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Transcriptome signatures of class I and III stress response deregulation in *Lactobacillus plantarum* reveal pleiotropic adaptation

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Abstract

Background: To cope with environmental challenges bacteria possess sophisticated defense mechanisms that involve stress-induced adaptive responses. The canonical stress regulators CtsR and HrcA play a central role in the adaptations to a plethora of stresses in a variety of organisms. Here, we determined the CtsR and HrcA regulons of the lactic acid bacterium *Lactobacillus plantarum* WCFS1 grown under reference (28°C) and elevated (40°C) temperatures, using *ctsR*, *hrcA*, and *ctsR-hrcA* deletion mutants.

Results: While the maximum specific growth rates of the mutants and the parental strain were similar at both temperatures ($0.33 \pm 0.02 \text{ h}^{-1}$ and $0.34 \pm 0.03 \text{ h}^{-1}$, respectively), DNA microarray analyses revealed that the CtsR or HrcA deficient strains displayed altered transcription patterns of genes encoding functions involved in transport and binding of sugars and other compounds, primary metabolism, transcription regulation, capsular polysaccharide biosynthesis, as well as fatty acid metabolism. These transcriptional signatures enabled the refinement of the gene repertoire that is directly or indirectly controlled by CtsR and HrcA of *L. plantarum*. Deletion of both regulators, elicited transcriptional changes of a large variety of additional genes in a temperature-dependent manner, including genes encoding functions involved in cell-envelope remodeling. Moreover, phenotypic assays revealed that both transcription regulators contribute to regulation of resistance to hydrogen peroxide stress. The integration of these results allowed the reconstruction of CtsR and HrcA regulatory networks in *L. plantarum*, highlighting the significant intertwinement of class I and III stress regulons.

Conclusions: Taken together, our results enabled the refinement of the CtsR and HrcA regulatory networks in *L. plantarum*, illustrating the complex nature of adaptive stress responses in this bacterium.

Keywords: CtsR, HrcA, *Lactobacillus plantarum*, Heat, Stress regulons

Background

Lactic acid bacteria (LAB) are Gram-positive bacteria that occupy a variety of habitats. LAB are acid tolerant and produce lactate as a major metabolic end-product, thereby generating preservative characteristics to fermented foods and beverages. Due to their long history of use in food products, LAB are generally regarded as safe (GRAS) [1,2]. Next to their prominent role in food

fermentation, LAB can be found on plant materials and are among the natural inhabitants of the gastrointestinal (GI) tract of animals and humans [3-5]. Specific *Lactobacillus* strains are marketed as probiotics which are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' [6]. The gastrointestinal tract is the site of action where probiotics are predominantly considered to confer these health benefits, where they may inhibit colonization and infection by pathogens, or may strengthen the intestinal epithelial barrier, or modulate immune responses [7]. Probiotics encounter a variety of stresses during industrial production and storage, e.g. temperature shifts and low

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water availability during freeze- or spray-drying, or acid stress during storage. Moreover, during GI passage probiotic bacteria are exposed to acid stress in the stomach, as well as exposure to bile salts and digestive enzymes, while they also have to cope with severe nutrient-competition with the endogenous gut microbiota [8].

To persist under stress conditions, probiotics and LAB in general have an arsenal of molecular defense mechanisms [9-12]. Many stress conditions induce protein denaturation and aggregation, and bacteria, including lactobacilli, possess conserved chaperones and proteases to restore or remove misfolded or denatured proteins. This process has extensively been studied in the paradigm Gram-positive bacterium *Bacillus subtilis* using abruptly or constantly elevated temperatures as the inducing stress condition. The repertoire of heat shock responses in *Bacillus subtilis* was stratified in six classes depending on their mode of transcriptional regulation [13-15]. Several of these stress response classes observed in *Bacillus subtilis* are conserved among the LAB, including the highly conserved Class I regulon. Expression of the Class I stress regulon members is controlled by the repressor HrcA, which specifically binds to the inverted repeat element, CIRCE (controlling inverted repeat for chaperon expression), under non-stressed conditions. The highly conserved CIRCE element (TTAGCACTC-N9-GAGTGCTAA) is typically found in the promoter regions of the *groE* and *dnaK* operons, which encode the two chaperon complexes GroES-GroEL and HrcA-DnaK-GrpE-DnaJ, respectively [16]. The *hrcA* gene is commonly part of the *dnaK* operon, placing this gene under autorepression control. HrcA-repression is dependent on the availability of the GroELS complex and is relieved when the GroELS chaperon complex is not available, i.e. during stress conditions when non-native proteins arise [13]. The HrcA regulon is not only induced during heat shock, but is also activated by a variety of other stress conditions, including acid, bile, and salt stress [9-11,17]. The genes encompassed within the class III stress regulon appear to be less conserved among LAB, although the class III stress regulon repressor CtsR (class three stress gene repressor) appears to be quite consistently present in these bacteria. However, LAB appear to consistently lack the regulatory adaptor genes encoding for McsB and McsA [18,19]. CtsR specifically binds to a heptanucleotide repeat (A/GGTCAAA/T), referred to as the CtsR box [20]. This *cis*-acting regulatory element is commonly encountered in the promoter regions of *clpP* and several other, but not all, *clp* genes, which encode Clp-proteases that are involved in protein quality control during both stress and non-stress conditions [21]. ClpP mediated proteolysis removes misfolded proteins from the cell, but Clp proteases can also function in cellular differentiation processes [21]. In some organisms other transcription regulators, including HrcA, are involved in

co-regulation of the CtsR target genes [21,22]. In conclusion, HrcA and CtsR are key components in stress response regulation, which may include cross-regulation between their respective regulons.

Lactobacillus plantarum is encountered in several environmental niches, including fermented foods and the human GI tract, and specific strains are marketed as probiotics [23]. *L. plantarum* WCFS1, a single colony isolate of strain NCIMB 8826, has been shown to actively survive passage through the human digestive tract [24,25], and it was the first *Lactobacillus* species of which the complete genome sequence was determined [26]. Besides the genome sequence, advanced functional annotations, as well as sophisticated bioinformatics and mutagenesis tools have been developed, enabling the investigation of gene-regulatory mechanisms at the molecular level [27-29]. For example, the *hrcA* and *ctsR* regulon members could be predicted on basis of the conserved *cis*-acting elements involved, which has in part been confirmed experimentally [11,30-33]. Some of the HrcA and CtsR regulon members in *L. plantarum* WCFS1 have been detected through phylogenetic footprinting [32], large scale analysis of co-regulation of expression [33], or via DNA binding assays [30,31]. Moreover, gene-expression responses in *L. plantarum* have been unraveled for various stress conditions, including lactate [34], low pH [34], oxidative [35,36], solvent [37,38], bile [39], cold [37], and heat stress [37]. Analysis of available transcriptome data indicates that some but not all of the predicted HrcA and CtsR regulon members of *L. plantarum* WCFS1 are differentially expressed during these different stress challenges [33]. Despite the characterization of these stress responses, the exact regulons of HrcA and CtsR in *L. plantarum* remain not completely determined.

This paper describes the regulons of CtsR and HrcA at reference and elevated growth temperatures by determination of the whole-genome transcriptome patterns of *ctsR*, *hrcA*, and *ctsR-hrcA* deletion mutants [38]. The data revealed that the CtsR or HrcA deficient strains displayed altered transcription patterns of genes encoding functions involved in transport and binding of sugars and other compounds, primary metabolism, as well as cell envelope remodeling. Moreover, deficiency of both transcription factors elicited temperature-dependent and pleiotropic transcriptional adaptation of the cell. Finally, stress-phenotyping of the mutants revealed a role of both regulators in the regulation of oxidative stress tolerance.

Materials and methods

Strains and growth conditions

L. plantarum WCFS1 [26], Δ *ctsR* (NZ3410) [38], Δ *hrcA*::*cat* (NZ3425^{CM}) [38], and Δ *ctsR* Δ *hrcA*::*cat* (NZ3423^{CM}) [40] were grown in MRS (de Man-Rogosa-Sharpe) broth

(Difco, West Molesey, United Kingdom) in pH-controlled batch fermentations at 0.5 L scale in a Multifors mini-parallel fermentor system (Infors-HT Benelux, Doetinchem, the Netherlands). A single colony isolate of *L. plantarum* WCFS1 or its derivatives was used to inoculate 5 mL of MRS followed by overnight growth at 37°C. The full-grown culture was used to prepare a dilution range from 10⁻¹ to 10⁻⁶ in fresh medium and these dilutions were grown overnight. Subsequently, the culture density was assessed by determination of the optical density at 600 nm (OD₆₀₀) and the culture that had an OD₆₀₀ closest to 1.5 (representing logarithmically growing cells) was used to inoculate the fermentors at an initial OD₆₀₀ of 0.1. During fermentation the cultures were stirred at 125 rpm, the pH of the culture was maintained at 5.8 by titration of 2.5 M NaOH, and temperature was set at 28°C or 40°C. A biologically independent duplicate; i.e., derived from independent colonies and performed on separate days, was included for all strains and temperatures. Cells were harvested at an OD₆₀₀ of 1.0 for RNA isolation.

RNA isolation and microarray analysis

RNA extraction, labeling and hybridization, as well as data analysis were performed as described previously [41,42]. Briefly, following quenching and cell disruption by bead beating, RNA was isolated using the High Pure kit including 1 h treatment with DNaseI (Roche Diagnostics, Mannheim, Germany). The resulting RNA was reverse transcribed to obtain cDNAs which were labeled using Cyanine 3 or Cyanine 5 labels (AmershamTM, CyTMDye Post-labelling Reactive Dye Pack, GE Healthcare, UK). The cDNAs were hybridized (Additional file 1: Figure S1) on WCFS1-specific, custom-made Agilent arrays. Each microarray contained at least 2, but mostly 3 distinct probes for all of the genes detected within the genome. These probes were spotted in duplicate on each array, which was based on the Agilent 15 k format (GEO accession number GPL13984; <http://www.ncbi.nlm.nih.gov/geo/>). Subsequently, the slides were washed and scanned using routine procedures [41,42] and the obtained transcriptome profiles were normalized using Lowess normalization [43]. The data were corrected for inter-slide differences on the basis of total signal intensity per slide using Postprep [44]. The median intensity of the different probes per gene was selected as the gene expression intensity. This analysis resulted in genome-wide, gene expression levels for *L. plantarum* WCFS1, NZ3410, NZ3423^{CM}, and NZ3425^{CM}. CyberT was used to compare the different transcriptomes [45]. This analysis resulted in a gene expression ratio and false discovery rate (FDR) for each gene. Genes were considered significantly differentially expressed when FDR-adjusted p-values were < 0.05. The DNA microarray data is available under GEO accession number GSE31253.

Data analysis tools

Visualization of the genes displaying differential expression in the mutants as compared to the wild-type was performed by loading Excel files into the Cytoscape software suite [46]. Data were first ordered using the spring embedded sorting algorithm in the Cytoscape tool. Coloring of the edges (up- or downregulation of the mutants over wild type) and nodes (annotated main class) and structuring of the network were performed manually. The SimPheny™ software package (Genomatica InC., San Diego, USA) loaded with the *L. plantarum* WCFS1 genome-scale model [28] was used to visualize differentially expressed genes that encode enzymes in metabolic pathways. Over-represented main classes and subclasses in the transcriptome data were identified using the Biological Networks Gene Ontology (BiNGO) [47] Cytoscape plugin. MEME software [48] was used with default settings to predict conserved *cis*-acting motifs from 300 nt upstream regions preceding the predicted translation start of the first genes of the operons of all genes. Subsequently, MAST [49] was used to perform genome-wide searches for the MEME-predicted *cis*-acting elements of HrcA and CtsR [32,33].

Phenotypic assays

To determine growth efficiency of the different mutant strains, *L. plantarum* WCFS1 or its derivatives were grown in MRS at 28°C, 37°C, 40°C, or 42°C, and growth was monitored by OD₆₀₀ measurement during 72 hours (SPECTRAMax PLUS384, Molecular Devices, UK). The maximum specific growth rate was determined by taking the slope of 5 consecutive ln transferred OD data points that gave the highest number. To quantify the colony forming capacity at elevated temperature, the wild type and gene deletion derivatives were grown at 30°C, serially diluted on MRS agar plates, and incubated for 1 week at 30°C or 42°C. Hydrogen peroxide stress tolerance was measured as described before [38]. In short, PBS washed cultures (OD₆₀₀ = 1.0) were resuspended in PBS containing 40 mM hydrogen peroxide at RT and samples were taken from this suspension, every 5 min for 60 min, and colony forming units were enumerated by plating of serial dilutions. Bile resistance was monitored as described before [50]. Briefly, cultures were inoculated in MRS containing 0.1% porcine bile (Sigma, Zwijndrecht, The Netherlands) at 28°C and growth was monitored by OD₆₀₀ determination (SPECTRAMax PLUS384, Molecular Devices, UK). H₂O₂ inactivation data were compared by fitting a reparameterized Weibull model according to Metselaar *et al.* [51]:

$$\log_{10}(N_t) = \log_{10}(N_0) - \Delta \left(\frac{t}{t_{\Delta D}} \right)^\beta$$

in which Δ is the number of decimal reductions, $t_{\Delta D}$ the time needed to reduce the initial number of

microorganisms with Δ decimals (min), and β a fitting parameter that defines the shape of the curve. Δ was set at 4 and the other parameters were estimated using Excel 2010. Two-sided Student's *t*-test was used for statistical analysis and $p < 0.05$ was considered significant.

Results

HrcA and CtsR are involved in the heat stress response of *L. plantarum*

HrcA and CtsR are regulators of class I and class III stress responses, respectively, including heat induced stress [13]. The role of these repressors at reference and elevated temperature was investigated in *L. plantarum* and its previously constructed derivatives that are deficient in either CtsR or HrcA alone, or both [38]. The maximum specific growth rate of the Δ ctsR, Δ hrcA::cat, and Δ ctsR Δ hrcA::cat strains at 28, 37, and 40°C did not differ from the *L. plantarum* WCFS1 wild-type strain (Figure 1). These findings expand earlier observations demonstrating unaltered growth characteristics of another *L. plantarum* ctsR mutant relative to its parental strain at 28°C [31]. However, although the maximum specific growth rate of Δ hrcA::cat was comparable to the wild-type at 42°C, the Δ ctsR and Δ ctsR Δ hrcA::cat mutants displayed 2.0- and 4.1-fold ($p < 0.001$; Figure 1) decreased specific growth rates, respectively. This result indicates that CtsR is required to sustain normal specific growth rates at 42°C. When serial dilutions of stationary phase cultures grown at 30°C were spotted on MRS plates, followed by continued incubation at 30°C, the wild-type and mutant strains gave approximately equal numbers of colonies, which were in all cases within the range anticipated for full-grown cultures. This observation indicates

that HrcA and CtsR do not influence the colony forming unit (CFU) numbers of *L. plantarum* WCFS1 at 30°C. Notably, when the plates were incubated at 42°C, the wild type strain generated approximately 100-fold lower CFU as compared to incubation at 30°C ($p < 0.001$). Importantly, the CFU numbers obtained with the Δ ctsR mutant were even stronger reduced at 42°C ($p < 0.001$), and this effect was even more pronounced for the Δ ctsR Δ hrcA::cat mutant (Figure 2). Conversely, CFU numbers for the mutant lacking a functional hrcA were not significantly different at 30°C, and 42°C, indicating that this mutation contributes to increased robustness as compared to the wild-type at this elevated temperature (Figure 2).

Transcriptional response of *L. plantarum* during heat stress

To investigate the transcriptional response of *L. plantarum* to elevated temperature and the role of CtsR and HrcA herein, transcriptome profiles of *L. plantarum* WCFS1 at control and elevated temperatures were determined. The control temperature of 28°C and elevated temperature of 40°C were selected since *L. plantarum* wild type displays similar specific growth rates at these temperatures as compared to the CtsR and HrcA deficient derivatives (see above). This prevents blurring of the results by genes responding to differential specific growth rates. When comparing the transcriptomes obtained for the wild-type strain at the two temperatures, more than 1000 genes were significantly differentially expressed and 488 genes (exclusive genes with phage and prophage related functions) were more than 2-fold up-regulated or downregulated (Additional file 2: Table S1).

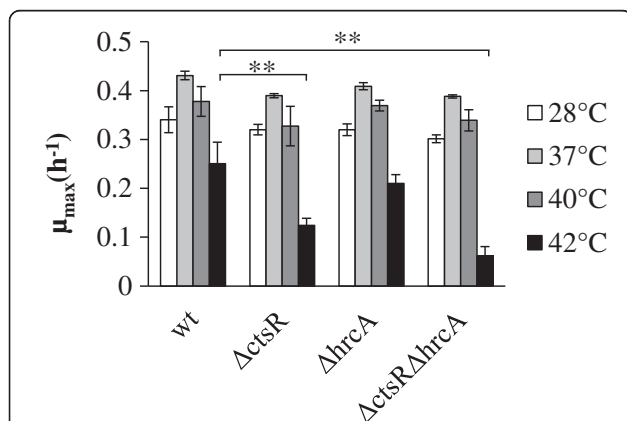


Figure 1 Maximum specific growth rates of *L. plantarum* WCFS1 (wt), NZ3410 (Δ ctsR), NZ3425^{CM} (Δ hrcA), and NZ3423^{CM} (Δ ctsR Δ hrcA). Specific growth rates are shown for reference (28°C) and elevated (37°C, 40°C, and 42°C) temperatures as indicated in the figure legend. Asterisks indicate p -value < 0.001 . Data shown are mean \pm standard deviation of 3 independent experiments.

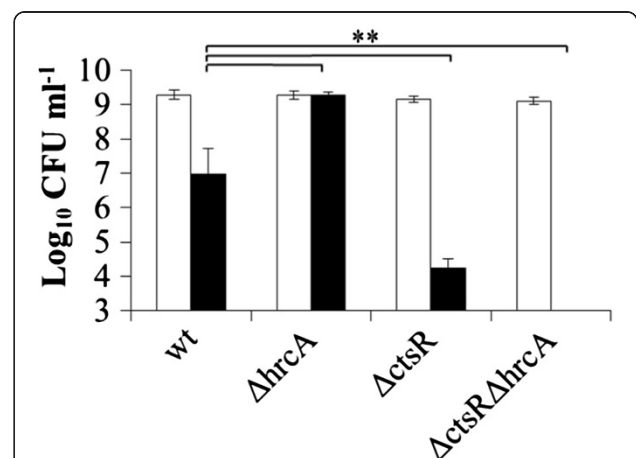


Figure 2 Involvement of CtsR and HrcA in the ability to form colonies at elevated temperature. *L. plantarum* WCFS1 (wt), NZ3410 (Δ ctsR), NZ3425^{CM} (Δ hrcA), and NZ3423^{CM} (Δ ctsR Δ hrcA) cultures were serially diluted on MRS plates and incubated at control (30°C; white bars) or elevated temperature (42°C; black bars). Asterisks indicate p -value < 0.001 . Data shown are mean \pm standard deviation of 3 independent experiments.

At 40°C *hrcA* expression was reduced, while that of *groEL* and *groES* were induced. In addition, *clpP*, *clpB*, and *clpE*, expression levels were induced at the elevated temperature. Of the other (predicted) HrcA or CtsR regulon members (see Table 1) only *hsp1* (small heat shock protein 1, which has been shown to be regulated by CtsR [31] and is also predicted to be regulated by HrcA [11]) was induced. In addition, at 40°C many genes coding for proteins with regulatory functions were transcribed at an elevated level, suggesting that their regulons contribute to maintenance of normal specific growth rates at this elevated growth temperature, while genes coding for proteins involved in degradation of proteins, peptides, and glycopeptides were repressed. Other transcriptional changes observed at elevated temperature were the downregulation of the capsular polysaccharide (*cps*)-clusters 1, 3, and 4, while many cell surface proteins, including *csell*, encoding one of 9 cell surface complexes (*lp_2173-lp_2175* (50)) were upregulated. Moreover, the majority of genes required for membrane lipid biosynthesis were down-regulated, including genes encoding fatty acid elongation proteins (*fab*), acyl carrier proteins (ACP), and acetyl-CoA carboxylases (ACC). The *fab*-locus encompasses 13 genes, which were all repressed at least 3.3-fold. In addition, expression levels of *dak1A*, involved in glycerolipid metabolism, and cyclopropane-fatty-acyl-phospholipid synthase (*cfa-1*) were increased, while its paralogue *cfa-2* was repressed. These results strongly suggest that *L. plantarum* adapts its cell envelope in response to growth at elevated temperature.

Impact of CtsR and HrcA deficiency on expression of their predicted regulons members

To unravel the role of HrcA and CtsR regulation in adaptation to growth at elevated temperatures, we evaluated the transcriptome profiles of the $\Delta ctsR$, $\Delta hrcA::cat$, and $\Delta ctsR\Delta hrcA::cat$ mutants grown at 28°C and 40°C (Figure 3). Relative to the wild-type strain, the expression of the *ctsR* gene was dramatically decreased in the mutants that lack a functional *ctsR* gene copy (161- to 984-fold), irrespective of the temperature of growth, confirming the integrity of the *ctsR* mutation in these strains (Table 1). Similarly, *hrcA* was decreased in the $\Delta hrcA::cat$, and $\Delta ctsR\Delta hrcA::cat$ mutants as compared to the wild type (145- to 241-fold; Table 1). The predicted HrcA and CtsR promoter binding motifs (*cis*-elements) [32,33] were used for MAST [49] analyses to predict the members of the HrcA and/or CtsR regulons, revealing several genes that appear to harbor the *cis*-acting motif of at least one of the transcription regulators (Table 1). Several of the CtsR regulon members that have previously been experimentally verified [31], were transcribed at higher levels in the $\Delta ctsR$ and $\Delta ctsR\Delta hrcA::cat$ mutants grown at 28°C as compared to the wild-type, including *clpP*, *clpE*, *clpB*, *clpC*, *hsp1*, and *spx1* (Figure 3 and Table 1). In addition, a gene with

unknown function (*lp_2061*) and an operon including 2 proteases (*hslU* and *hslV*) were expressed at elevated levels in the $\Delta ctsR$ strain. Of the predicted *hrcA* regulon members (Table 1), no altered expression pattern was detected for the *grpE*, *dnaK* and *dnaJ* genes, which are located in the same operon as *hrcA*, while *groEL* and *groES* expression patterns were increased in the $\Delta hrcA::cat$ mutant, at 28°C. A gene with unknown function (*lp_1880*) and an integrase/recombinase (*lp_1268*) were differentially expressed in the $\Delta hrcA::cat$ and $\Delta ctsR\Delta hrcA::cat$ strains. Remarkably, the *hrcA* operon seems to have 2 CIRCE elements and a CtsR-targeted *cis*-element in its promoter region, which may suggest dual control of this regulon by both regulators. However, *hrcA* was not differentially expressed in the $\Delta ctsR$ mutant at control or elevated temperature. When identifying possible dually regulated genes, only *hsp1* had CtsR and HrcA *cis*-acting elements in the promoter region of this gene (Table 1), as was described previously [11]. This was supported by the upregulation of this gene in all three mutants compared to wild type at 28°C (Figure 3A and Table 1). It might be that sequence or position of the *cis*-acting elements matches with their expression level in the mutants. However, no significant correlation could be detected. Together this indicates that the deregulation of class I and/or class III stress responses by mutation of their regulators induces a partial alteration of expression of their (predicted) regulon members under the conditions tested. Besides the predicted regulon members, the transcription of genes classified to various functional categories appeared to be affected by *ctsR* and/or *hrcA* mutation, which will be discussed below.

HrcA and CtsR mutation affect expression of genes encoding proteins with diverse functions

Additional genes coding for proteins from several functional categories were displaying altered transcription levels in the $\Delta hrcA::cat$ and $\Delta ctsR$ mutants as compared to the wild type. The *hrcA* mutation led to induced transcription of 29 transcription regulator encoding genes, including transcription regulators belonging to the AraC, LysR, MarR and TetR/AcrR family regulators. Several genes involved in primary metabolism were induced in the $\Delta ctsR$ strain compared to the wild type. These genes were involved in a variety of central metabolism reactions, centering around pyruvate dissipation and fermentation related reactions, including *pox*, *pfl*, *pdh*, *pps*, *mae*, *als*, and *cit* (Figure 4; abbreviations are addressed in the Additional file 3:Table S2). In addition, genes involved in pentose-5-phosphate pathway, producing D-xylulose-5-phosphate, which can be used for nucleotide synthesis or energy production, (including *xpkA*, *tkt1*, *deoM*, *rpiA1*, *gntK*, and *xfp*) were induced in the $\Delta ctsR$ strain compared to the wild type (Figure 4).

Table 1 Fold-changes of predicted and verified CtsR and HrcA regulon members^a in the NZ3410 (Δ ctsR), NZ3425^{CM} (Δ hrcA::cat), and NZ3423^{CM} (Δ ctsR Δ hrcA::cat) strains compared with the wild type

ID ^b	Name	Function	p-value ^c	28°C				40°C			
				Δ ctsR	Δ hrcA	Δ ctsR Δ hrcA		Δ ctsR	Δ hrcA	Δ ctsR Δ hrcA	
CtsR											
lp_0786	<i>clpP</i>	Endopeptidase Clp, proteolytic subunit		2.42^d	-1.09	2.33		1.27	-1.29	1.12	
lp_1269	<i>clpE</i>	ATP-dependent Clp protease, ATP-binding subunit ClpE	2.0·10 ⁻¹⁰	2.27	-1.02	2.12		-1.01	-1.20	-1.12	
lp_1903	<i>clpB</i>	ATP-dependent Clp protease, ATP-binding subunit ClpB	5.0·10 ⁻¹⁰	7.01	1.00	6.92		4.24	-1.33	3.71	
lp_1018	<i>ctsR</i>	transcription repressor of class III stress genes		-696	-1.15	-984		-526	-1.31	-161	
lp_1019	<i>clpC</i>	ATP-dependent Clp protease, ATP-binding subunit ClpC		1.92	-1.09	1.84		1.76	-1.30	1.58	
lp_0129	<i>hsp1</i>	Small heat shock protein	3.9·10 ⁻¹¹	5.57	3.16	12.70		1.12	-1.38	1.21	
lp_2945	<i>lp_2945</i>	Aromatic acid carboxylase, subunit C (putative)	3.5·10 ⁻¹⁰	1.27	-1.08	1.57		1.21	1.20	1.46	
lp_2451	<i>lp_2451</i>	Prophage P2a protein 6; endonuclease	4.9·10 ⁻⁷	1.05	1.11	1.12		1.03	1.40	1.32	
lp_2926	<i>lp_2926</i>	Unknown	2.8·10 ⁻⁶	1.08	-1.08	-1.10		1.30	-1.19	1.05	
lp_2426 ^e	<i>lp_2426</i>	Prophage P2a protein 31; phage transcriptional regulator, ArpU family	2.8·10 ⁻⁶	-1.18	-1.56	-2.07		8.85	-1.87	1.31	
lp_2540	<i>lp_2540</i>	Unknown	4.0·10 ⁻⁶	1.09	-1.31	4.11		-1.27	1.27	-1.14	
lp_2541	<i>lp_2541</i>	ABC transporter, substrate binding protein	4.0·10 ⁻⁶	-1.15	-1.03	1.01		1.07	1.31	1.44	
lp_2542	<i>lp_2542</i>	ABC transporter, permease protein (putative)	4.0·10 ⁻⁶	-1.03	-1.12	-1.06		-1.02	1.09	1.15	
lp_2543	<i>lp_2543</i>	ABC transporter, ATP-binding protein	4.0·10 ⁻⁶	-1.18	1.02	1.27		-1.14	1.15	1.01	
lp_3530	<i>treP</i>	Trehalose phosphorylase	4.0·10 ⁻⁶	-1.20	-1.25	-1.05		2.30	-1.32	-1.13	
lp_2061	<i>lp_2061</i>	Unknown	4.0·10 ⁻⁶	1.38	1.53	1.47		-1.21	1.10	1.07	
lp_2029	<i>hrcA</i>	Heat-inducible transcription repressor HrcA	5.8·10 ⁻⁶	-1.32	-241	-147		1.15	-176	-145	
lp_2028	<i>grpE</i>	Heat shock protein GrpE	5.8·10 ⁻⁶	-1.04	1.48	1.23		-1.21	1.26	-1.27	
lp_2027	<i>dnaK</i>	Chaperone, heat shock protein DnaK	5.8·10 ⁻⁶	-1.23	1.30	1.16		-1.28	1.09	-1.43	
lp_2842	<i>lp_2842</i>	Transcription regulator, LysR family	6.7·10 ⁻⁶	1.08	1.14	-1.04		-1.17	-1.34	1.03	
lp_1843	<i>lp_1843</i>	Aldose 1-epimerase family protein	9.8·10 ⁻⁶	-1.06	-1.14	1.06		1.50	1.19	1.20	
lp_1845	<i>hslU</i>	ATP-dependent Hsl protease, ATP-binding subunit HslU	9.8·10 ⁻⁶	1.10	-1.02	1.23		1.65	1.08	1.44	
lp_1846	<i>hslV</i>	ATP-dependent protease HslV	9.8·10 ⁻⁶	1.16	1.14	1.31		1.78	1.11	1.50	
lp_1847	<i>lp_1847</i>	Integrase/recombinase, XerC/CodV family	9.8·10 ⁻⁶	1.22	1.22	1.36		1.73	1.11	1.36	
HrcA											
lp_0727	<i>groEL</i>	GroEL chaperonin	5.9·10 ⁻⁹	-1.19	2.00	1.59		-1.46	1.06	-1.50	
lp_0728	<i>groES</i>	GroES co-chaperonin	5.9·10 ⁻⁹	-1.21	2.13	1.62		-1.55	1.14	-1.50	
lp_2029	<i>hrcA^f</i>	Heat-inducible transcription repressor HrcA	2.9·10 ⁻¹⁴	-1.32	-241	-147		1.15	-176	-145	
lp_2028	<i>grpE</i>	Heat shock protein GrpE	2.9·10 ⁻¹⁴	-1.04	1.48	1.23		-1.21	1.26	-1.27	
lp_2027	<i>dnaK</i>	Chaperone, heat shock protein DnaK	2.9·10 ⁻¹⁴	-1.23	1.30	1.16		-1.28	1.09	-1.43	
lp_2026	<i>dnaJ</i>	Chaperone protein DnaJ		-1.13	1.05	1.17		-1.07	1.08	1.14	
lp_0726	<i>lp_0726</i>	Membrane-bound protease, CAAX family	1.0·10 ⁻⁷	1.90	-1.07	1.56		2.26	-1.22	2.44	
lp_0129	<i>hsp1</i>	Small heat shock protein		5.57	3.16	12.70		1.12	-1.38	1.21	
lp_0413	<i>plnQ</i>	Plantaricin biosynthesis protein PlnQ	6.9·10 ⁻⁷	-1.03	1.23	1.51		-2.14	-1.16	1.16	
lp_3578	<i>kat</i>	Catalase	1.0·10 ⁻⁶	1.02	1.02	1.03		1.28	-1.20	-1.13	
lp_3617	<i>tal3</i>	Transaldolase	1.7·10 ⁻⁶	-1.19	-1.04	1.22		1.26	1.14	-1.28	
lp_3618	<i>pts37A</i>	Sorbitol PTS, EIIA	1.7·10 ⁻⁶	1.03	1.02	1.33		4.51	1.33	1.26	
lp_3619	<i>pts37BC</i>	Sorbitol PTS, EIIBC	1.7·10 ⁻⁶	2.15	1.31	2.50		2.68	-1.40	-1.23	
lp_3620	<i>pts37C</i>	Sorbitol PTS, EIIC	1.7·10 ⁻⁶	1.00	-1.33	-1.10		1.88	1.19	1.46	
lp_3621	<i>srlM1</i>	Sorbitol operon activator	1.7·10 ⁻⁶	1.39	1.17	2.13		2.22	1.08	1.40	
lp_3622	<i>srlR1</i>	Sorbitol operon transcription antiterminator, BglG family	1.7·10 ⁻⁶	-1.36	-1.13	-1.05		2.17	1.01	1.30	

Table 1 Fold-changes of predicted and verified CtsR and HrcA regulon members^a in the NZ3410 (Δ ctsR), NZ3425^{CM} (Δ hrcA::cat), and NZ3423^{CM} (Δ ctsR Δ hrcA::cat) strains compared with the wild type (Continued)

lp_3623	<i>srID1</i>	Sorbitol-6-phosphate 2-dehydrogenase (EC 1.1.1.140)	1.7·10 ⁻⁶	-1.10	-1.35	-1.43	1.37	-1.16	1.88
lp_1268	<i>lp_1268</i>	Integrase/recombinase	3.7·10 ⁻⁶	-2.21	-1.10	-1.56	-3.07	1.49	-3.38
lp_0387	<i>lp_0387</i>	Unknown	2.4·10 ⁻⁶	1.18	1.04	1.25	1.06	-1.00	1.35
lp_1879	<i>hbsU</i>	DNA-binding protein	9.9·10 ⁻⁶	-1.14	1.04	-1.14	-1.23	1.04	-1.27
lp_1880	<i>lp_1880</i>	Unknown	9.9·10 ⁻⁶	-1.13	1.11	-1.14	-1.59	1.20	-1.71

^aAdapted from [1].

^bThe lp_number indicates gene number on *L. plantarum* WCFS1 chromosome [2].

^cp-value of the best match on the upstream sequence after comparing the canonical regulatory factor binding site. Values lower than 1.0·10⁻⁵ were included.

^dFold-changes in bold are significant (FDR adjusted p-value < 0.05).

^eThe cis-element is predicted to be in front of this operon that contains *lp_2426* until *lp_2431*, which all encode proteins of prophage P2a. Fold-changes are only given for *lp_2426*.

^fThe upstream region of *hrcA* contains two CIRCE elements. The second has a p-value of 8.3·10⁻⁹.

Moreover, genes involved in sugar metabolism, such as *scrB* (sucrose), *pbg* (glucose), *lac* (galactose), *ara* (ribulose), and *iol* (inositol), were induced in this strain, as were genes involved in transport of other unspecified carbohydrate substrates and organic acids. These genes included sucrose (*pts26BCA*), glucose (*pts32*), maltodextrin (*mdx*, *msmX*), mannitol (*pts2A*), mannose (*lp_3643*, *pts9*), arabinose (*araP*), trehalose (*pts4ABC*) and sorbitol (*pts37A*, *pts38BC*) transporters. These results illustrate the impact of CtsR deregulation on the expression of metabolic genes, mainly affecting functions of primary carbohydrate import and central metabolic pathways, which was not observed in the *hrcA*-deficient strain. Nevertheless, the *hrcA*-mutation led to repression of genes involved in transport and binding functions, like those involved in transport of phosphate (*pst*), amino acids (*cho*, *sdA*, *lp_1722*, and *lp_3324*), and unknown substrates. Taken together these observations illustrate that deregulation of CtsR or HrcA elicits different response-profiles of transport and metabolism functions.

In addition, the mutations of *hrcA* and/or *ctsR* appeared to play a role in the control of expression of some of the genes and functions that were affected by the temperature of growth in the wild-type strain (see above). Temperature-mediated regulation appeared to be (partially) lost in the Δ ctsR mutant (*cps1*), in the Δ hrcA::cat mutant (*fab* operon, *dak1A*, and *cfa2*), or in the Δ ctsR Δ hrcA::cat mutant [*lp_0988* (lipoprotein precursor), *cps1*, and *cfa2*] compared to that seen in the wild-type strain (Figure 5). This indicates that inactivation of both class I and III transcription regulation leads to deregulation of different combinations of cell envelope biosynthesis processes compared to deregulation of one of the regulators in a temperature-dependent way. Taken together, these findings indicate that some of the more prominent adaptations that the wild-type strain employs to combat elevated growth temperatures, appear to be deregulated in the HrcA and CtsR mutant strains.

Combined HrcA and CtsR deficiency elicits pleiotropic deregulation of the stress control network

To characterize the gene-regulation consequences of the *hrcA* and *ctsR* single mutation relative to the double mutation, the significant regulatory profiles were reconstructed in gene-regulation networks for these strains relative to the wild-type strain at both 28°C (Figure 3A) and 40°C (Figure 3B). A relatively large number of genes displayed significant differential expression when comparing the Δ ctsR Δ hrcA::cat and wild type strains grown at either 28°C (513 genes) or 40°C (603 genes). At 28°C, these genes included almost all differentially expressed genes of the Δ ctsR and Δ hrcA::cat strains (Figure 3A). Conversely, less than one quarter and less than one third of the genes differentially expressed in the double mutant at 28°C were affected in the *ctsR* and *hrcA* single mutation at 40°C, respectively. Genes that are not differentially expressed in the other mutants than the Δ ctsR strain comprised for instance induction of energy metabolism (genes associated with TCA cycle, sugars, and glycolysis) and transport and binding proteins (e.g. the PTS system) and comprised 24 genes associated with regulatory functions for the Δ hrcA::cat strain. Overlapping genes of the *ctsR* or *hrcA* single mutation grown at 40°C with the double mutant grown at both temperatures included genes associated with the pentose phosphate pathway (*tkt1A* and *tkt1B*) and cell division (*ftsQ*, *parB1*, *parA*, and *parB2*), for the *ctsR* mutation and included genes associated with transport and binding proteins (e.g. ABC transporters and multidrug transporter proteins) for the *hrcA* mutation. In addition, genes associated with the cell envelope (such as genes encoding cell surface proteins and genes involved in fatty acid biosynthesis) were differentially expressed in all three mutants at 40°C. All three mutants affect temperature-independently the *dak1B* operon that is involved in glycerolipid metabolism. Moreover, approximately one third of the genes appeared to be consistently affected by the Δ ctsR Δ hrcA::cat mutation at both growth temperatures. The genes consistently affected by

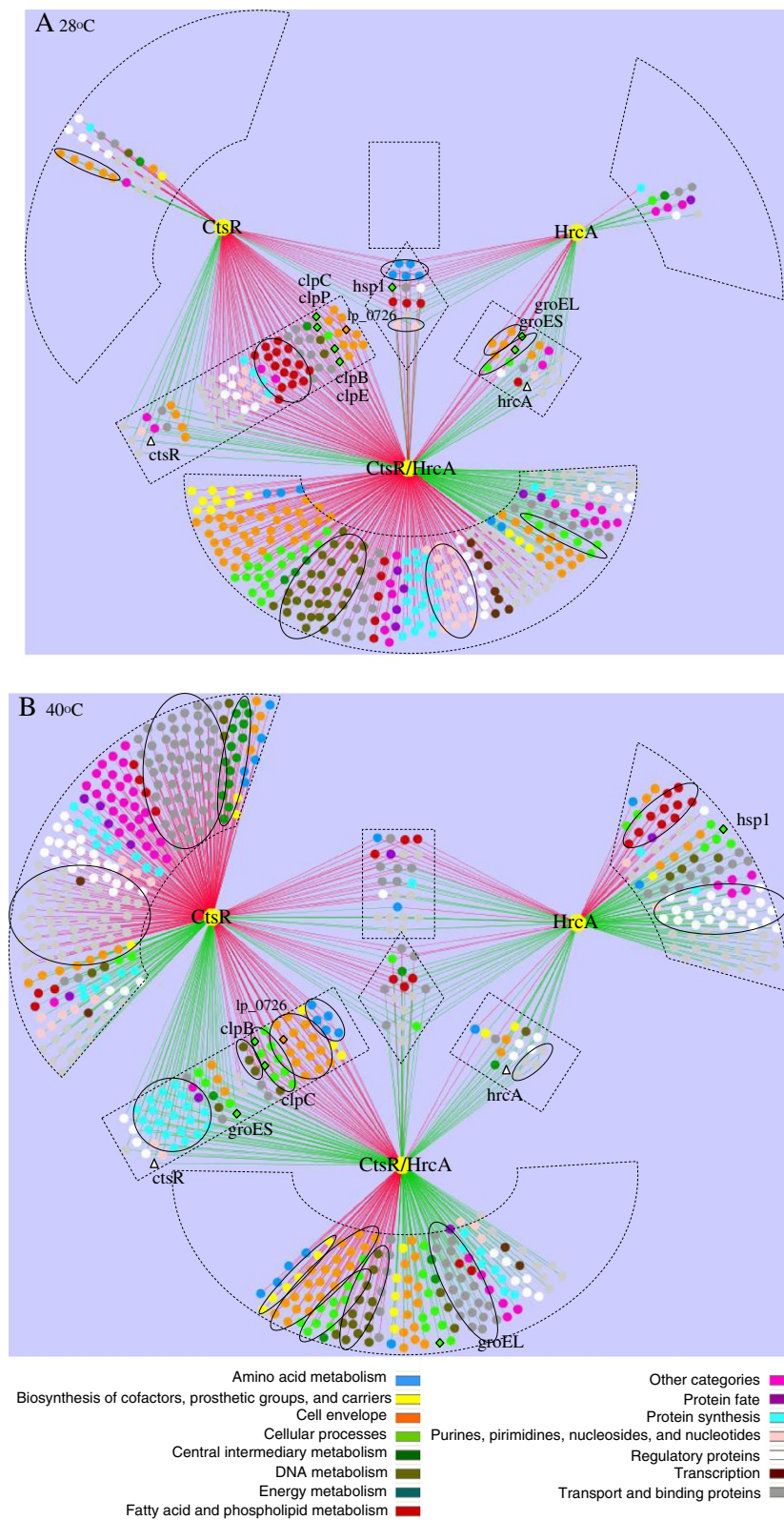


Figure 3 (See legend on next page.)

(See figure on previous page.)

Figure 3 Significantly differentially transcribed genes in NZ3410 (Δ *ctsR*), NZ3425^{CM} (Δ *hrcA*), and NZ3423^{CM} (Δ *ctsR* Δ *hrcA*) as compared to the wild-type grown at 28°C (A) or 40°C (B). Yellow colored octangular nodes represent the mutants and other colored nodes indicate main classes. The red and green lines indicate up- or downregulation, respectively. Triangle nodes indicate the CtsR or HrcA transcription regulator, diamond nodes indicate genes that are predicted to be part of the CtsR and/or HrcA regulon, whereas black ovals indicate over-represented main classes or subclasses in that particular main class. The main class "hypothetical proteins" was excluded.

the Δ *ctsR* Δ *hrcA*::*cat* mutation included induction of genes associated with the cellular processes (such as cell division protein-encoding genes *ftsZ*, *ftsA*, and *ftsQ*), DNA metabolism (DNA ligase *ligA*, DNA helicase *pcrA*, and DNA-directed DNA polymerase I *polA*), transport and binding proteins (Na⁺/H⁺ antiporter *napA2*, mannose PTS *pts9D*, and 10 ABC transporters), and cell envelope remodeling (*cps*-cluster 1, *fab*-locus, lipoprotein precursors *lp_1146* and *lp_1539*).

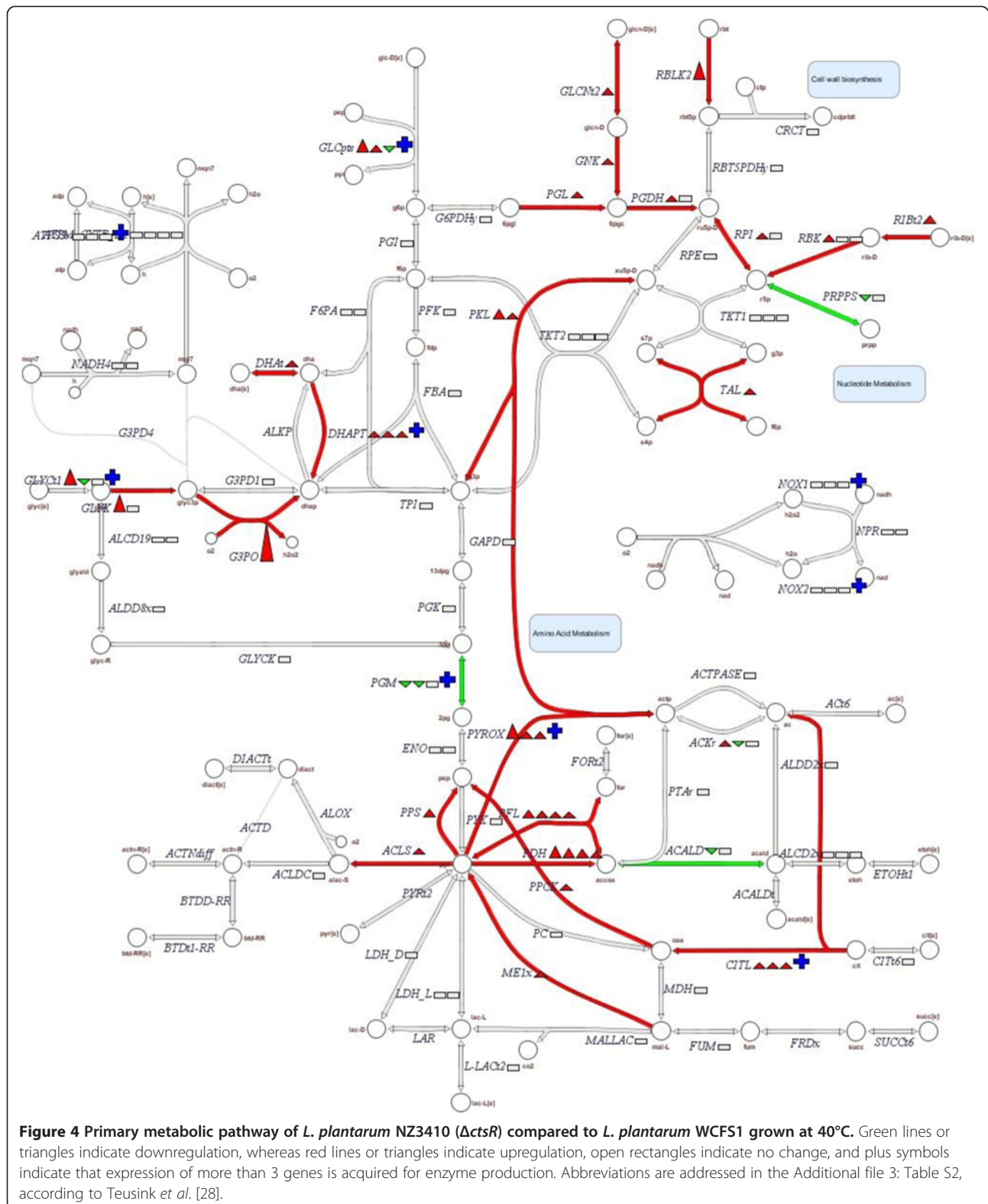
To further analyze the transcriptome profile of the Δ *ctsR* Δ *hrcA*::*cat* mutant grown at 28°C and 40°C, over-representative functional classes were identified (Figure 3). The BiNGO analysis tool was used to compare the Δ *ctsR* Δ *hrcA*::*cat* strain to the wild type, indicating that functional classes associated with cell envelope remodeling were induced, including the main class "cell envelope" with the sub-class "surface polysaccharides, lipopolysaccharides and antigens", which were induced at both temperatures of growth. In addition, the main classes "cellular processes" and "DNA metabolism" were temperature-independently induced. Temperature specific cell envelope remodeling was also apparent from over-representation of the main class "fatty acid and phospholipid metabolism" when grown at 28°C, while several subclasses of cell surface proteins ("LPxTG anchored", "membrane bound", and "other") were over-represented at 40°C. The main class "protein synthesis" was reduced in the *ctsR* and *hrcA* deficient strain only when grown at 40°C (Figure 3). Taken together, these data indicate that the cell employs highly adaptable, temperature-dependent systems involving many cell envelope associated functional classes to compensate for the absence of CtsR and HrcA regulation and that the expression of a large variety of additional genes appeared to be modulated compared to deregulation of one of the transcription factors.

HrcA and/or CtsR are required for hydrogen peroxide resistance regulation in *L. plantarum*

Besides involvement of CtsR and HrcA to combat temperature stress, it is known that the transcription factors are associated with other stresses. To evaluate whether *ctsR* and/or *hrcA* may be involved in gastrointestinal (GI)-tract survival, the overlap between the differentially expressed genes in the constructed mutant and the genes identified as being induced in the murine intestine [52] were compared, revealing a substantial overlap (26%) with the genes that were induced in the *ctsR* deletion mutant

compared to the wild type grown at 40°C. In addition, *L. plantarum* WCFS1 genes differentially expressed in response to porcine bile exposure [53], were also affected by the *ctsR* gene deletion when grown at 40°C (27%), albeit in the opposite direction. The possible role(s) of CtsR and/or HrcA in bile-stress response and tolerance was investigated by determination of the relative bile-tolerance of the three mutants relative to the wild type, revealing no significant role of either *ctsR* or *hrcA* in growth in the presence of bile (MRS containing 0.1% porcine bile; data not shown), suggesting that the *ctsR* and *hrcA* regulators do not play a role in bile tolerance. Although we cannot rule out the occurrence of polar effects that may have altered the expression some genes. In addition, the 3 mutant strains also displayed similar survival characteristics as the wild type in an *in vitro* assay that aims to mimic conditions encountered in the GI-tract [40]. Overall, these data suggest that although deregulation of CtsR and HrcA affects the expression of genes that were also differentially expressed under conditions relevant for the GI-tract, no experimental support could be found for a role of the *ctsR* and/or *hrcA* responses in survival under these conditions.

Another comparison between gene expression profiles of the Δ *ctsR* Δ *hrcA*::*cat* strain grown at 28°C and the response of *L. plantarum* to hydrogen peroxide [36], also revealed overlapping responses (21%). Analogous to what was observed for the bile responses (see above), the direction of gene expression changes were opposite for a number of genes affected both by H₂O₂ exposure, i.e., H₂O₂ induced expression of *lp_1163*, *dak1B*, *dak2*, *dak3*, *lp_1539*, the *cps1*-cluster and the Δ *ctsR* Δ *hrcA*::*cat* mutation elicited their repression. To evaluate the potential involvement of *ctsR* and *hrcA* in the oxidative-stress response and cognate tolerance towards H₂O₂ exposure, the wild type and mutant strains were grown to the exponential phase of growth (OD₆₀₀ of 1.0) and their rate of loss of survival upon lethal H₂O₂ exposure (40 mM H₂O₂ [54]), was followed over time by enumeration of colony forming units (Figure 6A). Compared to the wild-type strain, the Δ *ctsR* strain displayed similar rates of loss of survival, while the Δ *hrcA*::*cat* and especially the Δ *ctsR* Δ *hrcA*::*cat* strain were substantially reduced in their capability to tolerate H₂O₂ compared to the wild-type strain. This was already apparent after relatively short exposure to lethal peroxide stress levels, as is illustrated by the 10-fold reduced viability of the Δ *ctsR* Δ *hrcA*::*cat* strain after 10 min exposure to peroxide relative to the wild-type (Figure 6A).



To quantitatively compare the data, a reparameterized Weibull model was fitted to the inactivation data according to Metselaar *et al.* [51]. In this adjusted Weibull model, the

time to the first 4 decimal reductions (t_{4D}) was calculated (Figure 6B). Shaping parameter β was comparable between the wild type and the variants, ranging from 2.20 to 3.42.

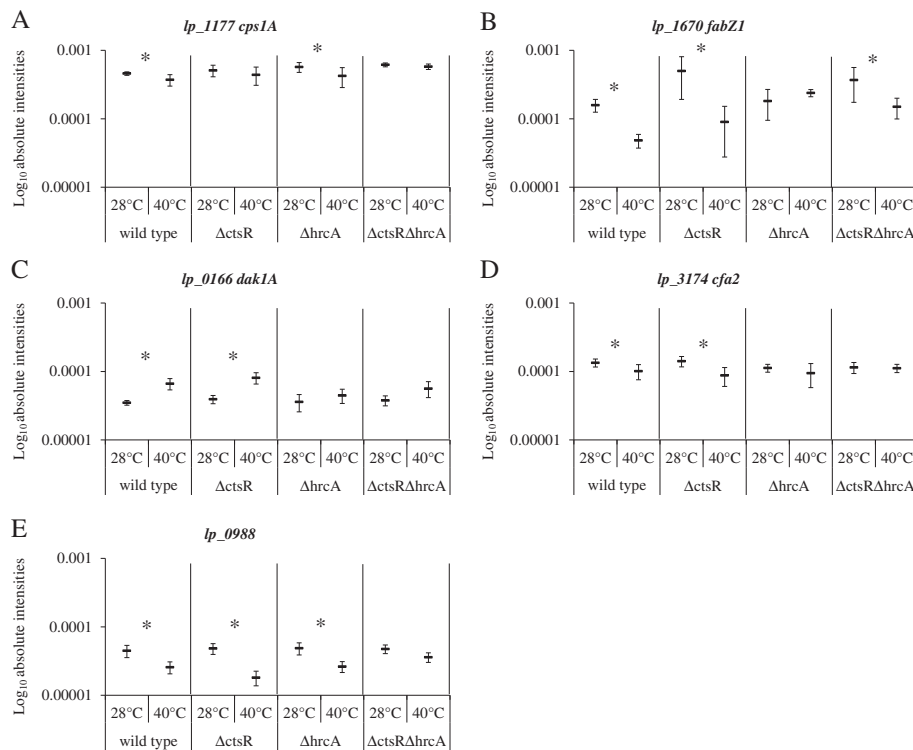


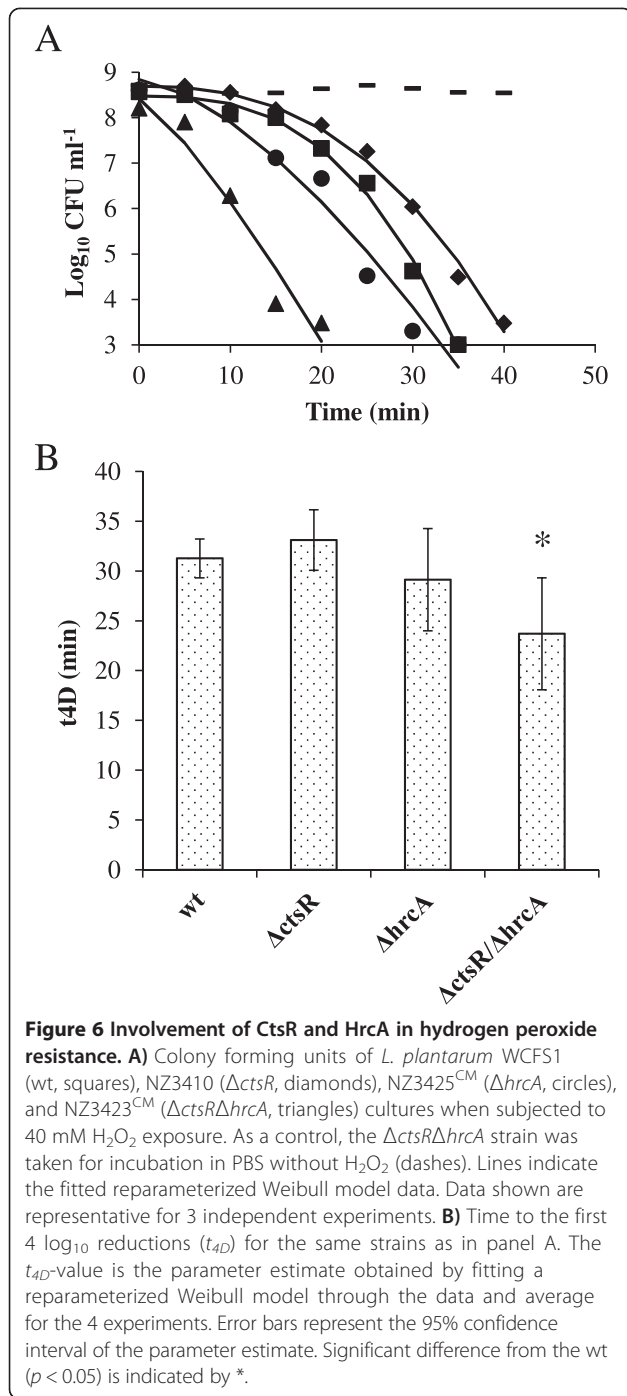
Figure 5 Box plots displaying the absolute intensity of the first gene of the *cps* cluster 1 (*lp*₁₁₇₇; A), the *fab*-operon (*lp*₁₆₇₀; B), *dak1A* (*lp*₀₁₆₆; C), *cfa2* (*lp*₃₁₇₄; D), and *lp*₀₉₈₈ (E) of *L. plantarum* WCFS1 (wild type), NZ3410 (Δ *ctsR*), NZ3425^{CM} (Δ *hrcA*), and NZ3423^{CM} (Δ *ctsR* Δ *hrcA*) grown at 28°C or 40°C. Asterisk indicates that (part) of the loci are significant differentially expressed when compared to the strains growth at the other temperature.

The Δ *ctsR* Δ *hrcA*::*cat* strain showed a significant lower t_{4D} compared to the wild type (Figure 6B). In conclusion, these data underline that deregulation of the HrcA and CtsR regulons influences H₂O₂ tolerance.

Discussion

In this paper, transcriptome profiles of *L. plantarum* WCFS1 were determined at reference and elevated temperatures. In the wild type strain, elevated temperature induced relatively major alterations in gene expression patterns. Many of these alterations suggest that adaptation of the cell envelope architecture is among the most important adaptive responses to elevated temperature. Relative to growth at 28°C, growth at 40°C induced the expression of several of the predicted CtsR and/or HrcA regulon members, e.g., *groES*, *groEL*, *clpP*, *clpB*, *clpE*, and *hsp1* [32,33]. This is in accordance with a study by Russo *et al.* that performed a global proteomic analysis of *L. plantarum* WCFS1 and a Δ *ctsR* mutant strain under optimal and heat stressed conditions [55]. Growth characteristics of the HrcA and CtsR deficient strains were considerably different from those of the wild-type, which was especially apparent from the mutants' phenotype at 42°C. At this temperature, CtsR appeared to be

required for maximum specific growth rates, while HrcA deletion increased colony forming capacity. Although the mechanism underlying the latter observation remains to be elucidated, it is most likely explained by culture-robustness heterogeneity, which in the *hrcA* deletion strain had shifted towards an average higher robustness level. While in several other organisms, *ctsR* mutation has been shown to enhance survival under stress conditions [56-59] this seemed not to be the case for *L. plantarum*, which is in agreement with previous studies in this organism [31]. Conversely, the enhanced colony forming capacity of the *hrcA* mutant at 42°C can be related to the deregulation of the class I stress response network, which is in agreement with the observation that similar mutations in other species enhanced their robustness under stress conditions [59,60]. However, in *Listeria monocytogenes*, *hrcA* deletion is suggested to be associated with increased heat sensitivity [61]. Overall, the impact of deregulation of the class I and class III stress responses on bacterial robustness is not very consistent and seems to vary considerably between species, which implies that extrapolation of the results obtained in specific species or strains to other organisms should be performed with great care.



To understand the HrcA and CtsR mediated stress adaptation, transcriptome analyses were performed comparing the transcriptional profiles of the HrcA- and CtsR-deficient strains at 28°C and 40°C. In addition, to unravel the intertwinement of the class I and class III stress response networks, a strain that lacked both repressors was included in this study. Transcriptome analyses of similar single mutants of either *hrcA* or *ctsR* have been reported for other species [4,62-66], and mutants

lacking both repressors have been constructed in *Listeria monocytogenes* [62] and in *Staphylococcus aureus* [66]. Nevertheless, to the best of our knowledge, this study presents the first transcriptome analysis of a strain that is deficient for both regulators. Of the predicted *hrcA* regulon members, no altered expression pattern was detected for the *grpE*, *dnaK* and *dnaJ* genes, which may be due to the involvement of additional regulatory factors in the control of expression of this chaperone genecluster. For example, it has been demonstrated that carbon catabolite control mediated through CcpA can affect the expression of the *groELS* and *dnaK* operons in *L. plantarum*, and that in a CcpA-deficient strain the expression of these functions could not be fully induced leading to reduced stress tolerance levels. Although these observations may not completely explain the lack of activation of *dnaK* operon expression in the *hrcA* mutant, they clearly imply that CcpA-activation could contribute to expression of the *dnaK* operon [67,68]. Moreover, in other organisms, e.g. in *Streptococcus pneumoniae*, the transcription of the *dnaK* and *groEL* operons is regulated by the medium concentration of Ca²⁺ as well as by HrcA [69], suggesting that additional environmental factors may modulate *hrcA* regulation of specific target genes and operons of its regulon. Although *lp_0726* is a predicted *hrcA* regulon member, its transcription level was increased in the Δ ctsR and Δ ctsR Δ hrcA::cat mutants. Besides transcriptional changes in the predicted regulons, *hrcA* and *ctsR* mutation led to a differential expression of genes involved in many functional classes during control and elevated temperature.

One of the deteriorating consequences encountered by cells growing at temperatures that can be considered as stress temperatures is denaturation and aggregation of proteins [70]. Lack of appropriate control of both the protein folding support (chaperones) and protein quality (Clp proteolysis) may elicit complementing gene expression responses involving genes belonging to different functional classes and affecting numerous cellular processes. These responses may include altered levels of regulator proteins in the cell, which may elicit changes in expression of a variety of regulons. Moreover, the levels of regulator protein may be differentially affected by the temperature of growth, leading to temperature-specific response of various regulatory networks, as was observed in this study. The drastic transcriptome changes elicited in the strain that lacks both CtsR and HrcA at control temperature is illustrative for the magnitude and complexity of the response required for the compensation for the deregulation of both class I and III stress responses. In addition, the results pinpoint that cell envelope remodeling plays an important role in the temperature adaptation in the wild-type strain, but is also prominently affected by the disruption of class I and III stress response networks. Intriguingly, it has been proposed that in prokaryotes heat shock responses

are predominantly controlled by the membrane physical state [71-73], which is in agreement with the finding that adaptive responses include many membrane and envelope modulating functions. Moreover, HrcA has been proposed to be a membrane-associated protein in *Helicobacter pylori*, and even an integral membrane protein in *Streptococcus pneumoniae*. In addition, the *hrcA*-regulon member GroELS of *Escherichia coli* is involved in folding of both soluble and membrane-associated proteins, while concomitantly stabilizing lipid membranes [49,74,75].

To understand the role of HrcA and CtsR in other stress conditions besides elevated temperature, the deregulation responses in the *hrcA* and *ctsR* mutant strains were compared with responses in the wild-type *L. plantarum* strain upon its exposure to specific stress conditions. The mutant lacking both *ctsR* and *hrcA* displayed significant decreased H₂O₂ tolerance levels compared with the wild type, suggesting that appropriate class I and III stress-regulation are required for optimal peroxide stress adaptation in *L. plantarum*. Downregulation of genes encoding proteins involved in membrane lipid synthesis (*dak1B*, *dak2*, *dak3*, and *lp_1539*) and cell wall (*cps1* cluster) in this mutant possibly induce cell envelope modifications that weaken the cell when exposed to peroxide stress. Furthermore, class I and class III stress responses were previously reported to be involved in oxidative stress tolerance in *Fusobacterium nucleatum*, which was associated to induction of ClpB and DnaK in response to H₂O₂ stress [76]. A potentially more indirect link may exist between the Clp protease and H₂O₂ stress responses in *B. subtilis*, where Clp protease activity is involved in regulation of Spx [21], which in its turn was shown to be induced upon H₂O₂ exposure [77].

Overall, deregulation of the CtsR and HrcA regulons in *L. plantarum* elicits compensatory responses that can be characterized by differential transcriptome analyses. These analyses reveal the modulation of several major functional classes, which appears to be temperature-dependent. Therefore, proper control of the CtsR and HrcA regulons are essential for maintaining optimal cell function in changing environments. Moreover, gene regulatory network reconstructions are essential to survey the full regulatory response of an organism. In these networks, the role of the canonical class I and III stress response regulators will be of great importance, because of their pleiotropic character.

Additional files

Additional file 1: Figure S1. Hybridization scheme for DNA microarrays using cDNA derived from *L. plantarum* WCF51 (WT), NZ3410 (Δ *ctsR*; dC), NZ3425CM (Δ *hrcA::cat*; dH), and NZ3423CM (Δ *ctsR* Δ *hrcA::cat*; dCdH). Temperature in °C is indicated after the slash. Duplicates were included (between brackets) and circled number indicates hybridization number. Tail and head of the arrow represent Cy3 and Cy5 labeling, respectively.

Additional file 2: Table S1. Differentially regulated genes in *L. plantarum* WCF51 grown at 40°C compared to 28°C.

Additional file 3: Table S2. Abbreviations used in Figure 4.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HvB-vdV, MW, PAB, and MK designed the experiments, HvB-vdV and RB performed the experiments, HvB-vdV, RB, MW, PAB, and MK interpreted the data, and HvB-vdV, PAB, and MK drafted and revised the manuscript. All authors approved the version of the manuscript to be published.

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