Refinement of the *Listeria monocytogenes* σ^B regulon through quantitative proteomic analysis

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σ^B is an alternative σ factor that regulates stress response and virulence genes in the foodborne pathogen *Listeria monocytogenes*. To gain further insight into σ^B -dependent regulatory mechanisms in *L. monocytogenes*, we (i) performed quantitative proteomic comparisons between the *L. monocytogenes* parent strain 10403S and an isogenic $\Delta sigB$ mutant and (ii) conducted a meta-analysis of published microarray studies on the 10403S σ^B regulon. A total of 134 genes were found to be significantly positively regulated by σ^B at the transcriptomic level with >75% of these genes preceded by putative σ^B -dependent promoters; 21 of these 134 genes were also found to be positively regulated by σ^B through proteomics. In addition, 15 proteins were only found to be positively regulated by σ^B through proteomics analyses, including Lmo1349, a putative glycine cleavage system protein. The Lmo1349 gene is preceded by a 5' UTR that functions as a glycine riboswitch, which suggests regulation of glycine metabolism by σ^B in *L. monocytogenes*. Herein, we propose a model where σ^B upregulates pathways that facilitate biosynthesis and uptake of glycine, which may then activate this riboswitch. Our data also (i) identified a number of σ^B -dependent proteins that appear to be encoded by genes that are co-regulated by multiple transcriptional regulators, in particular PrfA, and (ii) found σ^B -dependent genes and proteins to be overrepresented in the 'energy metabolism' role category, highlighting contributions of the σ^B regulon to *L. monocytogenes* energy metabolism as well as a role of PrfA and σ^B interaction in regulating aspects of energy metabolism in *L. monocytogenes*.

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INTRODUCTION

The general stress response alternative σ factor σ^B is an important transcriptional regulator in the facultative intracellular pathogen *Listeria monocytogenes* (O'Byrne & Karatzas, 2008). *L. monocytogenes* is not only the causative agent of listeriosis, a life-threatening foodborne infection in humans and animals, but is also commonly found in many non-host environments and has the ability to survive under a variety of environmental stress conditions that are lethal to other bacteria. σ^B contributes to the resistance of *L. monocytogenes* to different stress conditions, including acid, osmotic and energy stress, and also plays an important role in survival during stationary phase (O'Byrne & Karatzas, 2008). σ^B has also been shown to directly regulate transcription of some virulence genes as well as other genes that contribute to *L. monocytogenes* growth and survival during gastrointestinal passage as well as intracellular survival and replication (Chaturongakul *et al.*, 2008;

Garner *et al.*, 2006; Kazmierczak *et al.*, 2005; van Schaik & Abee, 2005).

As a transcriptional activator, σ^B directly upregulates and indirectly downregulates *L. monocytogenes* genes involved in stress response and virulence. Several transcriptomic studies have probed the σ^B regulon of *L. monocytogenes*, including four studies with *L. monocytogenes* strain 10403S. A microarray study by Raengpradub *et al.* (2008), comparing transcript levels in *L. monocytogenes* 10403S and an isogenic $\Delta sigB$ null mutant grown to stationary phase or exposed to salt stress, identified 168 genes as positively regulated by σ^B [$P < 0.05$; fold change (FC) ≥ 2.0]. A microarray study by Ollinger *et al.* (2009) characterized transcript levels in stationary phase cells of *L. monocytogenes* 10403S and $\Delta sigB$ mutant strains with a constitutively active PrfA protein, which forms a regulatory network with σ^B that contributes to virulence gene expression and host infection by *L. monocytogenes*. In this study >200 genes were identified as having significant σ^B effects with FC ≥ 1.5 between the parent and mutant strain. In addition, Oliver *et al.* (2010) used microarray analyses to compare transcript levels in parent and $\Delta sigB$ mutant strain pairs representing *L. monocytogenes* lineages I, II, IIIA and IIIB; lineages IIIA and IIIB have recently been reclassified as lineages III and IV (Ward *et al.*, 2008). In *L. monocytogenes* 10403S, a lineage II

Abbreviations: 2-DE, two-dimensional gel electrophoresis; FC, fold change; FDR, false discovery rate; iTRAQ, isobaric tags for relative and absolute quantification; RNA-Seq, RNA sequencing; UTR, untranslated region.

Two supplementary figures and three tables are available with the online version of this paper.

strain, 252 genes were found to be significantly positively regulated by σ^B in stationary phase cells, with a FC ≥ 1.5 . Furthermore, in an RNA sequencing (RNA-Seq) study of *L. monocytogenes* 10403S and an isogenic $\Delta sigB$ strain, Oliver *et al.* (2009) identified 96 genes as positively regulated by σ^B in stationary phase cells. Along with transcriptional analyses, proteomic studies have also been used to define the σ^B regulon in *L. monocytogenes* 10403S. Using non-gel based quantitative proteomics and two-dimensional gel electrophoresis (2-DE), Abram *et al.* (2008a) identified 17 proteins that showed higher expression levels in the parent strain than the $\Delta sigB$ strain, using bacteria grown to stationary phase in Brain Heart Infusion (BHI) medium with or without osmotic stress (0.5 M NaCl). Additionally, a 2-DE study of bacteria grown to exponential or stationary phase in chemically defined media with or without 0.5 M NaCl (Abram *et al.*, 2008b) identified 10 proteins that showed higher levels in the parent strain as compared to the $\Delta sigB$ mutant.

Overall, the studies detailed above show that σ^B in *L. monocytogenes* is responsible for positive regulation of a large regulon with >100 genes, with a considerable number of these genes directly regulated by σ^B , as supported by identification of σ^B consensus promoters upstream of many of these genes (Abram *et al.*, 2008a; Oliver *et al.*, 2009, 2010; Raengpradub *et al.*, 2008); however, a comprehensive analysis that formally integrates data on the σ^B regulon from multiple studies has been missing so far. Increasing evidence further suggests that σ^B also makes important contributions to gene regulation in *L. monocytogenes* via mechanisms other than direct regulation of a gene or operon through σ^B -dependent transcription from an upstream promoter, including, but not limited to, regulation of non-coding RNAs (ncRNAs), which can regulate gene expression at both the transcriptional and post-transcriptional stage (Mellin & Cossart, 2012; Nielsen *et al.*, 2008; Oliver *et al.*, 2009; Toledo-Arana *et al.*, 2009). We thus performed a non-gel based quantitative proteomic analysis of stationary phase *L. monocytogenes* 10403S and isogenic $\Delta sigB$ mutant cells to further refine the definition of the σ^B regulon for this *L. monocytogenes* strain and to explore additional σ^B -dependent mechanisms of gene regulation, including a meta-analysis of previous microarray studies on the σ^B regulon and a comparison with RNA-Seq data, all generated for the strain 10403S genetic background. Our hypothesis was that this approach would allow the identification of additional transcriptional and post-transcriptional regulatory pathways governed by *L. monocytogenes* σ^B .

METHODS

Bacterial strains and growth conditions. The *L. monocytogenes* parent strain 10403S and its isogenic $\Delta sigB$ mutant (Wiedmann *et al.*, 1998) were stored at -80°C in BHI medium containing 15% glycerol. Cells were grown to stationary phase with aeration at 37°C , as previously described (Raengpradub *et al.*, 2008).

Protein isolation, iTRAQ labelling, and nano-scale reverse phase chromatography and tandem mass spectrometry (nanoLC-MS/MS). Proteins were isolated from stationary phase

L. monocytogenes cells from 25 ml of culture using the method of Abram *et al.* (2008a) with slight modifications as previously described (Mujahid *et al.*, 2013). Protein concentrations were determined using a noninterfering protein assay kit (Calbiochem). Protein concentration and quality were verified using 1D SDS-PAGE.

Protein samples were analysed at the Cornell University Proteomics and Mass Spectrometry Core Facility using shotgun-based quantitative proteomics as previously described (Mujahid *et al.*, 2013). Briefly, a total of 100 μg protein of each sample was denatured, reduced, and the cysteine residues were blocked, after which protein samples were digested with sequence-grade modified trypsin at 37°C for 16 h. SDS-PAGE was used to assess the efficiency of protein digestion. The tryptic peptides from *L. monocytogenes* parent strain 10403S and the $\Delta sigB$ strain (along with two other strains) were labelled with 4-plex iTRAQ (isobaric tags for relative and absolute quantification) reagents, as per the manufacturer's protocols (documents 4351918A and 4350831C; Applied Biosystems). The labelled samples were combined and fractionated via IEF OffGel electrophoresis using an Agilent 3100 OFFGEL Fractionator (Agilent, G3100AA) using 24 cm, pH 3–10 NL IPG strips, as detailed previously (Mujahid *et al.*, 2013). nanoLC-MS/MS was carried out using an LTQ-Orbitrap Velos (Thermo-Fisher Scientific) mass spectrometer, which was operated in positive ion mode with nano spray voltage set at 1.5 kV and source temperature at 175°C (as detailed by Mujahid *et al.*, 2013). Two biological replicates of the parent strain 10403S and the isogenic mutant strain $\Delta sigB$ comparison were prepared and analysed independently.

Protein identification and data analysis. Data processing, protein identification and subsequent data analysis were carried out independently for each replicate, as described previously (Mujahid *et al.*, 2013). Briefly, Proteome Discoverer 1.1 was used to process all MS and MS/MS raw spectra from iTRAQ experiments for subsequent database search using in-house licensed Mascot Daemon. The *L. monocytogenes* protein sequence database downloaded from the Broad Institute was used for the database search. As detailed previously (Mujahid *et al.*, 2013), the default Mascot search settings included (i) one missed cleavage for full trypsin with fixed methyl methanethiosulfonate modification of cysteine, (ii) fixed 4-plex iTRAQ modifications on lysine and N-terminal amines and (iii) variable modifications of methionine oxidation and 4-plex iTRAQ on tyrosine. The peptide mass tolerance was 30 p.p.m. and the fragment mass tolerance was 0.15 Da. The false discovery rate (FDR) was estimated using an automatic decoy database search in Mascot. Only peptides with significance scores above the identity threshold (at the 95% confidence interval), defined by Mascot probability analysis (http://www.matrixscience.com/help/scoring_help.html#PBM), were used for protein identification. In addition, proteins that were identified in all the samples through at least two peptides with a *P* value of <0.05 (expectation value) as determined by Mascot probability analysis were quantified. The relative quantification ratios were normalized using the 'median ratio' procedure in each set of experiments.

The Wilcoxon signed rank test was applied to peptide ratios for each identified protein to determine significant changes between the parent strain 10403S and the $\Delta sigB$ mutant strain. Fisher's combined probability test was used to combine FDR adjusted Wilcoxon *P* values from each replicate into one test statistic for every protein to obtain a combined *P* value (*P^c* value). Proteins with peptide ratios exhibiting a Fisher's combined probability test *P^c* value <0.05 and a mean iTRAQ protein ratio ≥ 1.5 were considered significantly differentially expressed. Statistical analyses were conducted using the R language and environment for statistical computing (<http://www.r-project.org/>).

Meta-analysis of published studies. A meta-analysis of three published microarray studies was performed to identify genes that

showed differential transcript levels between the *L. monocytogenes* parent strain 10403S and an isogenic $\Delta sigB$ strain. Two of these studies used 10403S and an isogenic $\Delta sigB$ strain (Oliver *et al.*, 2010; Raengpradub *et al.*, 2008), while one study (Ollinger *et al.*, 2009) used an *L. monocytogenes* 10403S *prfA*^{*} strain, which constitutively expresses an active form of the virulence gene transcriptional activator PrfA, and an isogenic *prfA*^{*} $\Delta sigB$ strain. Two of these studies (Ollinger *et al.*, 2009; Raengpradub *et al.*, 2008) used the same set of microarray probes, which were designed based on the genome sequence of strain EGD-e, while the other study (Oliver *et al.*, 2010) used a set of microarray probes designed based on four different genomes. The meta-analysis used the Fisher's combined probability test to combine *P* values reported in each microarray study into one test statistic (combined microarray *P* value, *P*^c value) for every gene. Genes with a *P*^c value <0.05 and FC ≥ 1.5 in each of the three array studies were considered significantly differentially expressed. To further refine the σ^B regulon in *L. monocytogenes* 10403S, microarray meta-analysis data were also compared to RNA-Seq data collected to compare transcript levels between the *L. monocytogenes* parent strain 10403S and an isogenic $\Delta sigB$ strain (Oliver *et al.*, 2009). As previously described, genes were considered positively regulated by σ^B in the RNA-Seq study if they had a *Q*-value <0.05 and FC ≥ 2.0 in all four comparisons of parent and $\Delta sigB$ strain transcript levels (Oliver *et al.*, 2009).

Promoter mapping and analysis of 5' untranslated regions (UTRs). Data for σ^A and σ^B promoters as well as 5' UTRs were obtained from an analysis of a manually annotated genome sequence for *L. monocytogenes* 10403S (Broad Institute; see http://www.broadinstitute.org/annotation/genome/listeria_group/MultiHome.html) and RNA-Seq transcriptome data (Oliver *et al.*, 2009; R. H. Orsi, S. Chaturongakul, H. F. Oliver, L. Ponnala, M. Wiedmann, K. J. Boor., unpublished data). 5' UTR nucleotide sequences for selected σ^B -dependent genes were examined for self-binding ability through the RNAfold Server (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>). The lengths of 5' UTRs among different groups of genes were compared using a two-sided and one-sided Wilcoxon rank sum test, as the Shapiro-Wilk test for normality showed that 5' UTR lengths were not normally distributed.

Association of σ^B -dependent genes and proteins with JCVI role categories. A Monte-Carlo simulation of Fisher's exact test was used to determine whether the distribution of role categories among the 149 genes identified as positively regulated by σ^B by RNA-based or proteomic approaches was different from the role category distribution that would be expected by chance (based on the role category primary annotation for all *L. monocytogenes* EGD-e genes, available at <http://cmr.jcvi.org>). Individual Fisher's exact tests were subsequently used to determine whether individual role categories were over- or under-represented among the σ^B -dependent genes. While 21 comparisons were performed (based on 21 role categories), we did not adjust *P* values for multiple comparisons, rather we reported actual *P* values to allow readers to apply corrections if deemed appropriate. Analyses were performed using all role categories assigned to a given gene in the JCVI-CMR *L. monocytogenes* EGD-e database.

RESULTS AND DISCUSSION

Proteomics identified 36 proteins that show higher expression levels in the presence of σ^B , suggesting positive regulation by σ^B

Our quantitative proteomics comparison identified 36 proteins that show higher expression levels in the parent as compared to the $\Delta sigB$ strain (see Table S1, available in *Microbiology Online*, for details), suggesting σ^B -dependent

regulation, including (i) 21 proteins for which the corresponding genes were found to be positively regulated by σ^B through microarray meta-analysis and/or RNA-Seq and (ii) 15 proteins found to be σ^B -dependent through proteomics only (Group D; see Figs 1 and 2 and Table S2); these 15 proteins are discussed in further detail below. The 21 proteins for which the corresponding genes were found to be positively regulated by σ^B through microarray meta-analysis and/or RNA-Seq include (i) 15 proteins encoded by genes that were found to be under positive regulation by σ^B through RNA-Seq and microarray meta-analysis (Group A; Table S2); (ii) three proteins encoded by genes that were found to be positively regulated by σ^B through microarray meta-analysis but not RNA-Seq (Group E; Table S2); and (iii) three proteins [Lmo1426 (*OpuCC*), Lmo0819 and Lmo0265] encoded by genes that were found to be positively regulated by σ^B through RNA-Seq but not microarray meta-analysis (Group F; Table S2). The genes encoding the three proteins in Group F likely represent false negatives in the microarray analysis as both lmo0819 and *opuCC* had a significant combined microarray *P* value (*P*^c value) and positive FC in all three microarray studies, but missed the FC cut-off of ≥ 1.5 in one and two microarray studies, respectively. *opuCC* (i) is also preceded by a putative σ^B -dependent promoter and (ii) belongs to the four-gene *opuC* operon that has been identified in

Fig. 1. Genes or proteins identified as positively regulated by σ^B through microarray meta-analysis, RNA-sequencing or proteomics. Venn diagram of genes and proteins identified as positively regulated by σ^B in *L. monocytogenes* 10403S stationary phase cells, through (i) proteomic data reported here (*P*^c <0.05; mean FC ≥ 1.5), (ii) a meta-analysis of previous microarray studies (Oliver *et al.*, 2010; Ollinger *et al.*, 2009; Raengpradub *et al.*, 2008) (*P*^c <0.05; FC ≥ 1.5 in each of the three microarray studies) and (iii) previously reported RNA-Seq data (Oliver *et al.*, 2009) (*Q* <0.05; FC ≥ 2.0 in each of the four comparisons). Group names (A–G) correspond to the groups in Fig. 2 and Table S2.

Gene	Gene name or protein encoded	Microarray FC reported by			RNA-Seq fold changes (Oliver, 2009)				Proteomics FC (this study)	
		Ollinger, 2009	Raengpradub, 2008	Oliver, 2010	Comp. 1	Comp. 2	Comp. 3	Comp. 4	Rep. 1	Rep. 2
Group A. Genes or proteins identified as positively regulated by SigB through microarray, proteomics, and RNA-sequencing (15)										
lmo0134	hypothetical protein	8.70	6.20	5.64	72.00	72.00	52.00	52.00	12.82	2.96
lmo0539	locD	15.40	13.70	7.50	18.00	13.79	14.93	11.44	4.35	3.29
lmo0554	similar to NADH-dependent butanol dehydrogenase	3.80	4.40	6.03	106.00	53.00	214.00	107.00	2.16	2.26
lmo0654	hypothetical protein	4.60	2.10	3.77	5.32	6.73	7.21	9.13	7.87	3.73
lmo0722	pyruvate oxidase	3.10	2.30	5.39	21.25	9.44	33.63	14.94	12.20	2.13
lmo0783	mannose-specific PTS system IIB component	12.60	10.80	11.99	57.00	114.00	64.00	128.00	3.33	2.84
lmo0794	hypothetical protein	15.80	5.50	12.83	32.71	114.50	26.86	94.00	5.13	2.81
lmo0796	YceI-like family protein	4.60	3.70	4.03	4.31	3.30	5.22	4.00	4.33	2.44
lmo0913	succinate-semialdehyde dehydrogenase	11.40	5.80	13.43	365.00	182.50	516.00	258.00	1.98	1.74
lmo1602	hypothetical protein	18.60	5.10	4.56	4.71	3.07	8.55	5.56	2.41	1.77
lmo1830	short chain dehydrogenase	3.20	2.40	7.15	23.00	46.00	19.25	38.50	4.29	4.65
lmo2158	stress response protein	8.80	15.60	7.60	737.00	8.77	1158.00	13.79	3.76	2.49
lmo2213	similar to antibiotic biosynthesis monooxygenase subfamily	12.40	11.60	2.30	91.00	91.00	98.00	98.00	2.70	3.00
lmo2398	ltrC	4.60	2.30	2.38	2.03	3.22	2.29	3.64	2.43	1.63
lmo2748	general stress protein 26	9.50	8.90	10.02	281.00	281.00	134.00	134.00	8.70	2.84
Group B. Genes identified as positively regulated by SigB through microarray only (35)										
lmo0210	ldh1	5.20	3.10	2.63	-1.67	4.58	-2.67	2.86	1.15	1.11
lmo0342	transketolase	1.60	3.10	2.14	13.73	2.29	7.09	1.18	1.24	-1.01
lmo0343	tal2	1.50	3.80	2.01	9.60	1.71	4.80	-1.17	1.26	-1.01
lmo0345	ribose 5-phosphate isomerase B	2.20	3.30	1.53	14.00	3.50	3.50	-1.14	1.41	1.01
lmo0346	tpiA2	1.90	2.40	1.89	34.00	2.43	16.00	1.14	2.30	1.03
lmo0347	dihydroxyacetone kinase L subunit	1.80	2.90	2.11	8.75	3.18	3.50	1.27	-1.53	-1.03
lmo0348	dihydroxyacetone kinase	2.00	2.80	1.76	66.00	2.54	33.00	1.27	1.57	-1.08
lmo0406	lactoylglutathione lyase	1.50	1.60	1.63	1.83	5.50	4.50	13.50		
lmo0408	hypothetical protein	2.80	1.50	1.55	1.04	2.00	2.08	4.00		
lmo0524	similar to putative sulfate transporter	3.00	1.90	2.33	1.69	1.26	4.39	3.29	1.45	1.00
lmo0579	hypothetical protein	3.00	1.50	1.51	1.19	2.27	2.10	4.00	1.50	
lmo0580	weakly similar to carboxylesterase	3.30	1.60	1.61	1.24	1.64	1.88	2.49		
lmo0590	hypothetical protein	1.50	1.90	1.66	-1.61	-1.01	1.71	2.71	-1.37	
lmo0648	corA	1.90	1.60	1.65	1.91	3.50	5.91	10.83		
lmo0896	rsbX	2.30	2.20	2.34	2.89	1.82	5.27	3.33		1.04
lmo0956	N-acetylglucosamine-6-phosphate deacetylase	3.90	2.30	2.06	1.22	1.93	1.44	2.28	1.21	1.18
lmo0957	naaB	3.20	1.70	1.56	1.15	1.86	1.37	2.21	-1.06	1.06
lmo0995	YkrP protein	3.90	2.80	3.45	2.20	-1.09	15.20	6.33		
lmo1261	hypothetical protein	2.80	1.80	3.02	2.62	1.58	6.50	3.93	5.46	2.09
lmo1376	6-phosphogluconate dehydrogenase, decarboxylating	2.30	1.50	1.68	-1.13	1.07	1.68	2.05	1.00	1.00
lmo1388	tcsA	2.00	1.60	1.68	3.18	1.14	3.73	1.34	-1.21	-1.26
lmo1432	hypothetical protein	4.50	2.10	1.86	1.34	1.30	2.46	2.39	1.41	1.20
lmo1580	universal stress protein	2.30	2.60	1.96	2.77	2.06	2.32	1.72	1.23	1.30
lmo1605	murC	2.00	1.70	2.22	2.04	1.83	3.55	3.18	1.04	1.00
lmo1636	ABC-2 type transport system ATP-binding protein	1.50	1.50	1.89	-1.21	-2.04	5.28	3.12	-1.13	1.04
lmo1666	lapB	2.20	1.50	1.65	3.20	1.66	7.06	3.67		1.26
lmo1929	ndk	2.10	1.50	1.84	3.31	1.95	6.31	3.73	1.37	1.21
lmo1930	heptaprenyl diphosphate synthase component II	1.70	1.50	1.59	1.00	-1.04	3.92	3.76		1.00
lmo1933	folE	2.10	2.10	1.95	1.50	3.43	2.81	6.43	1.03	1.06
lmo2041	mraW	2.40	1.60	1.79	1.29	-1.15	3.57	2.41	-1.03	1.07
lmo2169	hypothetical protein	1.50	2.00	1.73	1.20	1.11	1.82	1.68		
lmo2191	spxA	3.20	2.70	3.03	1.79	3.23	1.03	1.86	1.26	1.21
lmo2386	hypothetical protein	2.40	1.60	1.91	-1.05	2.17	1.28	2.90	1.44	1.40
lmo2389	similar to NADH dehydrogenase	1.70	1.50	1.77	-1.40	2.41	-1.52	2.22	1.07	1.03
lmo2539	alvA	1.90	1.50	1.51	1.68	-1.06	2.69	1.51	1.12	1.00
Group C. Genes identified as positively regulated by SigB through RNA-sequencing only (13)										
lmo0122	similar to phage proteins	-1.25	1.10	1.08	4.60	4.60	3.20	3.20		
lmo0133	hypothetical protein	3.20	1.20	5.05	71.00	71.00	34.00	34.00		1.90
lmo0274	hypothetical protein	1.20	1.20	1.98	3.33	3.33	3.00	3.00		
lmo0372	beta-glucosidase	1.00	1.10	1.15	5.50	2.54	6.17	2.85		
lmo0405	PII family inorganic phosphate transporter	2.10	2.10	1.38	4.42	10.60	3.25	7.80		
lmo0433	inlA	-2.50	1.30	3.08	8.46	7.52	3.96	3.52		
lmo0434	inlB	-3.33	1.40	1.46	8.00	8.00	4.00	4.00		
lmo0439	weakly similar to a module of peptide synthetase	2.40	1.40	5.63	35.00	35.00	55.00	55.00		1.21
lmo1421	bilEA	1.40	1.50	2.19	34.00	2.83	71.00	5.92	3.34	1.65
lmo1866	phosphotransferase	2.70	-1.11	-1.07	2.80	2.80	2.47	2.47		
lmo2003	similar to transcription regulator GntR family	-1.11	1.00	-1.11	33.00	11.00	11.00	3.67	-1.55	-1.28
lmo2572	similar to chain A, dihydrofolate reductase	7.00	3.50	1.18	7.40	8.22	7.85	8.72		2.69
lmo2733	similar to PTS system, fructose-specific IIA component	1.20	1.10	1.57	14.00	2.80	12.50	2.50		
Group D. Proteins identified as positively regulated by SigB through proteomics only (15)										
lmo0398	fructose-specific PTS system IIA component	-2.00	11.60	13.48	1452.00	19.11	29.00	-2.62	2.04	1.65
lmo0399	fructose-specific PTS system IIB component	-2.00	13.50	10.52	374.20	22.01	8.00	-2.13	1.92	2.41
lmo0643	transaldolase	-3.33	1.30	1.06	4.17	-5.08	9.50	-2.23	2.05	1.53
lmo1046	moaC	1.10	1.20	1.36	7.88	-1.46	18.00	1.57	1.53	1.49
lmo1349	acvPA	-1.11	1.10	-1.14	7.87	-2.39	18.32	-1.02	1.70	1.69
lmo1422	similar to glycine betaine/carnitine/choline ABC transporter	1.50	1.30	1.40	5.00	1.25	52.00	13.00	2.40	2.04
lmo2047	rpmF2	-1.11	-1.43	-1.03	-1.56	-1.39	1.10	1.23	2.04	1.27
lmo1539	glycerol uptake facilitator protein	1.30	-5.00	-1.95	8.76	2.91	3.19	1.06	1.53	1.67
lmo2743	tal1	1.00	1.60	1.35	4.01	1.53	2.05	-1.28	1.67	1.41
lmo2697	dihydroxyacetone kinase	1.10	1.90	1.46	20.35	-1.23	20.10	-1.25	3.00	2.40
lmo2696	dihydroxyacetone kinase L subunit	1.30	1.50	1.15	18.30	-1.07	18.30	-1.07	4.29	2.57
lmo2695	dihydroxyacetone kinase DhA subunit	1.30	1.30	1.27	16.20	-1.19	16.61	-1.16	3.55	1.93
lmo2666	galactitol-specific PTS system IIB component	-10.00	1.40	-1.50	9.55	-7.18	9.09	-7.54	2.18	1.81
lmo0110	esterase/lipase	-1.43	1.10	1.31	4.66	-1.33	8.89	1.43	4.08	2.83
lmo1730	similar to sugar ABC transporter binding protein	-1.67	-1.43	-1.80	5.85	-3.80	8.54	-2.60	1.77	1.58

Group E. Genes or proteins identified as positively regulated by SigB through microarray and proteomics and not RNA-sequencing (3)

lmo1601	similar to general stress protein	12.00	4.20	4.01	3.36	1.86	6.68	3.70	2.91	1.86
lmo2205	<i>apmA</i>	2.70	2.20	2.05	2.40	2.94	1.93	2.37	3.95	2.20
lmo1428	<i>opuCA</i>	2.30	2.90	2.87	1.85	2.49	4.26	5.73	4.93	2.33

Group F. Genes or proteins identified as positively regulated by SigB through RNA-sequencing and proteomics and not microarray (3)

lmo1426	<i>opuCC</i>	1.20	1.00	3.12	3.05	2.08	5.90	4.03	6.54	1.81
lmo0819	hypothetical protein	1.80	1.40	1.65	2.79	2.39	3.69	3.16	1.47	1.63
lmo0265	<i>dapE</i>	-1.11	1.00	8.25	226.00	226.00	183.00	183.00	4.93	2.67

Group G. Genes identified as positively regulated by SigB through RNA-sequencing and microarray and not proteomics (65)

lmo0169	sugar uptake protein, similar to a glucose uptake protein	2.40	2.10	3.37	13.67	20.50	14.83	22.25		
lmo0170	hypothetical protein	5.40	1.90	3.05	2.14	3.90	3.38	6.16	1.02	1.11
LMRG_02646	<i>inlC2</i> (lmo0263)	7.10	6.90	3.73	278.00	92.67	144.00	48.00		
LMRG_02851	<i>inlD</i> (lmo0263)	7.10	6.90	5.29	16.00	8.00	25.00	12.50		
lmo0321	hypothetical protein	5.80	4.30	5.31	4.00	20.00	3.60	18.00		
lmo0445	putative M protein <i>trans</i> -acting positive regulator	3.30	3.00	5.03	22.25	89.00	19.00	76.00		
lmo0515	universal stress protein	4.30	2.10	3.41	19.00	31.67	7.60	12.67	1.31	
lmo0555	<i>dtoT</i>	9.10	4.10	4.11	7.50	6.08	11.00	8.92	1.62	
lmo0593	formate/nitrite transporter	3.80	4.30	5.69	3.95	9.22	2.95	6.89		
lmo0596	membrane protein	5.70	12.30	22.75	137.00	137.00	204.00	204.00		
lmo0602	weakly similar to transcription regulator	10.20	3.00	3.66	136.00	136.00	95.00	95.00	1.94	4.31
lmo0610	internalin, putative peptidoglycan bound protein	2.00	2.30	3.66	3.87	11.60	4.00	12.00		
lmo0628	hypothetical protein	3.40	3.80	3.31	30.00	30.00	18.00	18.00		
lmo0629	hypothetical protein	3.20	2.30	1.61	3.77	4.08	2.38	2.58		1.47
lmo0655	serine/threonine protein phosphatase 1	2.60	2.00	2.90	3.83	3.83	4.92	4.92		
lmo0669	uncharacterized oxidoreductase	19.70	34.40	15.79	25.80	43.00	88.10	146.83	2.53	-1.22
lmo0670	hypothetical protein	13.50	8.30	11.06	56.00	56.00	155.00	155.00		1.14
lmo0781	mannose-specific PTS system IID component	5.60	3.30	15.62	33.88	90.33	31.13	83.00	2.50	2.29
lmo0782	mannose-specific PTS system IIC component	13.20	12.60	13.47	19.00	13.93	24.82	18.20	4.98	2.84
lmo0784	mannose-specific PTS system IIA component	7.90	10.20	5.73	112.00	56.00	124.00	62.00		
lmo0880	similar to wall associated protein precursor	9.90	13.00	6.72	155.00	155.00	383.00	383.00		
lmo0911	hypothetical protein	2.80	2.70	2.15	3.59	4.76	3.37	4.46	-1.70	-1.33
lmo0937	predicted protein	3.90	3.80	10.41	26.00	13.00	119.00	59.50		
lmo0953	hypothetical protein	17.10	3.00	6.46	211.00	211.00	123.00	123.00		
lmo0994	hypothetical protein	14.00	7.30	14.06	82.00	82.00	81.00	81.00		
lmo1140	hypothetical protein	13.00	5.90	3.53	11.18	18.92	6.55	11.08		
lmo1241	hypothetical protein	7.00	2.60	2.22	10.50	28.00	12.88	34.33		
lmo1295	<i>hfa</i>	7.50	3.90	3.44	3.84	2.21	8.42	4.85		
lmo1375	similar to aminotripeptidase	3.30	3.40	3.02	8.89	11.43	16.67	21.43		
lmo1425	<i>opuCD</i>	1.50	1.80	3.43	3.16	2.34	5.03	3.72	3.32	2.68
lmo1433	glutathione reductase	5.80	2.60	4.25	11.00	22.00	25.00	50.00	2.17	1.21
lmo1526	hypothetical protein	3.30	1.90	3.99	9.75	4.88	16.75	8.38	3.85	1.89
lmo1606	similar to DNA translocase	4.20	2.80	5.57	7.18	4.40	12.38	7.58	1.02	1.15
lmo1694	similar to CDP-abeauose synthase	9.60	2.60	11.26	55.00	220.00	69.25	277.00	2.30	
lmo1698	ribosomal-protein-alanine <i>N</i> -acetyltransferase	2.10	2.00	3.52	2.10	3.06	4.57	6.67	3.76	2.07
lmo1883	chitinase	11.00	12.00	3.17	139.00	139.00	172.00	172.00		
lmo2067	choloylglycine hydrolase with bile hydrolase activity	4.80	4.40	3.52	22.67	136.00	15.00	90.00		1.79
lmo2085	peptidoglycan binding protein	6.50	11.10	12.19	106.50	106.50	102.25	102.25		1.02
lmo2130	hypothetical protein	2.80	2.50	2.55	4.25	2.04	7.83	3.76		
lmo2132	hypothetical protein	4.60	3.80	5.06	8.00	16.00	7.75	15.50		
lmo2157	<i>sedA</i>	10.20	11.60	14.96	24.57	49.14	41.36	82.71		1.04
lmo2230	arsenate reductase	44.20	21.80	18.66	355.00	355.00	467.00	467.00		2.16
lmo2231	cation efflux family protein	2.70	2.30	3.06	18.00	18.00	25.00	25.00		
lmo2269	hypothetical protein	1.90	5.70	5.70	13.00	26.00	14.00	28.00		
lmo2387	hypothetical protein	3.50	2.10	4.02	51.00	17.00	37.00	12.33		
lmo2391	hypothetical protein	19.40	7.50	9.08	7.28	22.36	4.28	13.14	1.16	1.32
lmo2434	glutamate decarboxylase	1.90	3.40	2.75	204.00	102.00	132.00	66.00		-1.18
lmo2454	hypothetical protein	4.10	4.40	4.62	110.00	110.00	59.00	59.00		
lmo2463	similar to transport protein	2.40	2.30	3.90	7.60	8.44	10.60	11.78	4.31	1.08
lmo2484	membrane protein	5.60	5.20	5.09	4.40	3.14	5.67	4.05		
lmo2485	PspC domain-containing protein	5.40	3.70	4.42	2.88	2.56	5.44	4.83		
lmo2494	<i>phoU</i>	3.00	2.70	2.34	5.75	7.67	8.75	11.67		1.43
lmo2570	hypothetical protein	3.00	1.90	4.51	12.80	5.82	18.60	8.45		
lmo2571	nicotinamidase	8.50	4.00	5.84	5.84	12.17	6.92	14.42		
lmo2573	similar to zinc-binding dehydrogenase	8.90	4.00	4.58	5.39	10.58	4.76	9.35		
lmo2602	Mg ²⁺ transporter-C family protein	4.00	2.20	7.00	27.50	55.00	16.00	32.00		
lmo2603	amidase	3.40	3.80	6.75	47.50	47.50	41.00	41.00		
lmo2670	hypothetical protein	2.10	1.90	2.32	3.00	3.64	2.41	2.93		
lmo2671	hypothetical protein	2.40	2.80	2.20	2.83	2.43	3.90	3.34		
lmo2672	weakly similar to transcription regulator	2.40	1.70	2.67	4.13	3.35	3.63	2.95		
lmo2673	universal stress protein	18.70	13.20	9.70	211.00	211.00	90.00	90.00		2.15
lmo2674	similar to ribose 5-phosphate epimerase	7.40	2.70	3.76	6.48	8.64	2.81	3.74	-1.10	-1.04
lmo2724	DNA binding 3-demethylubiquitin-9-3-methyltransferase domain-containing protein	3.40	1.80	2.86	4.42	11.78	3.17	8.44	3.28	2.26
lmo0019	transmembrane protein	2.30	3.50	2.92	31.00	15.50	48.00	24.00		
lmo0043	<i>arcA</i>	3.90	3.50	3.68	119.00	59.50	81.00	40.50		2.72

Fig. 2. Heat map comparing FC of genes or proteins identified as positively regulated by σ^B through microarray, RNA-Seq or proteomics. Heat map comparing FC of genes and proteins identified, in *L. monocytogenes* 10403S stationary phase cells, as positively regulated by σ^B through (i) proteomics data reported here (reported as both replicate 1 and 2; shown as Rep. 1 and Rep. 2), (ii) a meta-analysis of previous microarray studies (Raengpradub *et al.*, 2008; Ollinger *et al.*, 2009; Oliver *et al.*, 2010) and (iii) previously reported RNA-Seq data [all four comparisons reported by Oliver *et al.* (2009) are shown; indicated as Comp. 1 through 4]. Blank cells indicate that a given protein was not identified in the proteomic experiments. The heat map colour scale applies to microarray data columns, proteomics data columns, and RNA-Seq data columns separately; red indicates negative FC, while green indicates positive FC values; the darker the respective colour the larger the absolute FC value is. Detailed descriptions of genes and encoded proteins can be found in Table S2.

multiple studies as σ^B -dependent (Fraser *et al.*, 2003; Sue *et al.*, 2003). lmo0265, which had a significant P^c value and a FC of 8.0 reported in the Oliver *et al.* (2010) microarray study, did not show evidence for differential expression in the two other array studies (Ollinger *et al.*, 2009; Raengpradub *et al.*, 2008), which used an EGD-e array that had an lmo0265 probe with low (71 %) homology to the 10403S lmo0265 gene, likely leading to a false-negative result in these two studies.

Two previous proteomic studies aimed to identify σ^B -dependent genes in *L. monocytogenes* 10403S. One study only used 2-DE (Abram *et al.*, 2008b), and although the other study used iTRAQ as well as 2-DE, it used *L. monocytogenes* cells grown to stationary phase in 0.5 M salt for iTRAQ analysis, which is different from the growth conditions used to generate the microarray data that were used for the meta-analysis detailed below. The study that used both iTRAQ and 2-DE (Abram *et al.*, 2008a) identified 17 proteins as positively regulated by σ^B , compared to 36 proteins identified here; 14 of these 36 proteins had also been reported as positively regulated by σ^B in this previous study. The 2-DE study by Abram *et al.* (2008b), on the other hand, found 10 proteins to be positively regulated by σ^B ; 7 of these proteins were also found among the 36 proteins we identified here as positively regulated by σ^B . Our study thus identified substantially more proteins as positively regulated by σ^B as compared to previous studies. Importantly, the σ^B -dependent proteins newly identified here provide novel insight into the direct and indirect regulation of cellular pathways by σ^B , as discussed below.

Consistent with previous transcriptional studies, proteomics identified few genes that show evidence for negative regulation by σ^B

Our quantitative proteomic comparison conducted in this study found only six proteins that displayed significantly lower levels in the parent strain as compared to the $\Delta sigB$ mutant; three of these proteins are encoded by a single operon (lmo1997, a mannose-specific PTS system IIA component; lmo1998, a sugar isomerase domain-containing protein; lmo2002, a mannose-specific PTS system IIB component; these showed FC of -1.56 , -1.60 and -1.59 , respectively). The other proteins with lower expression levels were lmo0427 (fructose-specific PTS system IIB

component), lmo0484 (haem-degrading monooxygenase, lsdG) and lmo2648 (similar to phosphotriesterase), which showed FC of -1.56 , -1.53 and -1.60 , respectively. The genes encoding these six proteins also had significant P^c values in the microarray meta-analysis and displayed negative FC ranging from -1.6 to -1.1 in three (lmo2648; lmo1998; lmo2002) or two (lmo0484; lmo0427; lmo1997) microarray studies in $\Delta sigB$ stationary phase cells (Oliver *et al.*, 2010; Ollinger *et al.*, 2009; Raengpradub *et al.*, 2008). No genes were identified as negatively regulated by σ^B in the RNA-Seq study (Oliver *et al.*, 2009). These findings are similar to previous analyses that show that few genes are consistently identified as being negatively regulated by σ^B (Palmer *et al.*, 2011), which is likely due to the fact that negative regulation would be indirect. The only example of negative regulation by σ^B for which the mechanism has been characterized was described for genes required for the synthesis of flagella, which are negatively regulated by σ^B through an antisense RNA mechanism (Toledo-Arana *et al.*, 2009).

Meta-analysis of microarray data and comparison with RNA-Seq data identified 134 genes that show evidence for σ^B -dependent transcript levels

A meta-analysis of three microarray studies (Oliver *et al.*, 2010; Ollinger *et al.*, 2009; Raengpradub *et al.*, 2008) and comparison with RNA-Seq data (Oliver *et al.*, 2009) that also probed the stationary phase σ^B regulon in *L. monocytogenes* 10403S identified 134 genes that show strong evidence for being positively regulated by σ^B . These 134 genes include (i) 118 genes that were identified by our meta-analysis as being under positive control by σ^B and (ii) 16 genes that were identified as showing σ^B -dependent transcript levels by RNA-Seq but not by the microarray meta-analysis (Figs 1 and 2). The 118 genes identified by our meta-analysis as being under positive control by σ^B include (i) 65 genes identified by both microarray meta-analysis and RNA-Seq, but not by proteomics (Group G; Table S2); (ii) 35 genes identified by microarray meta-analysis, but not by RNA-Seq or proteomics (Group B; Table S2); (iii) 15 genes identified by microarray meta-analysis, RNA-Seq and the proteomics comparison in this study (Group A; Table S2); and (iv) 3 genes identified by microarray meta-analysis and proteomics, but not by RNA-Seq (Group E; Table S2). Among these 118 genes, 101 were preceded by putative

σ^B -dependent promoters, including 63/65 genes in Group G, 20/35 in Group B, 15/15 in Group A and 3/3 in Group E. The 16 genes that were identified as showing σ^B -dependent transcript levels by RNA-Seq but not by the microarray meta-analysis included (i) 13 genes identified by RNA-Seq but not by microarray meta-analysis and proteomics (Group C; Table S2) and (ii) 3 genes identified by RNA-Seq and proteomics but not by microarray meta-analysis (Group F; Table S2).

Overall, the 134 genes identified here as showing σ^B -dependent transcript levels include two groups that are only supported by a single experimental approach, i.e. 35 genes that were identified only by microarray meta-analysis (Group B; Table S2) and 13 genes only identified by RNA-Seq (Group C; Table S2). A number of pieces of evidence support that the genes in Group B are indeed σ^B -dependent, including (i) 20/35 genes in Group B are preceded by putative σ^B consensus promoters and (ii) 4/35 genes in Group B are in operons that include genes that were identified as σ^B -dependent by other experimental approaches (i.e. RNA-Seq or proteomics). In addition, we found that the 38 genes identified as σ^B -dependent by microarray, but not by RNA-Seq (representing the 35 genes in Group B as well as three genes in Group E), show significantly higher RNA-Seq FC values ($P < 0.05$; one-sided Wilcoxon ranked sum test) as compared to genes not identified as σ^B -dependent by microarray or RNA-Seq, suggesting that many of these genes represent false negatives for σ^B dependence in RNA-Seq. A number of pieces of evidence also support that the genes in Group C (identified as positively regulated by σ^B through RNA-Seq but not by microarray meta-analysis or proteomics) are indeed σ^B -dependent, including (i) 8/13 genes in Group C are preceded by σ^B consensus promoters, (ii) 3/13 genes in Group C are in operons that include genes that were identified as σ^B -dependent by other experimental approaches (i.e. microarray meta-analysis or proteomics) and (iii) 7/13 genes had a significant P^c value and FC above 1.0 in all three microarray studies, but missed the FC cut-off of ≥ 1.5 in one or more microarray study. Two of the genes in Group C (*inlA* and *inlB*) are located in the same operon and are co-regulated by PrfA and σ^B (Kim *et al.*, 2005; Oliver *et al.*, 2009; Ollinger *et al.*, 2009; Raengpradub *et al.*, 2008) and were thus not identified as positively regulated by σ^B in the one microarray study that used a *prfA** genetic background, which expresses a constitutively active PrfA (Ollinger *et al.*, 2009).

The 134 genes identified as positively regulated by σ^B by the meta-analysis reported here included 21 genes where the corresponding proteins were also identified here as showing higher expression levels in the parent strain, as compared to the $\Delta sigB$ strain; an additional 15 proteins were identified as σ^B -dependent by proteomics only, for a total of 149 σ^B -dependent genes and proteins (Fig. S1). Statistical analyses showed that role categories ($n=21$) were not randomly distributed among these 149 genes ($P=0.0210$; Monte-Carlo simulation of Fisher's exact test).

Individual Fisher's exact tests for each role category showed that the role category 'energy metabolism' was significantly overrepresented among the 149 σ^B -dependent genes and proteins (Table S3), similar to data reported by Oliver *et al.* (2010), who found σ^B -dependent genes to be overrepresented in the 'energy metabolism' and 'cellular processes' role categories. The contributions of the σ^B regulon to energy metabolism are also supported by phenotypic data. For example, under energy stress in glucose-limiting media, a *L. monocytogenes* $\Delta sigB$ mutant was shown to grow more rapidly than the parent strain, while the parent strain displayed greater survival after the carbon source was depleted (Chaturongakul & Boor, 2004). In addition to 30 genes that grouped into the 'energy metabolism' category, the 149 σ^B -dependent genes and proteins identified here also represented 18 other role categories, including transport and binding proteins (22 genes) and cellular processes (14 genes) (Table S3), illustrating the extensive network of cellular functions regulated by σ^B .

Genes identified as positively regulated by σ^B by proteomics but not by transcriptional analyses largely represent false negatives in transcriptional approaches, but also include at least some genes that may show indirect regulation by σ^B

Considerable evidence supports that the 15 proteins that were identified as positively regulated by σ^B through proteomics only (Group D; Table S2) represent a number of genes that were likely false negatives for σ^B -dependent transcript levels in the microarray meta-analysis and/or the RNA-Seq data analysis. Evidence supporting this notion includes (i) 4/15 genes encoding these proteins are preceded by a putative σ^B -dependent promoter, (ii) 8/15 genes had a significant P^c value and FC above 1.0 in at least two microarray studies, but missed the FC cut-off of ≥ 1.5 in one or more microarray study and (iv) 14/15 genes only failed one or two FC or Q-value cut-offs in the RNA-Seq study by Oliver *et al.* (2009), which considered genes as σ^B -dependent if they met both Q-value and FC cut-offs in all four comparisons carried out in that study. When including studies that used *L. monocytogenes* strains other than 10403S [e.g. EGD-e (Hain *et al.*, 2008); FSL J1-194, FSL J1-208, FSL J2-071 (Oliver *et al.*, 2010)], we also found that eight genes in Group D had previously been reported as positively regulated by σ^B in at least one study; the only seven genes not previously reported, in any study reviewed here, as positively regulated by σ^B are lmo0643, lmo1046, lmo1349, lmo2047, lmo2743, lmo0110 and lmo1730 (Table S2).

Interestingly, a number of proteins that were identified as showing σ^B -dependent expression levels and were classified into Group D are annotated as having functions that relate to energy metabolism and transport and binding functions. For example, Group D proteins Lmo2695, Lmo2696 and Lmo2697 are encoded as part of the *dhaK* operon, which includes a σ^B consensus promoter upstream of lmo2695. Among the genes encoding these three proteins, lmo2696

and lmo2697 had a significant P^c value (<0.05) in the microarray meta-analysis and this operon was previously reported to be σ^B -dependent in strain EGD-e (Hain *et al.*, 2008). σ^B thus appears to regulate transcription of these genes, which encode subunits of dihydroxyacetone kinase, an enzyme that plays a role in energy metabolism. The Group D protein Lmo1422 is encoded in an operon that includes lmo1421 (which was also identified as σ^B -dependent; Group C, Table S2). This operon is preceded by a σ^B consensus promoter upstream of lmo1421; and lmo1421 has been reported to be transcribed in a σ^B -dependent manner (Fraser *et al.*, 2003; Sue *et al.*, 2003). While lmo1421 and lmo1422 were initially annotated as glycine betaine/carnitine/choline ABC transporters, they have been reported to be part of a bile exclusion system co-ordinately regulated by σ^B and PrfA (Fraser *et al.*, 2003; Sleator *et al.*, 2005; Sue *et al.*, 2003). The gene encoding the Group D protein Lmo2666, annotated as a galactitol-specific PTS system IIB component, was previously found to be positively regulated by σ^B in *L. monocytogenes* strains representing lineages I and IIIB (which has been redesignated IV), but not in the lineage II strain 10403S (Oliver *et al.*, 2010). Lmo2666 has also been found to be differentially regulated in comparisons between *L. monocytogenes* EGD-e and mutants that did not express the regulators CodY and DegU (Bennett *et al.*, 2007; Williams *et al.*, 2005), suggesting regulatory interactions that govern transcription of this gene.

The Group D proteins Lmo0398 and Lmo0399 (annotated as fructose-specific PTS system IIA and IIB components) are also encoded by an operon. Lmo0398 and lmo0399 showed a significant P^c value in our microarray meta-analysis and showed FC values >10 in comparisons of wild-type and $\Delta sigB$ strains reported by Raengpradub *et al.* (2008) and Oliver *et al.* (2010) (P value <0.01 in both studies), but were not found to have significantly different transcript levels in the study by Ollinger *et al.* (2009), which used a *L. monocytogenes* *prfA*^{*} strain. As described above for the *inlAB* operon (Group C), these genes thus are likely co-regulated by PrfA and σ^B and hence were not identified as positively regulated by σ^B in the microarray study with the *prfA*^{*} strain. Lmo0398 and lmo0399 were previously proposed to play a role in σ^B -dependent regulation of carbohydrate metabolism during low nutrient energy stress conditions encountered during stationary phase (Chaturongakul *et al.*, 2008). Interestingly, the gene encoding Lmo1539, a Group D protein involved in the uptake of glycerol, which *L. monocytogenes* can utilize as a carbon source for growth (Joseph *et al.*, 2008; Premaratne *et al.*, 1991), was found to be negatively regulated by σ^B in the 10403S microarray analyses conducted by Raengpradub *et al.* (2008) and Oliver *et al.* (2010) (FC=0.2 and 0.5, respectively; $P<0.01$ in both studies), but was not found to be significantly differentially expressed in the microarray comparison of a *prfA*^{*} and *prfA*^{*} $\Delta sigB$ strain (Ollinger *et al.*, 2009). However, lmo1539 was found to be upregulated during intracellular replication (Chatterjee *et al.*, 2006) and

in a σ^B -dependent manner in the intestine (Toledo-Arana *et al.*, 2009) using *L. monocytogenes* strain EGD-e, during stationary phase stress using *L. monocytogenes* strains FSL J1-194, FSL J2-071 and FSL J1-208 (Oliver *et al.*, 2010), and showed evidence for positive regulation by σ^B using *L. monocytogenes* strain 10403S when FPSS (fluorophenylstyrene sulfonamide) was used to inhibit σ^B activation (Palmer *et al.*, 2011). The gene encoding the Group D protein Lmo0643, annotated as a transaldolase, was found to be significantly downregulated in the *prfA*^{*} parent strain as compared to the *prfA*^{*} $\Delta sigB$ strain (FC=0.3, P value <0.05) (Ollinger *et al.*, 2009), whereas it was not found to be significantly differentially expressed in the parent and $\Delta sigB$ strain comparisons by Raengpradub *et al.* (2008) and Oliver *et al.* (2010). Overall, a number of the proteins classified in Group D thus appear to be encoded by genes that are co-regulated by multiple transcriptional regulators, in particular PrfA and σ^B , supporting previously proposed models that hypothesized that σ^B plays a role in a number of regulatory networks (Fig. S2) and highlighting a role of PrfA and σ^B interaction in regulating some aspects of energy metabolism in stationary phase *L. monocytogenes*.

Identification of a 5' UTR, upstream of lmo1349, that functions as a metabolite-binding riboswitch, which is linked to a σ^B -dependent glycine cleavage pathway

While some of the 15 proteins that were identified here as σ^B -dependent by proteomics only (Group D, Table S2) appear to actually be encoded by genes that show σ^B -dependent transcription (representing false negatives in the microarray meta-analysis and/or RNA-Seq analysis), we hypothesized that some of the proteins classified in Group D are regulated by σ^B through post-transcriptional mechanisms. Genes that are regulated post-transcriptionally would be expected to show discrepancies between mRNA expression levels and the expression levels of their protein products (Hegde *et al.*, 2003; Picard *et al.*, 2009; Zhang *et al.*, 2010). In our case, we thus initially analysed genes that showed σ^B -dependent protein levels, but not σ^B -dependent transcript levels (i.e. proteins in Group D). As transcripts of several *L. monocytogenes* genes are known to be regulated post-transcriptionally, particularly through mechanisms affecting the 5' UTR of the transcripts (Loh *et al.*, 2006), we initially compared 5' UTR lengths among three categories of genes including genes identified as positively regulated by σ^B through (i) RNA-based methods, but not by our proteomics data (Groups B, C, G; Table S2); (ii) proteomics data only (Group D, Table S2); and (iii) all three methods (RNA-Seq, microarray meta-analysis and proteomics) (Group A, Table S2). A two-sided and one-sided Wilcoxon ranked sum test found no significant differences in 5' UTR lengths between all three groups ($P>0.05$).

As one mechanism for post-transcriptional regulation is through riboswitches, we further evaluated the 5' UTR

Fig. 3. Glycine cleavage system in *L. monocytogenes* 10403S. (a) Operon map for glycine cleavage system genes, *gcvT*, *gcvPA* and *gcvPB*, including the putative promoter and the aptamer and putative terminator of the glycine riboswitch upstream of the genes. The FC and *P* values for the genes from Raengpradub *et al.* (2008), Ollinger *et al.* (2009) and Oliver *et al.* (2009, 2010) are included. *In the Oliver *et al.* (2010) study, the FC and *P* values for *gcvT* in lineage I, IIIA, and IIIB (IV) strains were FC=1.7, *P*=0.044; FC=0.5, *P*=0.006; and FC=1.4, *P*=0.625, respectively. (b) Extended operon map showing the sequences and positions for the glycine riboswitch (aptamer and putative terminator) based on RNA-Seq data (unpublished). (c) Structure of the glycine riboswitch aptamer based on the putative base pairing for lmo1348 provided by Mandal *et al.* (2004); the leader-linker interaction is based on the data for lmo1348 provided by Sherman *et al.* (2012). The terminator was identified using TransTermHP (Kingsford *et al.*, 2007).

regions of the genes encoding the nine Group D proteins that (i) were identified as positively regulated by σ^B in our proteomics analysis; (ii) are encoded by genes that were not identified by RNA-based methods as showing σ^B -dependent transcript levels; and (iii) are not preceded by a σ^B consensus promoter. One of these proteins, Lmo1349, was found to be encoded by the second gene in the three-gene *gcvT* operon. In addition to lmo1349 (*gcvPA*), which encodes the glycine dehydrogenase subunit 1, this operon includes lmo1348 (*gcvT*), which encodes the glycine cleavage system T protein, and lmo1350 (*gcvPB*), which encodes the glycine dehydrogenase subunit 2 (glycine cleavage system P2-protein). Interestingly, the first gene in this operon has a 5' UTR that has previously been annotated as a glycine riboswitch (Mandal *et al.*, 2004) (Fig. 3). This glycine riboswitch is conserved across many bacterial species and occurs upstream of genes encoding proteins of the glycine cleavage system, which facilitates use of glycine as an energy source (Butler *et al.*, 2011; Erion & Strobel, 2011; Huang *et al.*, 2010; Kwon & Strobel, 2008; Mandal *et al.*, 2004; Sherman *et al.*, 2012). In *Bacillus subtilis*, it has been shown that this riboswitch regulates transcription of the downstream *gcvT* operon (Mandal *et al.*, 2004; Phan & Schumann, 2007). In the absence of glycine, this riboswitch allows the formation of a terminator upstream of *gcvT*; consequently, approximately 70 % of the transcripts from the upstream promoter are represented by a short, ~200 nt, transcript, while the other 30 % of transcripts represent the full-length 4 kb *gcvT* operon. In the presence of glycine, which binds to the riboswitch, the terminator appears to be destabilized and consequently ~70 % of the transcript is full-length (Mandal *et al.*, 2004). Glycine-dependent activation of this riboswitch has also been reported in other species (Sherman *et al.*, 2012). We hypothesize that σ^B -dependent formation of glycine is responsible for activating transcription of the *gcvT* operon via this riboswitch, as we found lmo2539 (*glyA*; Group B, Table S2), which encodes a serine hydroxymethyltransferase that catalyses the interconversion of L-serine to glycine (Stauffer, 1996), to be positively regulated by σ^B . In addition to the σ^B -dependent production of glycine, *L. monocytogenes* also includes glycine betaine/carnitine/choline ABC transporters that are encoded by σ^B -dependent genes, suggesting that the multiple σ^B -dependent glycine synthesis and uptake systems may facilitate indirect σ^B -dependent activation of the glycine riboswitch upstream of the *gcvT* operon in *L. monocytogenes*.

As the mechanism proposed here for σ^B -dependent expression of the proteins in the *gcvT* operon involves transcriptional regulation through the glycine riboswitch, one might expect that the three genes in the *gcvT* operon should actually show differential expression in transcriptome analyses of *L. monocytogenes* $\Delta sigB$ strains; neither our microarray meta-analysis nor previous RNA-Seq data found evidence for σ^B -dependent transcription of genes in the *gcvT* operon, even though lmo1348 (*gcvT*) was found

to show significantly higher transcript levels in the parent strain as compared to a $\Delta sigB$ strain (FC=2.1; $P=0.002$) in one microarray study (Raengpradub *et al.*, 2008). We propose that the relatively minor differences in transcript levels for these three genes (as supported by *B. subtilis* data that show that even in the absence of glycine a considerable proportion of the *gcvT* operon transcripts were still of full length) were not detectable by microarray, but were detectable by the proteomics tools. Additional indirect σ^B -dependent post-transcriptional regulatory mechanisms cannot be excluded though and future detailed dissection of the σ^B -dependent regulation of glycine cleavage systems will be necessary, particularly since one previous proteomics study (Abram *et al.*, 2008b) also found evidence for σ^B -dependent protein levels in exponential phase *L. monocytogenes* grown in defined media with salt, for GcvH, which encodes another component of the glycine cleavage system outside the *gcvT* operon.

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