by inoculation of the overnight culture (1:100) into 20 mL of MBHI adjusted at pH 9 (test) or pH 7 (control), and incubation at 30°C until they reached OD₆₀₀ 0.4.

Cells from AS and AA studies were recovered and examined as described below.

Microarrays experiment

RNA was extracted from AS and AA samples using the Qiagen RNeasy mini kit (Qiagen) and purified as previously

described (Giotis *et al.*, 2008b). Briefly, 5 mL of culture was mixed with bacterial RNA protect solution (Qiagen) to stabilize the transcripts. The mixture was then centrifuged to collect the cells. Pellets were resuspended in 1 mL of Trizol (Invitrogen) and cells were disrupted using the FastPrep system (Qbiogene) at speed 6.0 for 40 sec. From the broken cell lysate, RNA was extracted as per the manufacturer's instructions. Residual DNA fragments were removed by DNase (Ambion) treatment, and extracted RNA was purified using the RNeasy mini kit (Qiagen). cDNA was synthesized from the purified RNA by the method described in Pathogen Functional Genomics Resource Center's microarray protocols (<http://pfgrc.jcvi.org/index.php/microarray/protocols.html>). In this process 5 µg of the purified RNA was annealed with random hexamers as primers (Invitrogen) (70°C/5 min and snap frozen in ice/1 min), and extended overnight at 42°C with SuperScript II reverse transcriptase (Invitrogen) in the presence of 0.1 M dithiothreitol 12.5 mM DNTP/aa-UTP (Ambion) mix. Residual RNA was removed and cDNA was purified with a QIAquick PCR purification kit (Qiagen). Purified aminoallyl-modified cDNA was then recovered, labeled with Cy3 or Cy5 monofunctional NHS ester cyanogen dyes (Amersham Pharmacia Biotech), and purified, using Qiagen PCR purification kit following the manufacturer's instructions. Three independent RNA batches from each condition were isolated and analyzed on the same day, and each RNA preparation was used to make probes for at least two-separate arrays with reversal of incorporated dye (Cy3/Cy5). Purified labeled *Listeria* cDNA from alkali-treated and control samples were hybridized on microarray slides (Version 1) consisting of 6347 70-mer oligonucleotides representing open-reading frames (ORFs) from *L. monocytogenes* strains EGD-e, 4b F2365, 1/2a F6854, and 4b H7858, obtained from Pathogen Functional Genomic Research Centre (http://pfgrc.jcvi.org/index.php/microarray/array_description/listeria_monocytogenes/version1.html). Hybridization was done as previously described (Riordan *et al.*, 2007; Giotis *et al.*, 2008b).

Since the *L. monocytogenes* 10403S genome was not represented in the microarray, oligo sequence of the each 70-mer probe present in the array was subjected to similarity search with *L. monocytogenes* 10403S whole-genome shotgun sequence available at www.broadinstitute.org using BLASTN as described by Zhang *et al.* (2000). Two primary selection criteria were used to select the locus IDs for further normalization and statistical analyses: (1) similarity bit score with 130 (i.e., whole 70-mer match), and (2) similarity identity score with 100%. While the majority of the locus IDs used for analyzes were chosen based on 100% similarity, we used minimum cutoff value for the similarity score in the range of 100%–85%. Similarity percentage score for each locus ID that finally passed in both BLAST analysis and statistical analysis is presented in the Supplemental Table S1 (available online at www.liebertonline.com).

Significant changes of gene expression were identified with significance analysis of microarrays software as previously described (Tusher *et al.*, 2001; Giotis *et al.*, 2008b). A *q*-value assigned to each gene corresponds to the lowest false discovery rate at which the gene is called significant. The differentially expressed genes identified by significance analysis of microarrays were further filtered on the basis of previously defined criteria (Gao *et al.*, 2004; Lin *et al.*, 2005). Only

differences of ≥ 2 -fold change in the levels of gene expression, which also had p -values of ≤ 0.05 , were recorded as significant. Several controls were employed to minimize technical and biological variations, and ensure the quality of the data: (1) each ORF was present in duplicate in each array, (2) each RNA preparation was used to make probes for at least two separate arrays with reversal of incorporated dye (Cy3/Cy5), and (3) three independent RNA batches from each condition were analyzed.

Quantitative real-time polymerase chain reaction

Real-time polymerase chain reaction (RT-PCR) was also carried out on the above purified RNA samples (from alkali-treated and control *L. monocytogenes*) to validate the microarray analysis. Primers (Table 1) for three genes identified in the microarray analysis as significant and considered likely to be involved in alkali response processes in *Listeria* were designed with Primer Express 3.0 software (Applied Biosystems) and purchased from Integrated DNA Technologies. Purified RNA samples were further treated with DNAase, using the Turbo DNA-Free kit (Ambion) to remove any residual traces of genomic DNA, and reverse transcribed using M-MLV reverse transcriptase (Promega) and random primers (Roche; 60 min at 37°C) as described in the manufacturer's instructions. The most efficient primer (250 nM) and cDNA template concentrations (100 ng) were determined by combining a range of primer concentrations (50–900 nM) with a range of cDNA templates (10–100 ng). RT-PCR was performed in a 25 μ L reaction volume containing 12.5 μ L 2 $\times$ SYBR PCR Master Mix (Applied Biosystems) and appropriate templates and specific primer pair concentrations for each target gene. Amplification and detection of specific products were performed in the real-time PCR system 7300/7500 (Applied Biosystems), using a cycle profile of one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, 40 cycles at 95°C for 15 sec, and 60°C for 1 min. Each amplification product was evaluated using dissociation curve analysis (Wilhelm *et al.*, 2000; Mackay *et al.*, 2002) to confirm specific and accurate analysis. The quantity of cDNA for each target gene (*opuCA*, *gbuA*, and *lmo2378*) was normalized to the quantity of cDNA from the housekeeping gene 16S rRNA (O'Driscoll 1997). Each gene was subjected to analysis in triplicate. Two-tailed Student's *t*-test was employed to compare quantitative PCR gene expression levels ($p < 0.05$).

Results

The data discussed in this article have been deposited in NCBI's Gene Expression Omnibus (GEO) (Edgar *et al.*, 2002) and are accessible through GEO Series accession number GSE7854 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE7854). Comparison of global gene expression in DNA microarrays from alkali-treated *Listeria* with DNA microarrays from control cultures (pH 7) allowed the identification of 1502 significantly (i.e., up- or downregulated ≥ 2 -fold and q -values of ≤ 0.05) differentially expressed individual ORFs (Supplemental Table S1) that comprise $\sim 24\%$ of the total ORFs tested and exhibit a wide distribution among functional role categories (Fig. 1). Significant quantitative and qualitative differences exist among the transcriptomic profiles at different times of AS and between the differentially expressed genes in AS and AA. Overall, under all alkali exposure conditions

TABLE 1. TRANSCRIPT FOLD CHANGE COMPARISON OF *LISTERIA* 10403S GENES BETWEEN QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION AND MICROARRAY ANALYSIS

Gene	mRNA fold change										Sense primer sequence (5'–3')	Antisense primer sequence (5'–3')
	RT-PCR				Microarrays							
	15	30	60	AA	15	30	60	AA				
<i>lmo2378</i>	1.40	1.56	1.79	2.02	1.77	1.83	2.64	2.73	2.73	TTACGAAATCCGCGCAAGTT	ATGAGGACAATCACGGATGGAGGT	
<i>gbuA</i> (Fraser <i>et al.</i> , 2003)	–8.1	–1.9	–2	2.3	–10	–2.4	1.77	2.77	2.77	GGAAGTCGACTTTGGTAC	GAACCATCACGCATAATC	
<i>OpuCA</i> (Fraser <i>et al.</i> , 2003)	4.12	6.30	2.70	—	4.38	6.91	3.09	—	—	CATCATGGCGGAAGATCC	GCTTCATCCATATCATGG	
16S rRNA (O'Driscoll, 1997)										TTAGCTAGTTGGTAGGT	AATCCGGACAACGCTTGC	

AA, alkali adaptation; RT-PCR, real-time polymerase chain reaction; —, not measured.

FIG. 1. Percentage functional classification of up- and downregulated genes displaying at least twofold increases in mRNA level, classified according to the The Institute for Genomic Research (TIGR) *L. monocytogenes* genome annotation files (www.tigr.org).

examined, more genes from each functional group were up-regulated than downregulated.

ORFs identified as differentially regulated included mainly those coding for transport proteins (11%), energy metabolism proteins (10%), regulatory functions (8%), hypothetical (15%), and unknown function (7%) proteins (Fig. 1).

An important alteration of cellular metabolism genes was indicated by our array analysis (Table 2). Many operons encoding enzymes such as pyruvate dehydrogenase (*pdh*), succinate semialdehyde dehydrogenase (*gab*), phosphoglycerate mutase (*pgam*), and sugar phosphotransferase system (*pts*) were upregulated after 15 min AS. Many of the induced enzymes are associated with pyruvate metabolism, that is, *pdh*, alanine dehydrogenase (*lmo1579*), lactate dehydrogenase (*ldh*), and pyruvate formate lyase (*pfl*). Similarly, upregulation was observed in pathways for the biosynthesis and degradation of valine, leucine, and isoleucine; glycerolipid metabolism (*glpD*, *pfl*, and *lmo1538-a glpK* homolog); and the citrate cycle (*citZ*, *citB*, and *malA*).

At least 179 ORFs coding for transport and binding proteins were differentially regulated in the presence of alkali. These included genes encoding Na⁺/H⁺ antiporters and ATP-binding cassette (ABC) type transporters (Table 3).

The study noted that AS induced the upregulation of a number of recognized general stress proteins (GSPs; e.g., GSP 26), other putative GSPs (encoded by *lmo2673*, *lmo1580*, *lmo1601*, and *lmo2217*), arginase deaminase, and a range of molecular chaperones, including GroES and GroEL, DnaK, HtrA (degP), *lmo211 (cte)*, and ClpC ATPase (Supplemental Table S1).

At least 68 ORFs involved in DNA metabolism and DNA repair systems were differentially regulated after alkali treatment (Supplemental Table S1). Expression of a number of genes involved in cell wall catabolism (*ffh*) and biosynthesis of peptidoglycan (genes of the *mraY* subfamily) and lipoteichoic acid (*dlt*) was significantly altered by AS, as was expression of a wide range of genes coding for membrane proteins. In addition, there was a significant alteration in expression of the *sigB* genes and genes belonging to the *sigB* operon. Other genes showing at least a twofold difference in expression were those involved in detoxification, general regulatory functions, two-component systems, and genes with a putative contribution in cellular adaptation mechanisms in *L. monocytogenes*.

To evaluate the validity of microarray analysis, quantitative RT-PCR was carried out to confirm expression of selected genes *gbuA*, *opuCA*, and *lmo2378*, and the results obtained are presented in Table 1. These show the same patterns of increase or decrease of target genes as observed in the microarray analysis ($P < 0.05$).

Discussion

The 10403S strain (serotype 1/2a) (Edman *et al.*, 1968) is the most widely used in recent investigations of the pathogenesis of *L. monocytogenes*. However, this strain is not represented on the microarray, which raises the possibility of the occurrence of false-positive errors (due to nonspecific hybridization) associated with possible genetic divergence between the strains used in the manufacture of the arrays and test strains. Such

TABLE 2. *LISTERIA* 10403S ENERGY METABOLISM-RELATED GENES' FOLD CHANGE IN RESPONSE TO ALKALI SHOCK AND ALKALI ADAPTATION

Locus ID	Symbol	AS period (min)			AA	Subfunctional category	Gene/protein name
		15	30	60			
LMO2365_0935	<i>gabD</i>	11	6.63	2.19		Amino acids & amines	Succinate-semialdehyde dehydrogenase
<i>lmo1579</i>	NA	2	3.22				Similar to alanine dehydrogenase
<i>lmo2434</i>	<i>GAD</i>	14.2	6.65				Highly similar to glutamate decarboxylases
<i>lmo0043</i>	NA	12.6	5.23				Similar to arginine deiminase
<i>lmo0781</i>	NA	4.72	2.59	2.05		Aromatic amino acid family	Similar to mannose-specific phosphotransferase system (PTS) component IID
<i>lmo1514</i>	NA	3.73	3	3.55		ATP-proton motive force interconversion	Similar to unknown protein
<i>lmo2530</i>	<i>atpG</i>	3.54	2.93	2.48			Highly similar to H ⁺ -transporting ATP synthase chain gamma
<i>lmo2529</i>	<i>atpD</i>		2.39	2.5			Highly similar to H ⁺ -transporting ATP synthase chain beta
LMO6854_0178	NA	12	6.64	3.33		Cations and iron-carrying compounds	Glucose uptake protein
<i>lmo0783</i>	NA	9.81		5.55			Similar to mannose-specific phosphotransferase system (PTS) component IIB
<i>lmo0784</i>	NA	7.21	4.11	5.14			Similar to mannose-specific phosphotransferase system (PTS) component IIA
LMO2365_0799	NA	6.45		4.16			PTS system, mannose/fructose/sorbose family, IIC component
LMO2365_2351	NA	2.79	2.63	3.1	3.44		Putative Na ⁺ /H ⁺ antiporter component A
<i>lmo2384</i>	NA	2.05		4.1	3.55	Fermentation	Similar to proteins involved in resistance to cholate and Na ⁺ , and in pH homeostasis
LMO2365_1426	<i>pflA</i>	10.4					Pyruvate formate-lyase activating enzyme
<i>lmo1406</i>	<i>pflB</i>	8.09					Pyruvate formate-lyase
<i>lmo0210</i>	<i>ldh</i>	2.42		-2		Glycolysis/glyconeogenesis	Similar to L-lactate dehydrogenase
<i>lmo2458</i>	<i>pgk</i>	2.51	2.47	2.13			Highly similar to phosphoglycerate kinase
<i>lmo1567</i>	<i>citZ</i>	2.04	2			TCA cycle	Highly similar to citrate synthase subunit II
<i>lmo1641</i>	<i>citB</i>	2		-2.1			Highly similar to aconitate hydratases
<i>lmo2122</i>	<i>malA</i>	10.4					Similar to maltodextrin utilization protein MalA
<i>lmo1293</i>	<i>glpD</i>	5.35	4.47	3.4		Other	Similar to glycerol 3 phosphate dehydrogenase
<i>lmo1538</i>	NA	3.52	2.55				Similar to glycerol kinase
<i>lmo1407</i>	<i>pflC</i>	8.93				Protein modification & repair	Pyruvate-formate lyase activating enzyme
LMO2365_1073	<i>pdhA</i>	4.62	4.35			Pyruvate dehydrogenase	Pyruvate dehydrogenase complex, E1 component, Pyruvate dehydrogenase alpha subunit
<i>lmo1053</i>	<i>PdhB</i>	4.67	3.1				Highly similar to pyruvate dehydrogenase (E1 beta subunit)
<i>lmo2124</i>	NA	8.21	2.02			Sugars	Similar to maltodextrin ABC transport system (permease)

ABC, ATP-binding cassette; AS, alkali shock, NA, not available.

TABLE 3. *LISTERIA* 10403S TRANSPORTATION AND BINDING PROTEINS' FOLD CHANGE IN RESPONSE TO ALKALI SHOCK AND ALKALI ADAPTATION

Locus ID	Symbol	AS period (min)					Subfunctional category	Gene/protein name
		15	30	60	AA			
<i>lmo2497</i>	NA	20.2	10.5	9.37	5.56	Anions	Similar to phosphate ABC transporter (permease protein)	
<i>lmo1422</i>	NA	2.57	3.75	7.63	4.18	Amino acids,	Similar to glycine betaine/carnitine/choline ABC transporter (membrane protein)	
<i>lmo1421</i>	NA	2.39	8.14	10.5	2.45	peptides and amines	Similar to glycine betaine/carnitine/choline ABC transporter (ATP-binding protein)	
<i>lmo1428</i>	<i>opuCA</i>	4.38	6.91	3.09			Similar to glycine betaine/carnitine/choline ABC transporter (ATP-binding protein)	
<i>LMOJ2365_1446</i>	<i>opuCB</i>	4.41	5.3	3.73	2		Glycine betaine/L-proline ABC transporter, permease protein	
<i>lmo1426</i>	<i>opuCC</i>	4.13	5.47	4.51			Similar to glycine betaine/carnitine/choline ABC transporter (osmoprotectants-binding protein)	
<i>lmo1425</i>	<i>opuCD</i>	2.66	7	5.02			Similar to betaine/carnitine/choline ABC transporter (membrane p)	
<i>lmo1014</i>	<i>gbuA</i>	-10	-2.4		2.77		Highly similar to glycine betaine ABC transporter (ATP-binding protein)	
<i>lmo1015</i>	<i>gbuB</i>	-8.2	-4.5		2.64		Highly similar to glycine betaine ABC transporters (permease)	
<i>lmo1016</i>	<i>gbuC</i>	-13	-4.4		2.97		Highly similar to glycine betaine ABC transporters (glycine betaine-binding protein)	
<i>lmo2499</i>	NA	19.4	6.07	5.07		Anions	Similar to phosphate ABC transporter (binding protein)	
<i>lmo2495</i>	NA	18.3	14.6	4.89			Similar to phosphate ABC transporter (ATP-binding protein)	
<i>LMOJ2365_2470</i>	NA	15.4	14.6	5.29			Phosphate ABC transporter, permease protein	
<i>LMOJ2365_2469</i>	NA	14.9	14.1	8.45			Phosphate ABC transporter, ATP-binding protein	
<i>LMOJ6854_2797</i>	<i>kdpB</i>	-14	-8.3	-2.9		Cations and iron-carrying compounds	K ⁺ -transporting ATPase, B subunit	
<i>LMOJ2365_2660</i>	<i>kdpC</i>	-12	-5.4				K ⁺ -transporting ATPase, C subunit	

potential effects pose less concerns in this study, as, despite the identification of some unique strain-specific and serotype-specific genes, the genomes of *L. monocytogenes* strains have been shown to be remarkably similar in gene content (Nelson *et al.*, 2004; Zaytseva *et al.*, 2007). Nevertheless, those groups of genes that have been shown to be more prone to genetic diversity among strains/serotypes, such as the internalins (Bierne and Cossart, 2007) and other virulence factors (Wiedmann *et al.*, 1997), are not discussed in this study.

The significant number of differentially regulated genes under alkali conditions demonstrates the complexity of the alkali responses in *L. monocytogenes*. The significant differences between transcriptomic profiles in samples corresponding to different durations of alkali treatment also suggest the existence of cascades of responses of *Listeria* to alkali. Overall, the maximum number of altered genes was observed in samples recovered from cells that had been subject to AS for 15 min. This suggests that the largest reorganization of gene expression patterns (and metabolic changes) occurs within a very short time of transfer to alkali conditions. The data obtained from samples that have been subject to AS for longer periods (30 and 60 min) indicate that the cells are beginning to recover from the initial abuse, and move from an emergency response mode into perhaps a more sustainable longer-term pattern of coping with alkali challenge. Similarly, data from AA studies, that is, growth under alkali conditions (pH 9.0), show less comprehensive, but nevertheless still substantial, changes in gene expression patterns. It is not entirely clear why this is so, but it may be related to depletion of nutrients, acidification, or a shift to simpler fermentative metabolism during AA, and return to normal values of expression of genes involved in the initial response to alkali.

This study has also identified a large number of unknown genes that are strongly induced at alkali pH treatment. Additional functional annotation information and subsequent experimental validation is needed to characterize the unknown function transcripts.

Energy and metabolism

The most striking result of the transcriptome analysis was the significant alteration in the scale of expression of carbohydrate metabolism genes in response to AS (Table 2). It appears that short-term AS (15 and 30 min) favors metabolic pathways that lead to a rapid production of acids, perhaps as a means of off-setting the effects of higher pH. Further, induction of genes such as maltodextrin transporter (*lmo2122*, *lmo2123*, *lmo2124*, and *lmo2126*) suggests that initial uptake and processing of certain sugars, such as maltose, are favored under alkali conditions, perhaps because they can be easily and rapidly metabolized (by fermentation) to yield relatively large amounts of acidic products (Maurer *et al.*, 2005). This pattern of expression is quite different from that observed in AA cells, where most carbohydrate metabolism-related genes were expressed at normal levels. AA cells did, however, display increased expression of many members of the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) such as *fruA* (32-fold upregulation) and *fruB* (22.6-fold upregulation), which have active roles in sugar uptake by the cell (Deutscher *et al.*, 2002). The PTS sugar uptake system is incompletely understood in *Listeria* and more

work is needed to clarify the linkages between alkali stress and the PTS control mechanism identified in this study.

The study observed that genes associated with proton and electron transport across cell membranes were upregulated or downregulated in relation to their direction of action, that is, whether they imported or exported H^+ . For example, most of the genes in the *atp* operon, which encodes for F1F0-ATP synthase, acting to import H^+ during oxidative respiration (Cotter *et al.*, 2000; Padan *et al.*, 2005), were strongly upregulated at high pH. Other studies have suggested that *Listeria* (Cotter *et al.*, 2000) is one of a number of bacteria (Maurer *et al.*, 2005) that synthesize ATP using a proton-coupled F1F0-ATP synthase. Other microarray studies have associated ATP synthase with the import of protons in alkali-stressed *Escherichia coli*, *Bacillus subtilis* (Maurer *et al.*, 2005), and in acid-stressed *Listeria* (Cotter *et al.*, 2000). On the other hand, genes such as *Imo2638*, which codes for NADH dehydrogenase, acting to export H^+ , were unchanged or downregulated in the presence of alkali in this study (Supplemental Table S1).

This study observed that AS (15 and 30 min) induced the production of enzymes such as arginine deaminase (*Imo0043*, Table 2). This observation is in agreement with a number of previous reports (Maurer *et al.*, 2005; Padan *et al.*, 2005), which suggests that overexpression of deaminases and other enzymes responsible for the degradation of amino acids may assist in microbial adaptation to high pH. However, it is not yet clear if such processes have their effect(s) in lowering cytoplasmic pH, or lowering external pH (or both). In what might appear to be contrary to the above processes of (producing/excreting) acid products, this study observed different levels of induction of decarboxylases such as the lysine decarboxylase (*Imo2694*) and glutamate decarboxylase (GAD) (*Imo2434*), which produce alkaline end products (Cotter *et al.*, 2005) in AS and AA (Table 2). Induction of the latter (GAD) system is suggested to increase the pH of the immediate cell environment by a number of processes, including the export of γ -aminobutyrate (Conte *et al.*, 2002; Wemekamp-Kamphuis *et al.*, 2004). The GAD system has been extensively reported as a key system in the adaptation of *Listeria* and other pathogens (Olier *et al.*, 2004; Wemekamp-Kamphuis *et al.*, 2004) to acid challenge in the human stomach (Blankenhorn *et al.*, 1999). The upregulation of such systems that are known to increase pH, as observed in this study, and the detection of similar decarboxylases in other alkali-stressed bacteria (Blankenhorn *et al.*, 1999) suggest that they have a more complex role or impact than merely increasing the pH stress being suffered by already alkali-stressed organisms. However, the reasons for upregulation of higher pH inducing genes in cells that are already under alkali challenge remain unclear.

Transport and binding proteins

This study has shown the differential regulation of a large number of transporter genes, including genes encoding transporters of carbohydrates, organic acids, cations, peptides, amino acids, and metals (Table 3). Monovalent cation/proton antiporters and especially Na^+/H^+ antiporters have been implicated in a wide variety of important physiological roles, including export of undesirable (i.e., intracellularly toxic) concentrations of Na^+ , pH homeostasis, osmoregulation, and signaling (Krulwich, 1995). Active Na^+ excretion is essential in bacteria to maintain an appropriate

Na^+ gradient, which serves more widely as the driving force for many transport systems (Ito *et al.*, 2004), including those systems involved in the exchange of H^+ ions. Thus, the observed upregulation of Na^+/H^+ antiporters in *Listeria* is consistent with alkali responses observed in a number of other organisms (Padan *et al.*, 2005).

Alkali conditions (AS and AA) induced differential regulation of many ORFs coding for ABC transporters. These ABC transporters play an important role in various cellular physiological processes (Geourjon *et al.*, 2001), including the uptake of oligopeptides and other solutes during growth under alkaline pH conditions (Padan *et al.*, 2005). Previous studies in a range of organisms have shown that imported peptides can be broken down to free amino acids forming significant sources of protons (Padan *et al.*, 2005), suggesting a possible rationale for the increased uptake of peptides under conditions of alkali stress. Alternatively, rather than acting solely as a source of (amino) acids, such processes may contribute to structural adaptation within alkali-stressed bacteria, in which the derived free acidic amino acids can be incorporated into acidic (alkali resistant) polymers in the bacterial cell wall (Wood *et al.*, 2001; Takami *et al.*, 2002).

The study observed differential expression (mostly upregulation, but also some cases of downregulation) of osmolyte transporter systems responsible for the uptake and accumulation of potent osmolytes such as carnitine and choline (Wood *et al.*, 2001). Upregulation was particularly intense after 15 min exposure to alkali conditions. Such upregulation was also noted, but at lower levels, after 30 and 60 min exposure to alkali conditions. Upregulation of these systems were also noted in AA samples. These osmolyte transporter systems include the glycine betaine porter I (BetL), the glycine betaine porter II (GbuA), the carnitine transporter OpuC, and a putative osmolyte transporter homologous to the choline uptake system OpuB of *B. subtilis* (*Imo1421* and *Imo1422*) (Sleator and Hill, 2005). Upregulation of osmolyte transporter systems has been reported in the adaptation of *Listeria* to adverse osmotic conditions (Wood *et al.*, 2001), leading to the accumulation of the above compounds and maintenance of cell turgor (Booth *et al.*, 1994). Such osmolytes also accumulate under adverse (reduced) temperature conditions (Wood *et al.*, 2001), suggesting that they also have a role in stabilization of cold-sensitive cellular components (Mendum and Smith, 2002).

This study noted major upregulation of the OpuC carnitine transport system in response to AS. This system is encoded by 4 genes: the *opuCA* (coding for an ATPase binding protein), *opuCB*, *opuCD* (coding for two permease subunits), and *opuCC* (coding for a substrate binding protein) (Fraser *et al.*, 2000). Other strongly upregulated transporting systems that do not belong to the *opuC* operon include transcripts from *Imo1421* and *Imo1422*. Interestingly, GbuABC, which mediates the uptake of glycine betaine (Gerhardt *et al.*, 2000), was repressed during AS and highly increased at AA. The reasons for this difference between AA and AS are not clear.

In more general terms, it is not clear how or why alkali stress has such marked effects on systems that are more usually recognized as responses to other stresses, that is, osmotic and low temperature stress. We propose two possible explanations. Increased accumulation of osmolytes may protect the cytoplasmic proteins from alkali damage. Osmolytes have been reported to exert such broader stabilizing effects;

for example, carnitine has been shown to maintain protein configuration and stability and prevent precipitation of stress-damaged proteins (Welsh, 2000). Alternatively, these systems may be induced as Na^+ /solute symporters or antiporters, expressed in response to the presence of toxic levels of Na^+ (from the NaOH) in the cytoplasm, rather than as a direct response to the OH^- alkali ions. This suggestion is supported by reports that osmolytes such as glycine betaine can be co-transported with sodium ion in membrane vesicles in *L. monocytogenes*, in a sodium-driven process (Gerhardt *et al.*, 1996). Such coupling effects among not-specific $\text{Na}^+(\text{K}^+)/\text{H}^+$ antiporters (Hase *et al.*, 2001; Wilson and Ding, 2001) have been reported in the crucial control of Na^+ balance in a wide range of alkalophilic/alkali-tolerant bacteria (Abramson *et al.*, 2004; Beales, 2004). For example, the $\text{Na}^+(\text{K}^+)/\text{H}^+$ antiporter (and to a lesser degree ABC type exporters) is suggested to be the most important system in maintaining pH homeostasis and Na^+ extrusion in *B. subtilis* (Cheng *et al.*, 1997).

In this study, AS repressed the potassium transporting operon *Kdp*. The reasons for this are not clear, but such downregulation may be related to previous reports that K^+ is less effective than (the upregulated) osmolytes in the control of protein integrity and function (Wood *et al.*, 2001; Fujisawa *et al.*, 2004).

Adaptive mechanisms

The study noted significant upregulation of the *sigB* (*lmo0895*) gene in response to AS, but not in response to AA (Supplemental Table S1). The significance of this operon as a central mechanism in the sophisticated adaptive mechanisms to stress has been highlighted in a wide range of bacteria (Karlin *et al.*, 2004; Satorhelyi, 2004). In *L. monocytogenes*, it is involved in resistance against alkali, oxidative, osmotic, heat, bile, and acid stress (Ferreira *et al.*, 2004; Moorhead and Dykes, 2004; Sue *et al.*, 2004; Sleator *et al.*, 2005; Giotis *et al.*, 2008a) and is also important in virulence and the ability to invade host cells (Kim *et al.*, 2005; Rauch *et al.*, 2005; Garner *et al.*, 2006). As might be expected of such an important regulon, *sigB* is itself subject to a complex regulatory system, and this study observed that AS induced significant upregulation of three genes (*lmo0889*, *lmo0891*, and *lmo0892*) that encode for proteins involved in the regulation of *sigB*. AS also upregulated 4 regulatory proteins (*RsbV*, *RsbW*, *RsbX*, and *RsbS*) involved in the posttranslational regulation of the activity of *sigB*, suggesting multilevel modulation of the activities of this key regulon (Supplemental Table S1). Thus, the alkali-induced upregulation of *RsbV* and *RsbW* observed in this study is probably responsible, at least in part, for the observed upregulation of *sigB*, itself the central switch in the rapid activation of a range of mechanisms that allow *Listeria* to survive the alkali stress challenges posed in the human gastrointestinal tract and alkali-based disinfectants used in food-processing systems (Giotis *et al.*, 2008a).

The molecular chaperones that were induced transiently in AS (15 and 30 mins) interact with other proteins to protect them from the damaging effects of environmental stresses, to reverse misfolding (denaturation), and to maintain higher level organization among intracellular proteins (Dobson, 1993). Specific activities have been identified for some molecular chaperones, confirming our previous study (Giotis *et al.*, 2008b), including GroES and GroEL, which have been

shown to be vital in refolding stress-damaged proteins (Liu *et al.*, 2002; Folio *et al.*, 2004), including DnaK (essential for efficient phagocytosis of *Listeria*) (Hanawa *et al.*, 1999) and HtrA (degP), that are necessary for optimal growth under stress conditions of *Listeria* (Wonderling *et al.*, 2004). Some of these molecular chaperones, for example, GroES and GroEL, have cross protective effects against thermal stress (Gahan *et al.*, 2001), suggesting that a possible mechanism underlies the observation that short-term alkali stress induces cross protection to thermal stress in *Listeria* (Taormina and Beuchat, 2002a).

Conclusions

Our data support a model of a dynamic, time-dependent alkali tolerance system in *L. monocytogenes*, involving a large number of genes not previously shown to be involved in the reaction/adaptation of this pathogen to alkaline conditions. Abrupt shifts into alkaline environmental conditions were initially observed to dramatically upregulate multiple genes/operons associated with increasing or maintaining intracellular proton content. The study also revealed additional/alternative longer-term responses to extended exposure to longer-term alkaline stress and gradual AA, which involved a different overall mechanism. Such secondary mechanisms may offer more energy-efficient or sustainable means of maintaining acceptable pH values, after the initial emergency response to sudden onset alkali challenge. AA and AS induced upregulation of many operons involved in virulence and pathogenicity, implying an important role of alkali stimuli at critical stages of the pathogen's infection cycle. The study suggests considerable overlap/interaction between alkaline stress responses and the response of *Listeria* to numerous other adverse conditions, including low pH, low and high temperature, and cell wall and oxidative stress. In particular, systems involved in the acquisition and/or development of intracellular osmolytes appear to play a very active role in response to alkaline stress, perhaps because of their significant capacity as Na^+ symporters and their general protective effects.

The roles, mechanisms of actions, and significance of many of the known and unknown genes upregulated by high pH environments remain to be established in follow-up studies to provide a better understanding of the environmental and clinical significance of the alkali-tolerance response of *L. monocytogenes*. However, such information is likely to allow the development of new insights into and new methodologies for the control of the survival and growth of *Listeria* in human and animal infections, and high-risk food production and processing environments.

Acknowledgments

The authors would particularly like to thank Mudcharee Julotok for her technical advice and practical assistance. This study took place at the Illinois State University in Normal, IL, as part of a collaborative research visit by Efstathios S. Giotis, partly funded by a grant from the European Society of Clinical Microbiology and Infectious Diseases (ECCMID). The work was also supported by a National Research Initiative Competitive Grant (2006-35201-17386) to Prof. B.J. Wilkinson from the U.S. Department of Agriculture. Efstathios Giotis was also supported by a University of Ulster Vice-Chancellor Research

PhD Scholarship. The *L. monocytogenes* microarrays were obtained through NIAID's Pathogen Functional Genomics Resource Center, managed and funded by Division of Microbiology and Infectious Diseases, NIAID, NIH, DHHS and operated by The Institute for Genomic Research (TIGR).

Disclosure Statement

No competing financial interests exist.

References

- Abramson J, Kaback HR, and Iwata S. Structural comparison of lactose permease and the glycerol-3-phosphate antiporter: members of the major facilitator superfamily. *Curr Opin Struct Biol* 2004;14:413–419.
- Beales N. Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: a review. *Compr Rev Food Sci Food Saf* 2004;3:1–20.
- Bierne H and Cossart P. *Listeria monocytogenes* surface proteins: from genome predictions to function. *Microbiol Mol Biol R* 2007;71:377–397.
- Blankenhorn D, Phillips J, and Slonczewski JL. Acid and base induced proteins during aerobic and anaerobic growth of *Escherichia coli* revealed by two-dimensional gel electrophoresis. *J Bacteriol* 1999;181:2209–2216.
- Booth IR, Pourkomaillian B, McLaggan D, and Koo SP. Mechanisms controlling compatible solute accumulation—a consideration of the genetics and physiology of bacterial osmoregulation. *J Food Eng* 1994;22:381–397.
- Butler T, Frenck RW, Johnson RB, and Khakhria R. *In vitro* effects of azithromycin on *Salmonella typhi*: early inhibition by concentrations less than the MIC and reduction of MIC by alkaline pH and small inocula. *J Antimicrob Chemother* 2001;47:455–458.
- Cheng JB, Guffanti AA, and Krulwich TA. A two-gene ABC type transport system that extrudes Na⁺ in *Bacillus subtilis* is induced by ethanol or protonophore. *Mol Microbiol* 1997;23:1107–1120.
- Cheroutre-Vialette M, Lebert I, Hebraud M, Labadie JC, and Lebert A. Effects of pH or a_w stress on growth of *Listeria monocytogenes*. *Int J Food Microbiol* 1998;42:71–77.
- Conte MP, Petrone G, Di Biase AM, Longhi C, Penta M, Tinari A, Superti F, Fabozzi G, Visca P, and Seganti L. Effect of acid adaptation on the fate of *Listeria monocytogenes* in THP-1 human macrophages activated by gamma interferon. *Infect Immun* 2002;70:4369–4378.
- Cotter PD, Gahan CGM, and Hill C. Analysis of the role of the *Listeria monocytogenes* F₀F₁-ATPase operon in the acid tolerance response. *Int J Food Microbiol* 2000;60:137–146.
- Cotter PD, Ryan S, Gahan CGM, and Hill C. Presence of GadD1 glutamate decarboxylase in selected *Listeria monocytogenes* strains is associated with an ability to grow at low pH. *Appl Environ Microbiol* 2005;71:2832–2839.
- Deutscher J, Galinier A, and Martin-Verstraete I. Carbohydrate uptake and metabolism. In: *Bacillus Subtilis and Its Closest Relatives: From Genes to Cells*. Sonenshein AL, Hoch JA, and Losick R (eds.). Washington, DC: ASM Press, 2002, pp. 129–150.
- Dobson CM. Folding and Binding. *Curr Opin Struct Biol* 1993;3:57–59.
- Edgar R, Domrachev M, and Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* 2002;30:207–210.
- Edman DC, Pollock MB, and Hall ER. *Listeria monocytogenes* L forms. I. Induction maintenance, and biological characteristics. *J Bacteriol* 1968;96:352–357.
- Farber JM and Peterkin PI. *Listeria monocytogenes*, a food borne pathogen. *Microbiol Rev* 1991;55:476–511.
- Ferreira A, Gray M, Wiedmann M, and Boor KJ. Comparative genomic analysis of the *sigB* operon in *Listeria monocytogenes* and in other gram-positive bacteria. *Curr Microbiol* 2004;48:39–46.
- Folio P, Chavant P, Chafsey I, Belkorchia A, Chambon C, and Hebraud M. Two-dimensional electrophoresis database of *Listeria monocytogenes* EGDe proteome and proteomic analysis of mid-log and stationary growth phase cells. *Proteomics* 2004;4:3187–3201.
- Fraser KR, Harvie D, Coote PJ, and O'Byrne CP. Identification and characterization of an ATP binding cassette L-carnitine transporter in *Listeria monocytogenes*. *Appl Environ Microbiol* 2000;66:4696–4704.
- Fraser KR, Sue D, Wiedmann M, Boor K, and O'Byrne CP. Role of sigma(B) in regulating the compatible solute uptake systems of *Listeria monocytogenes*: osmotic induction of *opuC* is sigma(B) dependent. *Appl Environ Microbiol* 2003;69:2015–2022.
- Fujisawa M, Wada Y, and Ito M. Modulation of the K⁺ efflux activity of *Bacillus subtilis* YhaU by YhaT and the C-terminal region of YhaS. *FEMS Microbiol Lett* 2004;231:211–217.
- Gahan CGM, O'Mahony J, and Hill C. Characterization of the *groESL* operon in *Listeria monocytogenes*: utilization of two reporter systems (*gfp* and *hly*) for evaluating *in vivo* expression. *Infect Immun* 2001;69:3924–3932.
- Gao H, Wang Y, Liu X, Yan T, Wu L, Alm E, Arkin A, Thompson DK, and Zhou J. Global transcriptome analysis of the heat shock response of *Shewanella oneidensis*. *J Bacteriol* 2004;186:7796–7803.
- Garner MR, Njaa BL, Wiedmann M, and Boor KJ. Sigma B contributes to *Listeria monocytogenes* gastrointestinal infection, but not to systemic spread, in the guinea pig infection model. *Infect Immun* 2006;74:876–886.
- Geourjon C, Orelle C, Steinfels E, Blanchet C, Deleage G, Di Pietro A, and Jault JM. A common mechanism for ATP hydrolysis in ABC transporter and helicase superfamilies. *Trends Biochem Sci* 2001;26:539–544.
- Gerhardt PN, Smith LT, and Smith GM. Sodium-driven, osmotically activated glycine betaine transport in *Listeria monocytogenes* membrane vesicles. *J Bacteriol* 1996;178:6105–6109.
- Gerhardt PNM, Smith LT, and Smith GM. Osmotic and chill activation of glycine betaine porter II in *Listeria monocytogenes* membrane vesicles. *J Bacteriol* 2000;182:2544–2550.
- Giotis ES, Blair IS, and McDowell DA. Effects of short term alkaline adaptation on surface properties of *Listeria monocytogenes* 10403S. *Open Food Sci J* 2009;4:62–65.
- Giotis ES, Blair IS, and McDowell DA. Morphological changes in *Listeria monocytogenes* subjected to sublethal alkaline stress. *Int J Food Microbiol* 2007a;120:250–258.
- Giotis ES, McDowell DA, Blair IS, and Wilkinson BJ. Role of branched-chain fatty acids in pH stress tolerance in *Listeria monocytogenes*. *Appl Environ Microbiol* 2007b;73:997–1001.
- Giotis ES, Mudcharee J, Wilkinson BJ, Blair IS, and McDowell DA. Role of *sigB* factor in the alkaline tolerance response (AITR) of *Listeria monocytogenes* 10403S and cross protection to subsequent ethanol and osmotic stress. *J Food Prot* 2008a;71:1481–1485.
- Giotis ES, Muthaiyan A, Blair IS, Wilkinson BJ, and McDowell DA. Genomic and proteomic analysis of the alkali-tolerance

- response (AITR) in *Listeria monocytogenes* 10403S. BMC Microbiol 2008b;8:38.
- Hanawa T, Fukuda M, Kawakami H, Hirano H, Kamiya S, and Yamamoto T. The *Listeria monocytogenes* DnaK chaperone is required for stress tolerance and efficient phagocytosis with macrophages. Cell Stress Chaperones 1999;4:118–128.
- Hase CC, Fedorova ND, Galperin MY, and Dibrov PA. Sodium ion cycle in bacterial pathogens: evidence from cross genome comparisons. Microbiol Mol Biol Rev 2001;65:353–370.
- Ito M, Xu HX, Guffanti AA, Wei Y, Zvi L, Clapham DE, and Krulwich TA. The voltage-gated Na⁺ channel NavBP has a role in motility, chemotaxis, and pH homeostasis of an alkaliphilic *Bacillus*. Proc Natl Acad Sci USA 2004;101:10566–10571.
- Karlin S, Theriot J, and Mrazek J. Comparative analysis of gene expression among low G-C gram-positive genomes. Proc Natl Acad Sci USA 2004;101:6182–6187.
- Kathariou S. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. J Food Prot 2002;65:1811–1829.
- Kim H, Marquis H, and Boor KJ. sigma(B) contributes to *Listeria monocytogenes* invasion by controlling expression of *inlA* and *inlB*. Microbiology (UK) 2005;151:3215–3222.
- Krulwich TA. Alkaliphiles—basic molecular problems of pH tolerance and bioenergetics. Mol Microbiol 1995;15:403–410.
- Lin TL, Connelly MB, Amolo C, Otani S, and Yaver DS. Global transcriptional response of *Bacillus subtilis* to treatment with subinhibitory concentrations of antibiotics that inhibit protein synthesis. Antimicrob Agents Chemother 2005;49:1915–1926.
- Liu SQ, Graham JE, Bigelow L, Morse PD, and Wilkinson BJ. Identification of *Listeria monocytogenes* genes expressed in response to growth at low temperature. Appl Environ Microbiol 2002;68:1697–1705.
- Mackay IM, Arden KE, and Nitsche A. Real-time PCR in virology. Nucleic Acids Res 2002;30:1292–1305.
- Maurer LM, Yohannes E, Bondurant SS, Radmacher M, and Slonczewski JL. pH regulates genes for flagellar motility, catabolism, and oxidative stress in *Escherichia coli* K-12. J Bacteriol 2005;187:304–319.
- Mendum ML and Smith LT. Gbu glycine betaine porter and carnitine uptake in osmotically stressed *Listeria monocytogenes* cells. Appl Environ Microbiol 2002;68:5647–5655.
- Moorhead SM and Dykes GA. Influence of the *sigB* gene on the cold stress survival and subsequent recovery of two *Listeria monocytogenes* serotypes. Int J Food Microbiol 2004;91:63–72.
- Nelson KE, Fouts DE, Mongodin EF, Ravel J, DeBoy RT, Kolonay JF, Rasko DA, Angiuoli SV, Gill SR, Paulsen IT, Peterson J, White O, Nelson WC, Niernan W, Beanan MJ, Brinkac LM, Daugherty SC, Dodson RJ, Durkin AS, Madupu R, Haft DH, Selengut J, Van Aken S, Khouri H, Fedorova N, Forberger H, Tran B, Kathariou S, Wonderling LD, Uhlich GA, Bayles DO, Luchansky JB, and Fraser CM. 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 2004;32:2386–2395.
- O'Driscoll B. *Characterisation of the Acid Tolerance Response in Listeria monocytogenes*. Galway, Ireland: National University of Ireland, 1997.
- Olier M, Rousseaux S, Piveteau P, Lemaitre JP, Rousset A, and Guzzo J. Screening of glutamate decarboxylase activity and bile salt resistance of human asymptomatic carriage, clinical, food, and environmental isolates of *Listeria monocytogenes*. Int J Food Microbiol 2004;93:87–99.
- Padan E, Bibi E, Ito M, and Krulwich TA. Alkaline pH homeostasis in bacteria: new insights. Biochim Biophys Acta Biomembr 2005;1717:67–88.
- Rauch M, Luo Q, Muller-Altroch S, and Goebel W. SigB dependent *in vitro* transcription of *prfA* and some newly identified genes of *Listeria monocytogenes* whose expression is affected by PrfA *in vivo*. J Bacteriol 2005;187:800–804.
- Riordan JT, Muthaiyan A, Voorhies V, Price W, Christopher T, Graham JE, Wilkinson BJ, and Gustafson JE. Response of *Staphylococcus aureus* to salicylate challenge. J Bacteriol 2007;189:220–227.
- Satorhelyi P. *Microarray Analyse der pH Stressantwort von Listeria monocytogenes und Corynebacterium glutamicum*. München: Technischen Universität Eingereicht und Durch, 2004.
- Segal AW, Geisow M, Garcia R, Harper A, and Miller R. The respiratory burst of phagocytic cells is associated with a rise in vacuolar pH. Nature 1981;290:406–409.
- Sharma M, Taormina PJ, and Beuchat LR. Habituation of food-borne pathogens exposed to extreme pH conditions: genetic basis and implications in foods and food processing environments. Food Sci Technol Res 2003;9:115–127.
- Sleator RD and Hill C. A novel role for the LisRK two-component regulatory system in listerial osmotolerance. Clin Microbiol Infect 2005;11:599–601.
- Sleator RD, Wemekamp-Kamphuis HH, Gahan CGM, Abee T, and Hill C. A PrfA-regulated bile exclusion system (Bile) is a novel virulence factor in *Listeria monocytogenes*. Mol Microbiol 2005;55:1183–1195.
- Sue D, Fink D, Wiedmann M, and Boor KJ. Sigma(B)-dependent gene induction and expression in *Listeria monocytogenes* during osmotic and acid stress conditions simulating the intestinal environment. Microbiology (UK) 2004;150:3843–3855.
- Takami H, Takaki Y, and Uchiyama I. Genome sequence of *Oceanobacillus iheyensis* isolated from the Iheya Ridge and its unexpected adaptive capabilities to extreme environments. Nucleic Acids Res 2002;30:3927–3935.
- Taormina PJ and Beuchat LR. Survival and growth of alkali-stressed *Listeria monocytogenes* on beef frankfurters and thermotolerance in frankfurter exudates. J Food Prot 2002a;65:291–298.
- Taormina PJ and Beuchat LR. Survival of *Listeria monocytogenes* in commercial food-processing equipment cleaning solutions and subsequent sensitivity to sanitizers and heat. J Appl Microbiol 2002b;92:71–80.
- Tusher VG, Tibshirani R, and Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci 2001;98:5116–5121.
- Vasseur C, Rigaud N, Hebraud M, and Labadie J. Combined effects of NaCl, NaOH, and biocides (monolaurin or lauric acid) on inactivation of *Listeria monocytogenes* and *Pseudomonas* spp. J Food Prot 2001;64:1442–1445.
- Welsh DT. Ecological significance of compatible solute accumulation by micro-organisms: from single cells to global climate. FEMS Microbiol Rev 2000;24:263–290.
- Wemekamp-Kamphuis HH, Wouters JA, de Leeuw P, Hain T, Chakraborty T, and Abee T. Identification of sigma factor sigma(B)-controlled genes and their impact on acid stress, high hydrostatic pressure, and freeze survival in *Listeria monocytogenes* EGD-e. Appl Environ Microbiol 2004;70:3457–3466.
- Wiedmann M, Bruce JL, Keating C, Johnson AE, McDonough PL, and Batt CA. Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages

- with differences in pathogenic potential. *Infect Immun* 1997; 65:2707–2716.
- Wilhelm J, Hahn M, and Pingoud A. Influence of DNA target melting behavior on real-time PCR quantification. *Clin Chem* 2000;46:1738–1743.
- Wilson TH and Ding PZ. Sodium substrate cotransport in bacteria. *Biochim Biophys Acta Bioenerg* 2001;1505:121–130.
- Wonderling LD, Wilkinson BJ, and Bayles DO. The *htrA* (*degP*) gene of *Listeria monocytogenes* 10403S is essential for optimal growth under stress conditions. *Appl Environ Microbiol* 2004; 70:1935–1943.
- Wood JM, Bremer E, Csonka LN, Kraemer R, Poolman B, van der Heide T, and Smith LT. Osmosensing and osmoregulatory compatible solute accumulation by bacteria. *Comp Biochem Physiol A Mol Integr Physiol* 2001;130:437–460.
- Zaytseva E, Ermolaeva S, and Somov GP. Low genetic diversity and epidemiological significance of *Listeria monocytogenes* isolated from wild animals in the far east of Russia. *Infect Genet Evol* 2007;7:736–742.
- Zhang Z, Schwartz S, Wagner L, and Miller W. A greedy algorithm for aligning DNA sequences. *J Comput Biol* 2000;7: 203–214.
- Address correspondence to:
David A. McDowell, Ph.D.
Food Microbiology Research Group
School of Health Sciences
University of Ulster
Shore Road Jordanstown
Northern Ireland BT37 0QB
United Kingdom
E-mail: da.mcdowell@ulster.ac.uk