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The heat-shock response of *Listeria monocytogenes* comprises genes involved in heat shock, cell division, cell wall synthesis, and the SOS response

Stijn van der Veen,[1,2,3]† Torsten Hain,[4]† Jeroen A. Wouters,[2] Hamid Hossain,[4] Willem M. de Vos,[1] Tjakko Abee,[1,3] Trinad Chakraborty[4] and Marjon H. J. Wells-Bennik[1,2]

Correspondence
Stijn van der Veen
Stijn.vanderVeen@wur.nl

[1]Wageningen Centre for Food Sciences (WCFS), Diedenweg 20, 6703 GW Wageningen, The Netherlands

[2]Division of Health and Safety, NIZO Food Research, Kernhemseweg 2, 6718 ZB Ede, The Netherlands

[3]Laboratory of Food Microbiology, Wageningen University and Research Centre, Bomenweg 2, 6703 HD Wageningen, The Netherlands

[4]Institute of Medical Microbiology, Justus-Liebig University, Frankfurter Strasse 107, 35392 Giessen, Germany

The food-borne pathogen *Listeria monocytogenes* has the ability to survive extreme environmental conditions due to an extensive interacting network of stress responses. It is able to grow and survive at relatively high temperatures in comparison with other non-sporulating food-borne pathogens. To investigate the heat-shock response of *L. monocytogenes*, whole-genome expression profiles of cells that were grown at 37 °C and exposed to 48 °C were examined using DNA microarrays. Transcription levels were measured over a 40 min period after exposure of the culture to 48 °C and compared with those of unexposed cultures at 37 °C. After 3 min, 25 % of all genes were differentially expressed, while after 40 min only 2 % of all genes showed differential expression, indicative of the transient nature of the heat-shock response. The global transcriptional response was validated by analysing the expression of a set of 13 genes by quantitative PCR. Genes previously identified as part of the class I and class III heat-shock response and the class II stress response showed induction at one or more of the time points investigated. This is believed to be the first study to report that several heat-shock-induced genes are part of the SOS response in *L. monocytogenes*. Furthermore, numerous differentially expressed genes that have roles in the cell division machinery or cell wall synthesis were down-regulated. This expression pattern is in line with the observation that heat shock results in cell elongation and prevention of cell division.

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INTRODUCTION

The food-borne pathogen *Listeria monocytogenes* is a Gram-positive facultative anaerobic rod and the causative agent of listeriosis. Due to the severity of the disease and the fact that its incidence is increasing in numerous European countries, *L. monocytogenes* is of great public health concern (Doorduyn *et al.*, 2006; Health Protection Agency, 2005; Koch & Stark, 2006). This bacterium shows relatively high resistance to environmental insults compared with many other non-spore-forming food-borne pathogens. It is able to grow at a wide pH range (from

†These authors contributed equally to this work.

Abbreviation: Q-PCR, quantitative PCR.

Supplementary tables of the sequences of the Q-PCR primers and the genes that show differential expression at different time points after exposure to a temperature of 48 °C are available with the online version of this paper.

The microarray platform and microarray data discussed in this publication have been deposited in ArrayExpress (http://www.ebi.ac.uk/arrayexpress) under accession numbers A-MEXP-752 and E-MEXP-1118, respectively.

pH 5 to pH 9), at high salt concentrations (up to 12 %), and at a wide range of temperatures (−0.4 to 44 °C) (Kallipolitis & Ingmer, 2001; Karatzas *et al.*, 2005). The ability of *L. monocytogenes* to proliferate under adverse conditions and survive environmental insults is mediated by various mechanisms that allow for rapid responses and adaptations to changing environments. Due to consumers' demands for less heavily preserved foods and more convenience foods, processing conditions in the food industry are becoming milder. *L. monocytogenes* is able to adapt to such milder conditions, making it of major concern to the food industry.

Variation in temperature is a stress that is commonly encountered in nature and during the processing of foods. DNA microarrays provide an excellent tool to study the expression profiles of a complete genome during exposure to heat stress. Previous studies involving transcriptional analysis of the heat-shock response in various bacteria have shown induction of several protection mechanisms, including general protection mechanisms and specific heat-shock responses. The heat-shock response is a common phenomenon among bacteria that enables them to survive a wide variety of stresses, in particular heat stress. Most heat-stress-induced genes encode molecular chaperones or proteases that can either protect other proteins/enzymes against misfolding and damage or mediate degradation when this fails. Maintenance of protein quality is important for normal growth of cells and is essential under stress conditions. In *L. monocytogenes*, two specific heat-shock-response mechanisms and a general stress-response mechanism can be distinguished, namely, the class I and class III heat-shock response, and the class II stress response (Benson & Haldenwang, 1993; Kruger & Hecker, 1998; Schulz & Schumann, 1996). Class I heat-shock genes are controlled by the HrcA repressor, which binds to the CIRCE operator sequence (TTAGCACTC-N_9-GAGTGCTAA) preceding this class of genes. Class I heat-shock genes include *dnaK*, *dnaJ*, *groES* and *groEL*, encoding chaperones. Class III heat-shock genes encode chaperones and ATP-dependent Clp proteases, which degrade damaged or misfolded proteins. This class is regulated by the CtsR repressor, which binds specifically to a heptanucleotide repeat in the promoter region (A/GGTCAAANANA/GGTCAAA). The class II stress genes encode general stress proteins, of which the expression is regulated by the alternative sigma factor SigB. This sigma factor recognizes alternative −35 and −10 sequences (GTTT-$N_{13–17}$-GGGWAT) in the promoter region of the class II stress genes (Kazmierczak *et al.*, 2003).

The complete heat-shock regulon of *L. monocytogenes* that acts in response to a temperature increase has, apparently, not been investigated before, even though heating is an important preservation strategy for the food industry during minimal processing. The aim of this study was to determine the global transcriptional response of *L. monocytogenes* to heat stress. Our data show that exposure to elevated temperatures triggers the classical heat-shock genes, and, in addition, a transient effect on expression of genes involved in the cell replication machinery was observed. Another novel finding is that heat shock triggers the SOS response in *L. monocytogenes*.

METHODS

Strains and sample conditions. *L. monocytogenes* EGD-e (Glaser *et al.*, 2001) was grown in brain heart infusion (BHI) broth (Difco) with shaking (200 r.p.m., New Brunswick C24KC) using 10 ml culture medium in 100 ml conical flasks. An exponentially growing culture was used to inoculate 100 ml fresh pre-warmed BHI broth in a 500 ml flask. This culture was incubated at 37 °C with agitation at 200 r.p.m. until OD_{600} 1.0 was reached. At this point (designated time zero) a 5 ml aliquot was removed for RNA extraction and 10 ml aliquots were transferred to pre-warmed 100 ml flasks at 48 °C. The cultures were incubated in a shaking water bath at 48 °C (GFL type 1083, 60 % shaking speed), and samples for RNA extraction and microscopic analysis were taken after 3, 10, 20 and 40 min.

RNA isolation, labelling, hybridization, imaging and microarray analysis. Samples (0.5 ml) were rapidly removed and diluted in 1.0 ml RNAprotect (Qiagen). After incubation for 5 min at room temperature and centrifugation at 5000 ***g*** for 5 min pellets were stored at −80 °C. Microarray experiments (including microarray generation, total RNA extraction, labelling, hybridization, imaging and microarray analysis) were performed as described in detail previously (Chatterjee *et al.*, 2006). The Significance Analysis of Microarrays (SAM) program was used to analyse the data. The cut-off for significantly differentially expressed genes was set with a q value (false discovery rate) of $\leqslant$1 % and a fold-change of $\geqslant$2. In three independent experiments, the whole-genome expression profiles of cells that had undergone heat shock for 3, 10, 20 and 40 min were compared with those of cells at time zero in a dye-swap hybridization experiment.

Microscopy and image analysis. Samples (1 ml) were removed from cultures (see above) and centrifuged at 5000 ***g*** for 2 min. Cells were dissolved in nigrosin solution (Sigma-Aldrich) and dried on glass slides. Images of the cells were taken at ×100 magnification with a Dialux 20 microscope (Leica). The ImageJ program (http://rsb.info.nih.gov/ij/download.html) was used to analyse the images. The images were loaded in eight-bit type and the threshold was adjusted to black and white. The number of pixels per cell was counted and distribution graphs were constructed in Excel (Microsoft) by analyses of seven images from two cell preparations for each time point.

Quantitative PCR (Q-PCR). Superscript III reverse transcriptase (Invitrogen) was used to synthesize first-strand cDNA using 1 μg DNase-treated total RNA. RNA samples were controlled for DNA contamination by omitting this cDNA synthesis step. Q-PCR reactions were performed using 10 μl 2×Sybr Green PCR Master Mix (Applied Biosystems), 200 nM primers and 1 μl cDNA sample in a 20 μl final volume. For each primer set a standard curve was generated using both genomic DNA and cDNA, and negative control samples using pure water were included. Reactions were run on the 7500 Real-Time PCR system (Applied Biosystems) with an initial step of 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. To verify single-product formation, a dissociation cycle was added. Forward and reverse primers (Supplementary Table S1) were designed with an amplicon length of about 100 bp and a Netprimer rating (http://www.premierbiosoft.com/netprimer) of above 80.

RESULTS

Global gene expression analysis

Whole-genome expression profiles of cells at four time points (3, 10, 20 and 40 min) following the temperature shift from 37 to 48 °C were compared with those from cells harvested prior to the upshift (time zero). In total, 714 genes showed more than twofold differential expression at one or more of the four time points compared with the time zero samples with a q value ⩽1 % (significant values are given in Supplementary Table S2). Of these 714 genes, 427 showed increased expression and 287 showed decreased expression upon heat shock, constituting 15 and 10 % of the total of 2857 genes, respectively. The maximum level of gene induction observed was 50.3-fold (lmo1883), while the maximum level of gene repression was 31.6-fold (lmo0048). Most of the differentially expressed genes showed a transient pattern. The highest number of differentially expressed genes, which account for 24 % of all genes, was observed 3 min after the temperature upshift. After 40 min, only 2 % of all genes were differentially expressed. The differentially expressed genes were grouped into functional classes (Fig. 1). The classes containing the highest numbers of differentially expressed genes were carbohydrate transport and metabolism, amino-acid transport and metabolism, transcription and translation, with 84, 63, 59 and 52 genes, respectively. Most genes involved in carbohydrate transport and metabolism (82 %) and transcription (81 %) showed upregulation, and most genes involved in translation (92 %) showed down-regulation. Numerous genes belonging to the amino-acid transport and metabolism class showed differential expression, both up- and down-regulation (36 and 27 genes, respectively).

Stress response

The *L. monocytogenes* genome encodes different classes of (heat) stress-response genes and general mechanisms to survive a wide variety of environmental insults. During exposure to elevated temperatures these classes of genes show differential expression of a constant or transient nature. The number of differentially expressed genes belonging to different classes that are significantly upregulated and down-regulated at the measured time points is shown in Table 1. A detailed list of the differentially expressed genes belonging to these different classes is presented in Table 2 and described further below.

Stress-response genes

The specific heat-shock regulons, consisting of class I (molecular chaperones) and class III (ATP-dependent proteases) heat-shock genes, were induced during the heat-shock experiments. Genes belonging to the class I heat-shock response [*dnaJ*, *dnaK*, *grpE* and *hrcA* (lmo1472–lmo1475), and *groEL* and *groES* (lmo2068 and lmo2069)] showed between twofold and fourfold higher expression levels. In contrast, the class III heat-shock genes [*ctsR* (lmo0229), lmo0230, lmo0231, *clpC* (lmo0232), *clpE* (lmo0997), lmo1138, *clpY* (lmo1279), *clpB* (lmo2206) and *clpP* (lmo2468)] showed a transient differential expression pattern. Another transiently expressed gene [*htrA* (lmo0292)], encoding a serine protease, showed approximately fivefold higher expression levels (Supplementary Table S2). This protease does not belong to the class III heat-shock genes but to a general group of stress genes. Helmann *et al.* (2001) also detected fivefold induction of two *htrA* paralogues in *Bacillus subtilis*, and designated this general class of heat-shock genes class U.

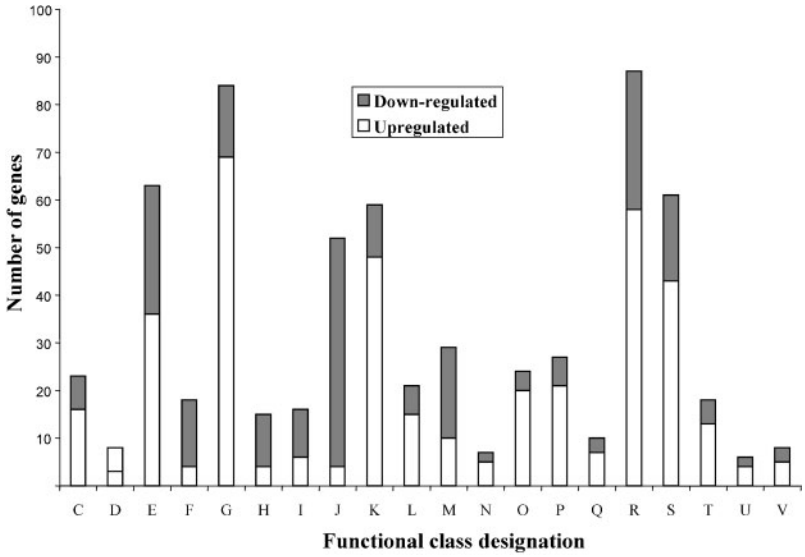

Fig. 1. Differentially expressed genes (fold-change ⩾2, q value ⩽1 %) grouped by functional classification according to the NCBI database (www.ncbi.nlm.nih.gov/COG/). Columns: C, energy production and conversion; D, cell cycle control, mitosis and meiosis; E, amino-acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; J, translation; K, transcription; L, replication, recombination and repair; M, cell wall/membrane biogenesis; N, cell motility; O, post-translational modification, protein turnover and chaperones; P, inorganic ion transport and metabolism; Q, secondary metabolite biosynthesis, transport and catabolism; R, general function prediction only; S, function unknown; T, signal transduction mechanisms; U, intracellular trafficking and secretion; V, defence mechanisms.

Table 1. Numbers of up- and down-regulated genes of *L. monocytogenes* at designated time points after exposure to 48 °C in comparison with time zero expression at 37 °C

Numbers are shown of up- and down-regulated genes that belong to different classes of the (heat) stress response and general mechanisms that are important for surviving a wide variety of environmental insults. Up, upregulated; down, down-regulated.

Response	Time point after exposure to 48 °C							
	3 min		10 min		20 min		40 min	
	Up	Down	Up	Down	Up	Down	Up	Down
Group I heat shock	6	0	2	0	6	0	5	0
Group III heat shock	9	0	4	0	6	0	4	0
Group II stress	54	0	33	0	21	0	5	0
SOS response and DNA repair	7	0	4	0	7	0	8	0
Cell division	0	12	0	2	0	1	0	0
Autolysis and cell wall hydrolases	0	8	0	3	0	3	0	1
Cell wall synthesis	7	7	3	4	3	4	2	4
Cell wall associated	6	2	1	1	3	0	0	0
Virulence associated	3	4	1	3	2	4	2	4

The class II stress response represents a general stress-response mechanism that is regulated by the alternative sigma factor SigB. The gene encoding SigB (lmo0895) did not show more than twofold differential expression on the microarrays during heat shock. Verification of *sigB* expression using Q-PCR showed 1.5-fold upregulation after 3 and 10 min (Fig. 2). In total, 51 genes previously identified as being SigB-regulated (Kazmierczak *et al.*, 2003) showed increased expression at one or more of the selected time points. Among these upregulated genes were *ctc* (lmo0211), lmo1601 and *ydaG* (lmo2748). These genes encode proteins that show sequence similarity with general stress proteins. Increased expression was also observed for the SigB-regulated *opuC* operon (lmo1425–lmo1428). This operon encodes a betaine/carnitine/choline ABC transporter and has previously been shown to be responsible for the accumulation of osmolytes in response to salt, acid and cold stress (Sleator *et al.*, 2001; Wemekamp-Kamphuis *et al.*, 2004). Notably, these osmolytes have also been shown to provide protection during heat exposure of *B. subtilis* (Holtmann & Bremer, 2004) and *Escherichia coli* (Caldas *et al.*, 1999), though no increase in their intracellular concentration was observed.

SOS response and DNA repair

The SOS response is a mechanism involved in the repair of DNA damage and restart of stalled replication forks (Maul & Sutton, 2005) or in the introduction of adaptive point mutations (Michel, 2005; Miller *et al.*, 2004). Numerous genes that are part of the SOS response and the DNA repair machinery showed increased expression after heat exposure. The *recA* (lmo1398) gene, encoding the major activator of the SOS response (RecA), showed a gradual increase in expression over time, from 1.4-fold to 5.2-fold induction between 3 and 40 min. Differential expression of *lexA* (lmo1302), the repressor of the SOS response, was not observed using microarrays, but Q-PCR showed a change from 1.9-fold repression at 3 min to 2.0-fold induction at 40 min (Fig. 2). Transcription of *dinB* (lmo1975) and *umuDC* (lmo2675 and lmo2676), coding for DNA polymerases IV and V, respectively, was also upregulated. These gene products constitute important alternative polymerases in the SOS response. The genes encoding the DNA repair proteins *radA* (lmo0233), *recN* (lmo1368), *uvrA* (lmo2488) and *uvrB* (lmo2489) showed increased expression as well. Two other genes belonging to the SOS response were induced, namely *ynzC* (lmo0496) and *yneA* (lmo1303). The latter is transcribed divergently from *lexA* and the product has been shown to be responsible for cell elongation and suppression of cell division (Kawai *et al.*, 2003). Thus, the expression of *yneA* is consistent with the observation that exposure of *L. monocytogenes* to elevated temperatures results in elongated cells (Fig. 3). After exposure of cells growing at 37 °C to a temperature of 48 °C, the OD_{600} continued to increase (Fig. 3a), whereas the concentration of cells (c.f.u. ml^{-1}) was found to remain constant (Fig. 3b). Cell size measurements of microscopic images showed increased cell size with time after heat exposure compared with cells that were grown continuously at 37 °C (Fig. 3c–e).

Cell division

Cell division is a complicated mechanism involving different pathways that are intertwined. The expression of two genes, lmo2687 and lmo2688, encoding proteins that show sequence similarity to the cell division protein FtsW, was induced after 3 min exposure to 48 °C. Ten other genes involved in cell division showed reduced expression, mainly in the initial phase of the heat shock. Repression of the genes lmo1071, *minDE* (lmo1544 and lmo1545), *mreDCB* (lmo1546–lmo1548), *divIVA* (lmo2020) and *pbpB* (lmo2039), encoding proteins similar to RodA and

Table 2. Differentially expressed genes of *L. monocytogenes* EGD-e at designated time points after exposure to 48 °C in comparison with time zero expression at 37 °C

Significant values (q value ⩽1 % and fold-change ⩾2) are given in bold type.

Gene	Fold-change at time (min)				Gene designation	Description of product
	3	10	20	40		
Class I heat-shock genes						
lmo1472	**2.67**	1.64	**2.15**	**2.50**	*dnaJ*	Heat-shock protein DnaJ
lmo1473	**3.14**	1.94	**2.52**	**2.41**	*dnaK*	Heat-shock protein (molecular chaperone) DnaK
lmo1474	**2.97**	1.48	**2.06**	2.10	*grpE*	Heat-shock protein GrpE
lmo1475	**3.04**	1.81	**2.42**	**2.30**	*hrcA*	Transcription repressor of class I heat-shock genes HrcA
lmo2068	**2.96**	**2.97**	**2.96**	**2.71**	*groEL*	Heat-shock protein (chaperone) GroEL
lmo2069	**3.06**	**2.66**	**3.26**	**2.94**	*groES*	Heat-shock protein (chaperone) GroES
Class III heat-shock genes						
lmo0229	**2.95**	1.02	1.13	1.22		Transcription repressor of class III heat-shock genes CtsR
lmo0230	**4.98**	1.13	1.57	1.56		Similar to *B. subtilis* YacH protein
lmo0231	**8.12**	1.81	**2.27**	1.95		Similar to arginine kinase
lmo0232	**6.86**	1.74	**2.23**	2.14	*clpC*	Endopeptidase Clp ATP-binding chain C
lmo0997	**29.01**	**3.42**	**4.37**	**3.32**	*clpE*	ATP-dependent protease
lmo1138	**7.10**	**2.75**	**3.13**	**2.49**		Similar to ATP-dependent Clp protease
lmo1279	1.97	1.28	1.18	−1.07	*clpY*; *hslU*	Highly similar to ATP-dependent Clp protease-like proteins
lmo2206	**16.12**	**8.20**	**6.42**	**4.00**	*clpB*	Similar to endopeptidase Clp ATP-binding chain B
lmo2468	**6.38**	**4.94**	**4.76**	**3.34**	*clpP*	ATP-dependent Clp protease, proteolytic subunit
Class II stress genes (SigB regulated)						
lmo0200	**6.63**	**4.29**	**5.06**	**4.35**	*prfA*	Listeriolysin positive regulatory protein
lmo0211	**2.32**	1.22	1.50	1.36	*ctc*	Similar to *B. subtilis* general stress protein
lmo0405	**5.69**	1.54	1.16	−1.01		Similar to phosphate transport protein
lmo0593	**2.04**	1.50	−1.04	−1.25		Similar to transport proteins
lmo0669	**5.30**	1.17	1.66	1.30		Similar to oxidoreductase
lmo0670	**6.49**	1.17	1.74	1.23		Hypothetical protein
lmo0781	**5.30**	1.49	1.30	−1.17		Similar to mannose-specific PTS* component IID
lmo0782	**4.83**	1.56	1.33	−1.16		Similar to mannose-specific PTS component IIC
lmo0783	**6.08**	1.83	1.47	−1.24		Similar to mannose-specific PTS component IIB
lmo0794	**11.61**	**2.03**	1.77	1.01		Similar to *B. subtilis* YwnB protein
lmo0880	**8.15**	1.43	1.37	−1.13		Similar to wall-associated-protein precursor (LPXTG motif)
lmo0911	**9.90**	**2.10**	**2.36**	1.20		Hypothetical protein
lmo0956	**7.93**	**3.30**	**4.29**	**2.80**		Similar to *N*-acetylglucosamine-6-phosphate deacetylase
lmo0957	**7.19**	**2.52**	**3.33**	2.41	*nagB*	Similar to glucosamine-6-phosphate isomerase
lmo0958	**5.39**	**2.56**	**3.27**	**2.47**		Similar to transcription regulator (GntR family)
lmo0994	**6.59**	1.59	1.62	−1.11		Hypothetical protein
lmo1421	**3.03**	1.19	1.15	−1.02	*bilEA*	Bile-exclusion system
lmo1425	**5.01**	**2.52**	1.56	−1.34	*opuCD*	Similar to betaine-carnitine-choline ABC transporter (membrane protein)
lmo1426	**5.09**	**2.03**	1.58	−1.27	*opuCC*	Similar to glycine betaine-carnitine-choline ABC transporter (osmoprotectant-binding protein)
lmo1427	**5.54**	1.49	1.35	−1.27	*opuCB*	Similar to glycine betaine-carnitine-choline ABC transporter (membrane protein)
lmo1428	**4.86**	1.44	1.28	−1.36	*opuCA*	Similar to glycine betaine-carnitine-choline ABC transporter (ATP-binding protein)
lmo1433	**4.10**	1.27	1.42	−1.19		Similar to glutathione reductase
lmo1538	**4.93**	1.09	1.12	−1.15	*glpK*	Similar to glycerol kinase
lmo1539	**7.02**	1.70	1.41	−1.04		Similar to glycerol uptake facilitator
lmo1580	**2.85**	−1.01	−1.14	−1.33		Similar to unknown protein
lmo1601	**2.79**	**2.63**	**2.03**	−1.48		Similar to general stress protein

Table 2. cont.

Gene	Fold-change at time (min)				Gene designation	Description of product
	3	10	20	40		
lmo1602	**3.03**	1.94	1.46	−1.38		Similar to unknown protein
lmo1694	**8.96**	**3.48**	2.27	1.18		Similar to CDP-abequose synthase
lmo1883	**50.28**	**2.48**	**3.22**	2.18		Similar to chitinases
lmo2067	**8.22**	**3.22**	**2.88**	1.84		Similar to conjugated bile acid hydrolase
lmo2085	**10.81**	**2.38**	**2.02**	1.43		Putative peptidoglycan-bound protein (LPXTG motif)
lmo2157	**10.54**	**3.76**	**5.04**	1.12	*sepA*	SepA
lmo2205	**11.94**	**7.47**	**5.55**	**3.05**	*gpmA*	Similar to phosphoglyceromutase 1
lmo2230	**10.95**	**5.12**	**4.59**	**2.44**		Similar to arsenate reductase
lmo2269	**2.66**	**2.14**	**2.36**	2.17		Hypothetical protein
lmo2386	**10.34**	**2.02**	1.92	1.42		Similar to *B. subtilis* YuiD protein
lmo2391	**15.53**	**2.35**	1.72	−1.06		Similar to *B. subtilis* conserved hypothetical protein YhfK
lmo2398	**3.93**	1.99	1.48	1.01	*ltrC*	Low-temperature-requirement C protein
lmo2434	**19.43**	**2.79**	**2.24**	1.51		Highly similar to glutamate decarboxylases
lmo2463	**12.75**	**2.89**	1.82	1.19		Similar to transport protein
lmo2484	**11.91**	**2.62**	**2.58**	1.34		Similar to *B. subtilis* YvlD protein
lmo2485	**9.04**	**2.35**	**2.18**	1.36		Similar to *B. subtilis* YvlC protein
lmo2511	**4.39**	1.71	1.27	−1.18		Similar to *B. subtilis* conserved hypothetical protein YvyD
lmo2570	**11.65**	**3.04**	**2.40**	1.41		Hypothetical protein
lmo2571	**12.60**	**3.13**	**2.37**	1.37		Similar to nicotinamidase
lmo2572	**15.24**	**2.86**	**2.07**	1.40		Similar to chain A, dihydrofolate reductase
lmo2573	**15.86**	**3.11**	**2.53**	1.23		Similar to zinc-binding dehydrogenase
lmo2602	**7.66**	**2.16**	1.69	1.14		Conserved hypothetical protein
lmo2673	**12.77**	**3.36**	**2.73**	1.61		Conserved hypothetical protein
lmo2695	**13.84**	**3.91**	2.27	−1.13		Similar to dihydroxyacetone kinase
lmo2696	**13.56**	**3.06**	1.90	−1.11		Similar to hypothetical dihydroxyacetone kinase
lmo2697	**11.82**	**3.00**	2.00	−1.22		Hypothetical protein
lmo2748	**11.87**	**2.34**	1.68	1.07		Similar to *B. subtilis* stress protein YdaG
SOS response and DNA repair						
lmo0233	**3.32**	1.04	1.19	1.29	*radA*; *sms*	Similar to DNA repair protein Sms
lmo0496	**6.51**	**3.58**	**5.04**	**3.36**	*ynzC*	Similar to YnzC of *B. subtilis*
lmo1303	**2.48**	1.27	**3.14**	**5.15**		Similar to *B. subtilis* YneA protein
lmo1368	**2.00**	1.14	1.11	1.10	*recN*	DNA repair and genetic recombination
lmo1398	1.40	**2.29**	**4.47**	**5.23**	*recA*	Recombination protein RecA
lmo1975	**3.99**	**3.15**	**4.53**	**5.52**	*dinB*	DNA polymerase IV
lmo2488	**2.29**	2.15	**2.55**	**4.54**	*uvrA*	Excinuclease ABC (subunit A)
lmo2489	**2.65**	**2.26**	**3.60**	**5.24**	*uvrB*	Excinuclease ABC (subunit B)
lmo2675	1.82	1.38	**2.00**	**2.90**	*umuD*	DNA polymerase V
lmo2676	1.60	1.30	1.41	**2.60**	*umuC*	DNA polymerase V
Cell division						
lmo2687	**3.40**	1.14	1.24	1.50		Similar to cell-division protein FtsW
lmo2688	**3.56**	1.12	1.27	1.59		Similar to cell-division protein FtsW
lmo1071	**−2.09**	−1.17	−1.24	−1.02		Similar to cell-division protein RodA and FtsW
lmo1544	**−2.45**	−1.44	−1.57	−1.65	*minD*	Highly similar to septum placement protein MinD
lmo1545	**−3.25**	−1.80	−1.95	−2.02	*minC*	Similar to septum placement protein MinC
lmo1546	**−3.52**	−1.45	−1.33	−1.58	*mreD*	Similar to cell-shape-determining protein MreD
lmo1547	**−2.98**	−1.55	−1.70	−1.81	*mreC*	Similar to cell-shape-determining protein MreC
lmo1548	**−2.86**	−1.46	−1.75	−1.64	*mreB*	Similar to cell-shape-determining protein MreB
lmo2020	**−4.32**	−1.62	−1.94	−1.88	*divIVA*	Similar to cell-division initiation protein
lmo2039	**−2.36**	−1.23	−1.39	−1.41	*pbpB*	Similar to penicillin-binding protein 2B, cell-division protein FtsI (penicillin-binding protein 3)
lmo2506	**−10.3**	**−2.67**	−2.34	−1.33	*ftsX*	Highly similar to cell-division protein FtsX
lmo2507	**−11.4**	**−2.68**	**−2.30**	−1.35	*ftsE*	Highly similar to cell-division ATP-binding protein FtsE

Table 2. cont.

Gene	Fold-change at time (min)				Gene designation	Description of product
	3	10	20	40		
Autolysins and cell wall hydrolases						
lmo0582	**−3.24**	−2.09	**−3.04**	**−3.15**	*iap*	P60 extracellular protein, invasion-associated protein Iap
lmo1076	**−2.67**	−1.52	−1.67	−1.45	*auto*	Similar to *N*-acetylmuramoyl-L-alanine amidase (autolysin)
lmo1216	**−5.45**	**−2.04**	**−2.06**	−1.50		Similar to *N*-acetylmuramoyl-L-alanine amidase (autolysin)
lmo1855	**−2.39**	−1.48	−1.59	−1.56		Similar to D-alanyl-D-alanine carboxypeptidases
lmo2505	**−8.80**	**−2.95**	−2.63	−1.30	*spl*	Peptidoglycan lytic protein P45
lmo2558	**−2.22**	−1.47	−1.69	−1.45	*ami*	Amidase (autolysin)
lmo2691	**−7.98**	**−2.78**	**−3.15**	−2.29	*murA*	Similar to *N*-acetylmuramidase (autolysin)
lmo2754	**−2.41**	−1.67	−1.96	−1.86		Similar to D-alanyl-D-alanine carboxypeptidase (penicillin-binding protein 5)
Cell wall synthesis						
lmo0877	**2.39**	1.13	1.04	−1.05		Similar to glucosamine-6-phosphate isomerase
lmo0956	**7.93**	**3.30**	**4.29**	**2.80**		Similar to *N*-acetylglucosamine-6-phosphate deacetylase
lmo0957	**7.19**	**2.52**	**3.33**	2.41		Similar to glucosamine-6-phosphate isomerase
lmo0958	**5.39**	**2.56**	**3.27**	**2.47**		Similar to transcription regulator (GntR family)
lmo1998	**3.36**	1.07	−1.02	1.03		Weakly similar to glucosamine-fructose-6-phosphate aminotransferase
lmo1999	**14.38**	1.22	1.39	1.31		Weakly similar to glucosamine-fructose-6-phosphate aminotransferase
lmo2108	**9.88**	1.49	1.86	1.76		Similar to *N*-acetylglucosamine-6-phosphate deacetylase
lmo0971	**−6.84**	**−2.89**	**−4.95**	**−5.02**	*dltD*	DltD protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid
lmo0972	**−6.08**	**−2.15**	**−3.27**	**−3.60**	*dltC*	D-Alanyl carrier protein
lmo0973	**−6.49**	**−1.96**	**−2.81**	**−2.61**	*dltB*	DltB protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid
lmo0974	**−10.1**	**−2.86**	**−4.40**	**−3.77**	*dltA*	D-Alanine-activating enzyme (*dae*), D-alanine-D-alanyl carrier protein ligase (*dcl*)
lmo1075	**−2.15**	−1.37	−1.45	−1.44		Similar to teichoic acid translocation ATP-binding protein TagH (ABC transporter)
lmo1420	**−2.15**	−1.21	−1.15	−1.33	*murB*	Weakly similar to UDP-*N*-acetylglucosaminyl-3-enol-pyruvate reductase
lmo2537	**−4.59**	−2.27	−2.61	−2.03		Similar to UDP-*N*-acetylglucosamine 2-epimerase
Cell-wall-associated						
lmo0576	**2.20**	1.11	1.28	1.20		Hypothetical cell-wall-associated protein
lmo0610	**6.17**	1.33	1.12	−1.13		Similar to internalin proteins, putative peptidoglycan-bound protein (LPXTG motif)
lmo0880	**8.15**	1.43	1.37	−1.13		Similar to wall-associated-protein precursor (LPXTG motif)
lmo1413	1.63	1.33	**2.07**	1.91		Putative peptidoglycan-bound protein (LPXTG motif)
lmo1666	**2.13**	1.34	1.07	−1.16		Peptidoglycan-bound protein (LPXTG motif)
lmo2027	**2.31**	1.44	**2.05**	1.74		Putative cell-surface protein, similar to internalin proteins
lmo2085	**10.81**	**2.38**	**2.02**	1.43		Putative peptidoglycan-bound protein (LPXTG motif)
lmo2504	**−4.35**	**−2.06**	−1.56	−1.53		Similar to cell-wall-binding proteins
lmo2522	**−5.91**	−1.42	−1.30	1.21		Similar to hypothetical cell-wall-binding protein from *B. subtilis*
PrfA regulated genes						
lmo0200	**6.63**	**4.29**	**5.06**	**4.35**	*prfA*	Listeriolysin positive regulatory protein
lmo0201	1.74	1.25	**2.13**	**3.86**	*plcA*; *pic*	Phosphatidylinositol-specific phospholipase C
lmo0433	**3.88**	1.07	1.09	−1.11	*inlA*	Internalin A
lmo0434	**2.30**	1.82	1.61	1.43	*inlB*	Internalin B
***agr* operon**						
lmo0048	**−31.6**	**−3.23**	**−6.34**	**−6.69**		Similar to *Staphylococcus* two-component sensor histidine kinase AgrB

Table 2. cont.

Gene	Fold-change at time (min)				Gene designation	Description of product
	3	10	20	40		
lmo0049	**−19.4**	**−3.84**	**−5.84**	**−8.11**		Similar to *Staphylococcus* two-component protein AgrD
lmo0050	**−11.8**	**−2.39**	**−3.68**	**−3.80**		Similar to *Staphylococcus* two-component sensor histidine kinase AgrC
lmo0051	**−8.89**	−1.92	**−3.26**	**−3.45**		Similar to two-component response regulator protein (AgrA from *Staphylococcus*)

*Phosphotransferase system.

FtsW, cell division inhibitors (septum placement), cell shape determining proteins, cell division initiation protein (septum placement), and cell division protein FtsI (penicillin-binding protein 3), respectively, was observed only after 3 min heat shock. Transcription of *ftsX* (lmo2506) and *ftsE* (lmo2507), whose products are highly similar to cell division protein FtsX and cell division ATP-binding protein FtsE, respectively, was transiently down-regulated for 10 and 20 min after heat exposure, respectively.

Cell-wall-associated genes

The bacterial cell wall is a complex structure and plays an important role as the first protection against environmental stresses. Cell wall hydrolases (including autolysins) play important roles in numerous cellular processes, including cell division and cell wall turnover. In line with the results described above, all genes encoding cell wall hydrolases, namely *aut* (lmo1076), lmo1216, lmo1855, *spl* (lmo2505), *ami* (lmo2558), *murA* (lmo2691) and lmo2754, showed a transiently repressed expression pattern, except for *iap* (lmo0582), which showed an almost constant threefold repression. The intermediate glucosamine-6-phosphate plays a central role in the regulation of cell wall biosynthesis vs glycolysis (Komatsuzawa *et al.*, 2004). Genes controlling the regulation between these two pathways showed induction after heat exposure. These genes are lmo0877 and lmo0957, for which the encoded products show sequence similarity to glucosamine-6-phosphate isomerases, lmo0956 and lmo2108, which encode putative *N*-acetylglucosamine-6-phosphate deacetylases, and lmo1998 and lmo1999, which encode products that show low sequence similarity to glucosamine-fructose-6-phosphate aminotransferases. The essential gene in the biosynthetic pathway, *murB* (lmo1420), was down-regulated after 3 min heat shock. Another operon that plays a role in cell wall synthesis and showed decreased expression upon heat shock was the *dlt* operon (lmo0971–lmo0974), which encodes proteins important in catalysing the incorporation of D-alanine residues into lipoteichoic acids and wall teichoic acids. Furthermore, the lmo1075 gene, which encodes a protein with 64 % sequence similarity to *B. subtilis* teichoic acid translocation ATP-binding protein TagH, was also down-regulated after 3 min of heat shock. Nine genes encoding putative cell-wall-associated proteins were differentially expressed during heat exposure. Five of these genes encode products containing an LPXTG peptidoglycan-binding motif, namely lmo0610, lmo0880, lmo1413, lmo1666 and lmo2085.

Virulence-associated genes

A constant induction was observed for *prfA* (lmo0200), which encodes the major regulator of the virulence genes. This gene is both SigB- and SigA-regulated, and its product induces genes containing a PrfA box in their promoter sequence. Accordingly, numerous PrfA-regulated virulence genes (Milohanic *et al.*, 2003) showed upregulation after heat exposure, including *plcA* (lmo0201), encoding a phospholipase, *inlA* and *inlB* (lmo0433 and lmo0434), encoding *Listeria*-specific internalins, lmo2067, encoding a conjugated bile acid hydrolase, and lmo0596, encoding an unknown protein. However, Rauch *et al.* (2005) have shown that PrfA has no direct influence on the transcription of lmo2067 and lmo0596. Significant down-regulation was found for the *agr* locus (lmo0048– lmo0051). This locus encodes a two-component system that has been shown to play a role in bacterial virulence (Autret *et al.*, 2003).

Validation of microarray gene expression

The microarray data were validated by Q-PCR analysis using a set of 13 genes which cover a range of expression values in the microarray data. The relative gene expression levels obtained by Q-PCR were normalized to that of three genes (*tpi*, *rpoB* and the 16S rRNA gene) that did not show fluctuating expression during temperature changes. Relative quantitative values were obtained using the comparative threshold cycle method ($\Delta\Delta C_T$) in which the C_T value corresponds to the cycle at which the fluorescent signal crosses the threshold line. The relative expression of the genes was determined in quadruplicate for three independent temperature upshift experiments. The resulting ratios were $\log_2$ transformed and plotted against the $\log_2$ values from the microarray analysis. Fig. 2 shows a strong correlation, with $r > 0.8$ for all time points, which is considered the threshold for strong correlation. Generally,

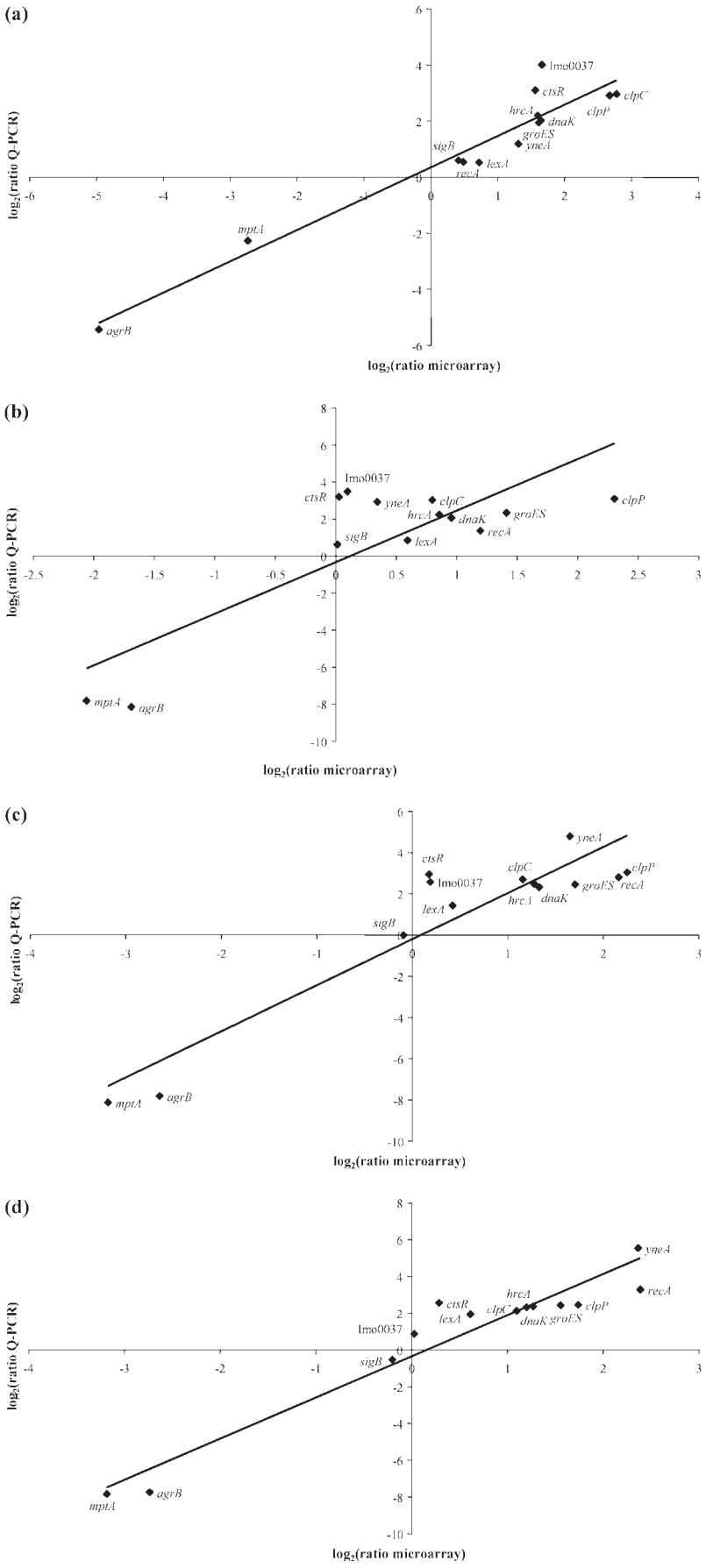

Fig. 2. Validation of microarray data by Q-PCR analysis. (a) Comparison of differentially expressed genes after 3 min; (b) comparison of differentially expressed genes after 10 min; (c) comparison of differentially expressed genes after 20 min; (d) comparison of differentially expressed genes after 40 min.

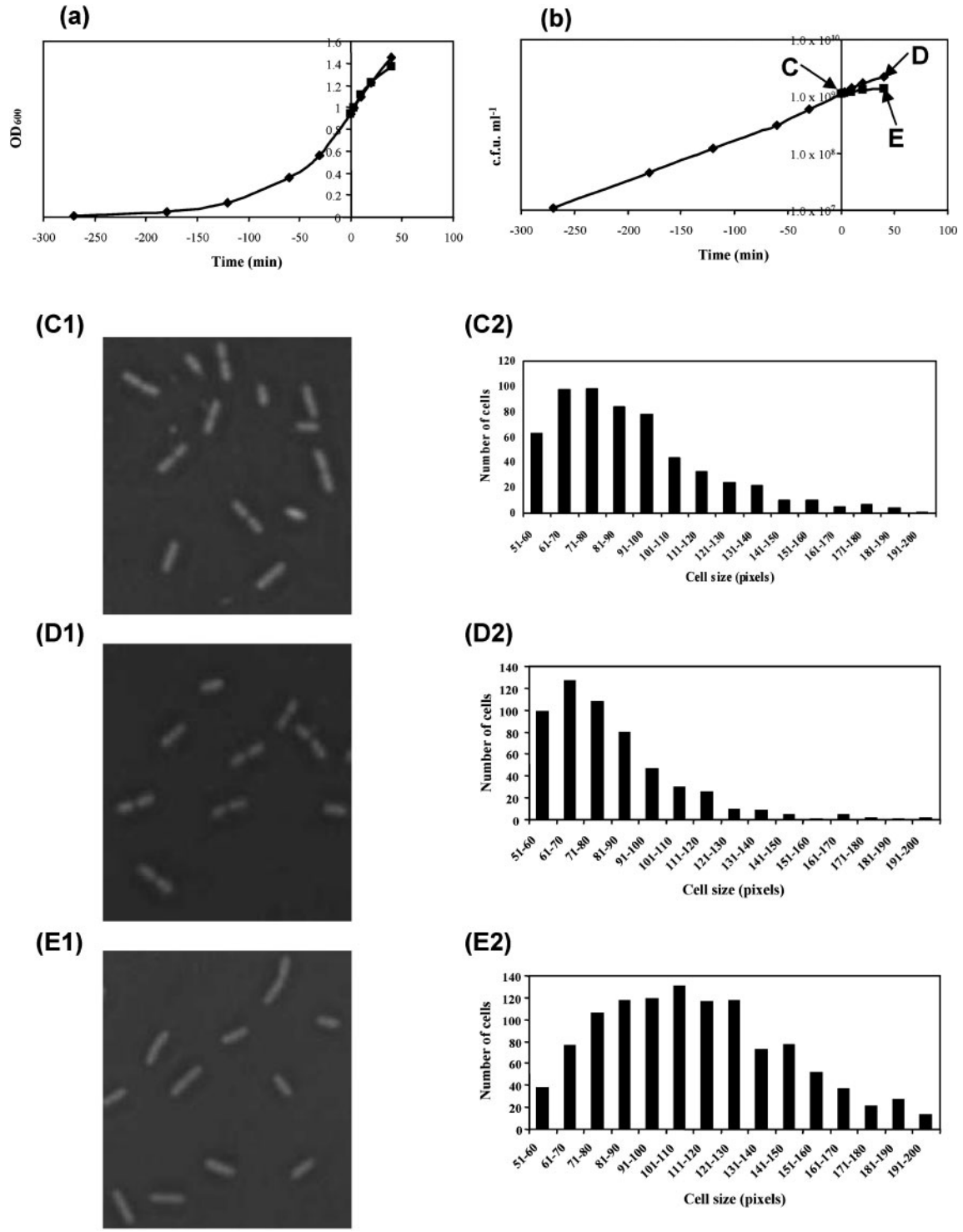

Fig. 3. Increasing cell size of *L. monocytogenes* cells exposed to 48 °C in comparison with cells continuously grown at 37 °C. The OD_{600} (a) and viability count (b) of a *L. monocytogenes* culture grown at 37 °C (◆) were measured. At time zero (point C) the culture was transferred to 48 °C (■) or grown on at 37 °C (◆). The cultures were incubated for 40 min and samples were taken from the culture at 37 °C (point D) and from the culture at 48 °C (point E). C1, D1 and E1: microscopic images of the cells collected at points C, D and E, respectively; C2, D2 and E2: analyses of microscopic images of cells at points C, D and E, respectively, using ImageJ. The graphs show the distribution of cell sizes in number of pixels per cell.

the microarray analysis underestimated the differential expression values by an average of fivefold. Similar observations have been reported previously when using ORF arrays in microbial transcriptome analysis (Gao *et al.*, 2004; Helmann *et al.*, 2001; Stintzi, 2003).

DISCUSSION

Variation in temperature is frequently encountered in nature, and bacteria have evolved to cope with such fluctuations by employing various mechanisms. Microarrays were used to investigate the whole-genome expression profiles of *L. monocytogenes* in response to a temperature upshift from 37 to 48 °C over a 40 min period. Our data show differential expression of genes involved in different cellular processes, including the SOS response, cell division and specific (heat) stress responses. For many differentially expressed genes, a transient expression pattern was observed between 3 and 40 min after temperature upshift. The highest number of differentially expressed genes was observed 3 min after temperature upshift. Whole-genome expression profiles in response to heat shock have been reported for *B. subtilis*, *Shewanella oneidensis* and *Campylobacter jejuni* (Gao *et al.*, 2004; Helmann *et al.*, 2001; Stintzi, 2003). These studies also showed transient expression patterns, albeit that peak numbers of differentially expressed genes were observed at later times after heat exposure. The ability of *L. monocytogenes* to rapidly alter transcript levels allows for a quick response to sudden environmental changes.

In *L. monocytogenes*, genes belonging to the class I and class III heat-shock regulons showed increased expression during heat exposure. Interestingly, the class I heat-shock genes showed a constant induction in expression levels over the 40 min period, whereas the class III heat-shock genes showed a transient expression pattern. This is likely related to the different roles that the two classes fulfil during heat shock. The Clp-ases are specifically required at the early stages of heat shock to remove the proteins initially damaged and misfolded. Following adaptation to heat, Clp-ase levels are reduced, while higher levels of chaperones are present to prevent accumulation of damaged and misfolded proteins. Q-PCR showed 1.5-fold induction of the gene encoding SigB after 3 and 10 min. Considering the role of SigB as a regulator, this slight increase in expression appears to be enough to induce genes that contain a SigB promoter, as evidenced by the transient nature of the class II stress response. When present in *B. subtilis*, *C. jejuni* and *S. oneidensis* (Gao *et al.*, 2004; Helmann *et al.*, 2001; Stintzi, 2003), genes belonging to the class I and class III heat-shock response and SigB-regulated class II stress response were induced upon heat shock. Given the genetic resemblance and similar heat-shock conditions applied, a direct comparison of upregulated genes of *L. monocytogenes* and *B. subtilis* (Helmann *et al.*, 2001) was possible. A total of 25 orthologues of the 130 induced class II stress genes in *B. subtilis* showed induction in *L. monocytogenes* as well. Of these 25 genes, eight had been previously identified in *L. monocytogenes* to be SigB-regulated (Kazmierczak *et al.*, 2003). In *B. subtilis*, 76 other genes, designated class U, show increased expression upon heat shock. In *L. monocytogenes*, nine orthologues of these genes showed induction, including *htrA*. Other research has shown that HtrA is important for survival during exposure to environmental and cellular stresses (Stack *et al.*, 2005; Wilson *et al.*, 2006).

In *L. monocytogenes*, several genes that play a role in the SOS response and DNA repair machinery showed increased expression after heat exposure, including *recA*, which is the major activator of the SOS response. RecA is involved in various processing steps of DNA replication proteins and repair proteins, and plays a role in the restart of stalled replication forks (Cox *et al.*, 2000), homologous recombination, repair of double-stranded DNA breaks (Lusetti & Cox, 2002) and introduction of adaptive point mutations (McKenzie *et al.*, 2001). Furthermore, it stimulates cleavage of the LexA repressor of the SOS response, activating expression of the SOS response genes (Fig. 4). The LexA repressor is autoregulatory, and binds to the operator sequence in the promoter region of the SOS response genes (CGAACATATGTTCG in *B. subtilis*; Au *et al.*, 2005). Several LexA-regulated genes showed increased expression in *L. monocytogenes*, including genes encoding the two alternative DNA polymerases *umuDC* and *dinB*. DNA polymerases Pol IV (DinB) and Pol V ($UmuD_2'C$) have previously been shown to respond to inhibition of replication fork progression in a damage-independent manner (Godoy *et al.*, 2006; Hare *et al.*, 2006). Elevated temperatures may cause the *L. monocytogenes* DNA replication fork to stall, leading to exposure and possible degradation of the arrested replication fork without rescue by Pol IV and Pol V. This theory is consistent with the observation that heat shock resulted in cell elongation and prevention of cell division. This effect is called 'nucleoid occlusion' and is generally interpreted as prevention of Z-ring formation in the vicinity of the nucleoid, thereby preventing cell division without complete replication and segregation of the nucleoid (Rothfield *et al.*, 2005). The expression profiles showed two mechanisms that might lead to the prevention of Z-ring formation after heat shock (Fig. 5). The first mechanism would be the result of increased expression of *yneA* (as part of the SOS response), which is responsible for suppression of cell division in *B. subtilis* by reducing the level of FtsZ proteins at the cell division site, thereby preventing FtsZ ring formation (Kawai *et al.*, 2003). The second mechanism is the result of a decreased expression of *minDE* and *divIVA*, which are involved in spatially correct septum placement. The MinCD complex negatively regulates formation of the cytokinetic Z-ring to midcell by preventing its formation near the poles (Zhou & Lutkenhaus, 2005). Another strategy of *L. monocytogenes* to suppress cell division might be to decrease expression of *fteX* and *ftsE*. These encode proteins with sequence similarity to ABC transporters and

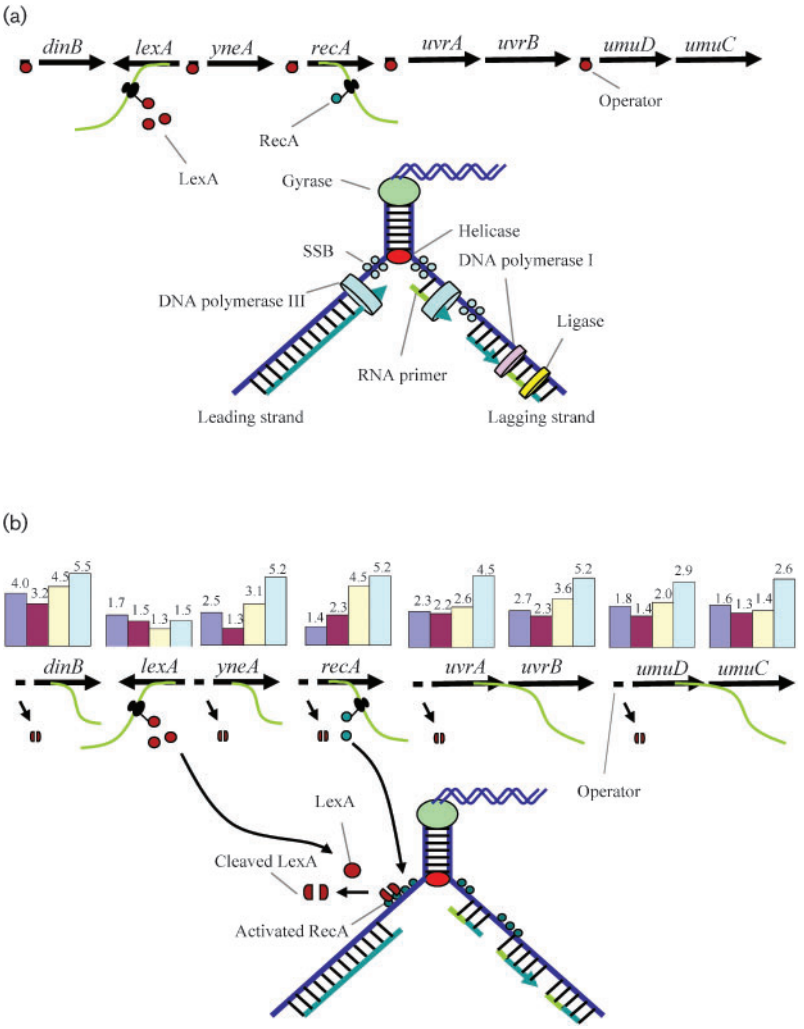

Fig. 4. Activation of the SOS response of *L. monocytogenes* during exposure to elevated temperatures. (a) In cells with an active replication fork (before exposure), LexA represses the transcription of *lexA*, *recA* and the other genes of the SOS response (*dinB*, *yneA*, *uvrAB* and *umuDC*) by binding to the operator in the promoter region. (b) When the replication fork is stalling after exposure to elevated temperatures, RecA is activated by the resulting ssDNA. Activated RecA promotes cleavage of LexA and consequently the SOS response genes are induced. The genes of the SOS response are illustrated schematically and are not to scale. The induction of the genes after heat shock is represented by the bar charts above each gene (blue, 3 min; purple, 10 min; yellow, 20 min; green, 40 min).

are localized at the division site. In *E. coli* they are directly involved in cell division and are important for septal ring formation (Schmidt *et al.*, 2004). Mutants for *ftsXE* constitutively showed induction of the SOS response in *E. coli* (O'Reilly & Kreuzer, 2004).

A possible strategy for *L. monocytogenes* cells to prevent continuous elongation after suppression of cell division is to reduce the expression of genes that encode proteins involved in cell wall biosynthesis and turnover. The down-regulated genes *rodA* and *mreDCB* encode proteins involved in cell wall synthesis and the determination of cell shape. The MreDCB complex forms actin-like cables beneath the cell surface and requires RodA for the control of cell shape (Kruse *et al.*, 2005). Research in *B. subtilis* has shown that cells depleted of RodA are impaired in cell division (Henriques *et al.*, 1998). Reduced expression levels were also observed for the genes encoding autolysins, for *murB*, which is an essential gene in the biosynthetic pathway of peptidoglycan, and for the *dlt* operon, which encodes products that catalyse the incorporation of D-alanine residues into teichoic acids. While most bacteria show slower growth rates after mutations in the *dlt* operon, *Streptococcus gordonii* shows abnormal septation and defective cell separation (Neuhaus & Baddiley, 2003).

PrfA and various other virulence genes showed induction upon heat exposure, which has apparently not been reported before. PrfA is the major virulence regulator in *L. monocytogenes* (Chakraborty *et al.*, 1992; Leimeister-Wachter *et al.*, 1990). Previous studies have shown maximal induction of the virulence genes at 37 °C (Johansson *et al.*, 2002; Leimeister-Wachter *et al.*, 1992). However, expression levels at temperatures above 37 °C were not measured in these studies. Destabilizing mutations in the secondary structure of the untranslated region of *prfA* mRNA resulted in increased levels of PrfA at 37 °C (Johansson *et al.*, 2002), indicating that this structure is not completely unfolded at this temperature. Increasing the temperature above 37 °C probably results in a more relaxed secondary structure, allowing more PrfA translation.

A comparative analysis of the transcription profiles of *L. monocytogenes* during heat shock and in the intracellular

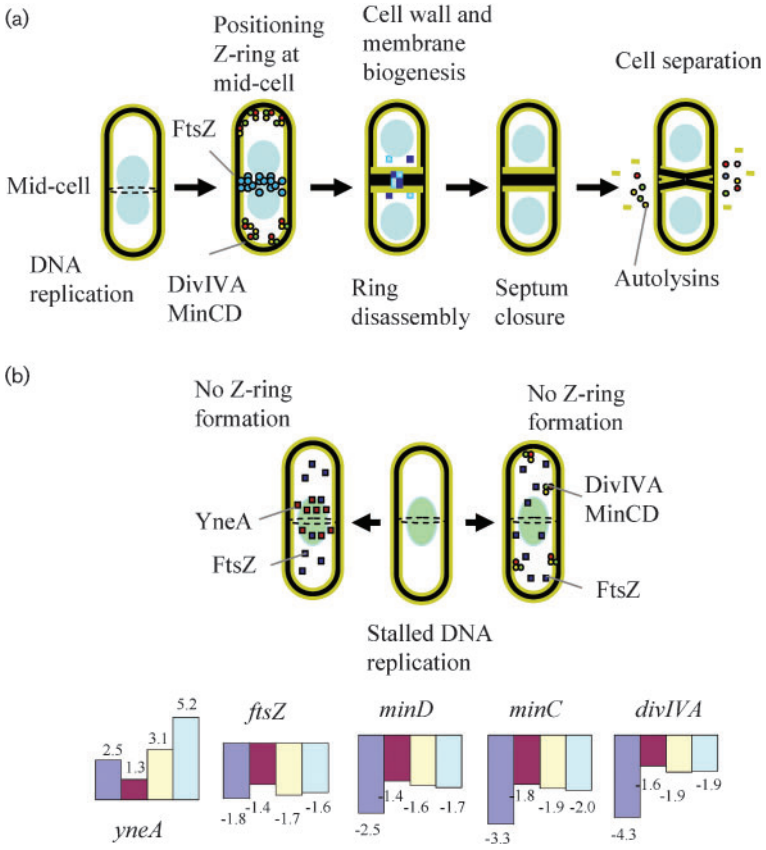

Fig. 5. Nucleoid occlusion. (a) Normal replicating cells; (b) stalling DNA replication results in perturbation of cell division. There are two possible explanations. (1) An activated SOS response results in increased amounts of YneA at the nucleoid in the mid-cell. YneA reduces the concentration of FtsZ, preventing septum formation. (2) A reduction in numbers of MinCD and DivIVA prevents the concentration of FtsZ at the mid-cell by allowing FtsZ at the poles. Differential expression of the genes after heat shock is represented by the bar charts (blue, 3 min; purple, 10 min; yellow, 20 min; green, 40 min).

environment (Chatterjee *et al.*, 2006) showed a high number of genes that were differentially expressed during both exposures. Of the 714 genes differentially expressed during heat shock, 236 showed differential expression in the intracellular environment as well. Among genes that were commonly regulated during both exposures, 172 showed increased expression and 64 showed decreased expression. Exposure of *L. monocytogenes* to the intracellular environment triggered numerous (heat) stress-response genes that were shown to be differentially expressed during heat shock in this study. All genes belonging to the class I and class III heat-shock-response classes and 24 of the SigB-regulated class II stress-response genes showed induction in the intracellular environment. Remarkably, similar expression profiles for SOS response genes, cell division genes and cell-wall-related genes were observed. This comparative analysis showed that numerous responses induced during heat exposure contribute to the pathogenicity of *L. monocytogenes* by allowing survival under the stressful conditions encountered intracellularly and during passage through the stomach and the intestinal tract.

This study shows the importance of the SOS response as a common protection mechanism during heat shock, and its possible role in suppression of cell division to prevent transection of the genome as a result of incomplete chromosomal segregation. Whether replication fork stalling is the actual cause that leads to both the SOS response and suppression of cell division remains to be elucidated. This opens up interesting possibilities for future research. Our transcriptome analysis has revealed several aspects of the heat-shock response of *L. monocytogenes* that may include targets for inactivation. The role of specific factors, such as the SOS response in *L. monocytogenes* stress survival and virulence, will be assessed in future studies.

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