Whole-Genome Transcriptional Analysis of *Escherichia coli* during Heat Inactivation Processes Related to Industrial Cooking

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Escherichia coli K-12 was grown to the stationary phase, for maximum physiological resistance, in brain heart infusion (BHI) broth at 37°C. Cells were then heated at 58°C or 60°C to reach a process lethality value ($F_{70}^{0.10}$) of 2 or 3 or to a core temperature of 71°C (control industrial cooking temperature). Growth recovery and cell membrane integrity were evaluated immediately after heating, and a global transcription analysis was performed using gene expression microarrays. Only cells heated at 58°C with $F^o = 2$ were still able to grow on liquid or solid BHI broth after heat treatment. However, their transcriptome did not differ from that of bacteria heated at 58°C with $F^o = 3$ (P value for the false discovery rate [P-FDR] > 0.01), where no growth recovery was observed posttreatment. Genome-wide transcriptomic data obtained at 71°C were distinct from those of the other treatments without growth recovery. Quantification of heat shock gene expression by real-time PCR revealed that *dnaK* and *groEL* mRNA levels decreased significantly above 60°C to reach levels similar to those of control cells at 37°C ($P < 0.0001$). Furthermore, despite similar levels of cell inactivation measured by growth on BHI media after heating, 132 and 8 genes were differentially expressed at 71°C compared to 58°C and 60°C at $F^o = 3$, respectively (P-FDR < 0.01). Among them, genes such as *aroA*, *citE*, *glyS*, *oppB*, and *asd*, whose expression was upregulated at 71°C, may be worth investigating as good biomarkers for accurately determining the efficiency of heat treatments, especially when cells are too injured to be enumerated using growth media.

Heat treatments, such as pasteurization or cooking, remain widely used in the food industry to limit the total number of microorganisms in food. Exposure to extreme temperatures leads to major physiological alterations, such as protein or membrane degradation, which ultimately result in cell inactivation and death (1). However, the time needed to reach cell inactivation depends on which microbial strains must be controlled, the extent of the initial contamination, and the temperatures used in the industrial process. Inactivation time also varies according to the food matrices and their heat transfer characteristics (2–4). In addition, the bacteria, which have already experienced a sublethal heat shock, become more resistant to further treatment at elevated temperatures (5–7). Recent studies have reported that some *Escherichia coli* strains can still grow above 65°C (3) and can survive in ground beef after a cooking period to the recommended internal temperature of 71°C (8).

Because of this ability to adapt and resist heat stress, the extent of bacterial death after heating must be evaluated very carefully. Unfortunately, the efficiency of antimicrobial systems used in the food industry is still tested *a posteriori* by detection of survivor growth on agar media. This method takes into account only those cells that are readily “culturable” under laboratory conditions. Populations of cells that are too injured or stressed to grow and form distinct colonies are, therefore, underestimated. However, failure to reproduce on agar plates does not necessarily prove that bacteria are not metabolically active. Viable but nonculturable (VBNC) cells can be generated by nutrient starvation, high pressure, and pH or temperature down- and upshifts (9). Since its first description (10), the VBNC phenotype has been shown in a large number of pathogenic and nonpathogenic bacteria, including *E. coli* (11–13), but the biological significance of this physiological phenomenon is still a matter of debate. Some researchers consider this physiological state to correspond to the result of a degenerative process leading to death, whereas others hypothesize a strat-

egy to adapt to inimical life conditions. Nevertheless, VBNC cells retain their pathogenic properties and can resuscitate whenever the appropriate growth conditions are restored (14). Thus, VBNC cells should be a major concern for food safety, especially during extended storage where recovery of VBNC cells could have important public health consequences. Hence, new means are required to assess the real efficacy of antimicrobial systems.

To overcome the limitations of cell enumeration, alternative methods based on fluorescent staining or ion flux measurements (15, 16) have been already proposed but molecular approaches remain the most powerful techniques to discriminate between live and dead cells after heat shock (17). Through these methods, the influence of sublethal temperatures has been well characterized in *E. coli* and more than 30 proteins implicated in the physiological response to heat stress have been identified (6). Thus, molecular chaperones, such as *dnaK* and *groEL*, are involved in protein folding and repair throughout the life of bacteria and their accumulation may be used to assess bacterial physiology (18). Furthermore, many global transcriptome analyses have been performed on *E. coli* after heat shock to understand molecular changes during cell adaptation (19–25) but none were carried out above 50°C and for a period as long as those used in industry to provide microbial control.

Our study was conducted to determine both viability and tran-

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scriptional changes at temperatures relevant to industrial thermal meat processing. Thus, heat treatments displaying different processed lethality values were used to challenge the thermal resistance and viability of *E. coli*. The identification of molecular biomarkers, expressed even when cells can no longer adapt, and of clusters of genes sharing similar expression patterns was investigated using microarray experiments to better understand bacterial behavior at extreme temperatures. We also propose new molecular tests to assess cell death and the efficacy of heating processes.

(Some of these results were presented at the 58th International Congress of Meat Science and Technology [ICoMST], Montreal, Canada, 12 to 17 August 2012.)

MATERIALS AND METHODS

Bacterial culture and heating conditions. Because the genome of *E. coli* K-12 MG1655 is well understood, this organism was used as a model to investigate physiological and molecular changes during cell adaptation and survival of cooking temperatures used in the food industry. For each biological replicate, frozen aliquots (500 μ l) of stock cultures were inoculated into 10 ml of brain-heart infusion (BHI) broth (BD Biosciences, Mississauga, Ontario, Canada) and incubated overnight at 37°C. Cells were then transferred (1% [vol/vol]) into 50 ml BHI broth and incubated at 37°C for 24 h with constant agitation (150 rpm). The bacterial suspensions were inoculated (1% [vol/vol]) into 200 ml BHI broth and grown under the same conditions to the stationary phase. Upon reaching an optical density at 600 nm (OD_{600}) of 0.9, cell cultures were placed immediately in a shaking water bath and heated to the target temperature along with another inoculated BHI flask in which a type T thermocouple was inserted. This thermocouple was connected to a MultiPac21 data logger (Datapaq Inc., Wilmington, MA) for real-time recording of temperature and computing process lethality value directly in the cell suspensions. The temperature of the BHI broth was measured every minute throughout the heating period. The corresponding partial process lethality values (F^o) were calculated according to a table previously published (26) for heat inactivation of *Enterococcus faecalis* (F_{70}^{10} with T°C reference temperature of 70°C; z value, 10°C). This heat-resistant bacterium has been considered to be the reference microorganism for evaluating the efficiency of meat-cooking processes (27, 28). Four different heat treatments were carried out in our experiment. Bacterial suspensions were heated at 58°C until the F reached 2 or 3 (58°C F^o = 2 or 58°C F^o = 3), at 60°C until F^o = 3 (60°C F^o = 3), or until an internal temperature of 71°C (F^o = 10) was reached (no holding time). This last condition corresponds to the core temperature desired for most meat at the end of its cooking process, according to the North American Food Safety Regulations (6). Biological replicates of heated and control (bacteria grown at 37°C to an OD_{600} of 0.9) cell suspensions were tested. After heat treatments, bacterial cultures were immediately cooled in an ice water bath with constant agitation at 150 rpm until the temperature dropped to ca. 37°C (but, to avoid cold stress, not below).

Evaluation of growth recovery and cell integrity after heating. Immediately after cooling to 37°C, a fraction of each cell suspension (about 4 ml) was collected for cell enumeration and testing membrane integrity. One ml of each cell suspension was spread onto five agar plates (200 μ l/plate) and incubated at 37°C for at least 48 h. Heated-cell suspensions were also inoculated (1% [vol/vol]) in 200 ml of fresh prewarmed BHI broth and incubated at 37°C with constant agitation (150 rpm) to assess recuperation and growth. Cell membrane integrity was evaluated with a LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Life Technologies Inc., Burlington, Ontario, Canada) according to the manufacturer's instructions. Bacteria were stained with green-fluorescent SYTO 9 and red-fluorescent propidium iodide to differentiate intact cells from those with membrane damage. Briefly, equal volumes of the two fluorescent dyes were mixed with 1 ml of the heated cell suspension. After 15 min in the dark at room temperature, 5 μ l of the stained suspension was mounted on a glass slide and observed consecutively with the fluorescein

isothiocyanate (FITC) and the Texas Red bandpass filters of an Olympus BX-51 microscope (Olympus, Center Valley, PA). The images obtained were processed with Image-Pro Plus 6.2 software (Media Cybernetics Inc., Bethesda, MD), and the percentages of intact (green) and damaged (red) cells were determined after enumeration of a minimum of 100 cells in five different fields randomly chosen from each picture.

RNA isolation for microarray analysis. Total RNA was prepared for microarray analysis using an RNeasy Midi kit (Qiagen Inc., Toronto, Ontario, Canada). All surfaces and pipettes were cleaned with RNaseZap solution (Ambion, Life Technologies Inc., Burlington, Ontario, Canada) before RNA preparation to avoid potential degradation by ribonucleases. RNA was isolated both from control cells and bacteria subjected to one of the different heat treatments (58°C F^o = 2 or 3, 60°C F^o = 3, 71°C core). After the heating and cooling phases, the remaining volume of bacterial suspension (about 180 ml) was centrifuged at $7,000 \times g$ for 2 min at room temperature. Cell pellets were then suspended in 5 ml of RNeasy Protect Bacteria Reagent (Qiagen Inc.) and incubated for 5 min at room temperature according to the manufacturer's instructions. After centrifugation at $5,000 \times g$ for 5 min, the supernatants were removed and the remaining pellets were immediately stored at -80°C . For total RNA extraction, frozen cell pellets were mixed in sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 1 mg/ml lysozyme (Sigma-Aldrich, Oakville, Ontario, Canada) and 20 μ l proteinase K (Qiagen Inc.). Enzymatic lysis was performed at room temperature for 10 min with regular high-speed vortexing. Cell lysates were mixed with RLT buffer (from the RNeasy Midi kit) and absolute ethanol before transfer into RNeasy Midi columns. The following RNA extraction steps were performed according to protocol no. 8 of the RNeasy Protect Bacteria Reagent handbook. RNA concentrations were measured with a NanoDrop ND 1000 spectrophotometer (Thermo Scientific, Waltham, MA), and their integrity was assessed using OD_{260}/OD_{280} ratios. To remove residual genomic DNA, RNA samples were incubated at 37°C for 20 min with an adequate quantity of DNase I (Ambion) (2 U per 10 μ g of total RNA) before a phenol-chloroform purification (25:24:1 phenol:chloroform:isoamyl alcohol; Sigma-Aldrich) and an overnight precipitation at -20°C in absolute ethanol containing LiCl at a final concentration of 0.25 M. After centrifugation, nucleic acid pellets were rinsed with 70% ethanol and centrifuged at $13,000 \times g$ for 10 min at 4°C. RNA pellets were air-dried in a vacuum concentrator (Vacufuge Plus; Eppendorf, Mississauga, Ontario, Canada) to remove traces of ethanol and suspended in DNase/RNase-free water. The RNA integrity number (RIN) and concentration of each sample were determined with a model 2100 Bioanalyzer (Agilent Technologies Inc., Mississauga, Ontario, Canada).

Preparation of microarray probes and hybridization. Indirect labeling of RNA templates was based on the initial protocol described by Seidel and modified by Schroeder, Sniesrud, and Yang for the J. Craig Venter Institute standard operating procedure (29). A 5- μ g volume of total RNA was mixed with 2 μ g of random hexamers (Invitrogen, Life Technologies Inc., Burlington, Ontario, Canada) and incubated at 70°C for 10 min. After a snap-freeze step in a dry ice-ethanol bath and a brief centrifugation, samples were reverse transcribed using the Superscript II enzyme with 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.3 mM dTTP (Invitrogen), and 0.2 mM 5-(3-aminoallyl)-dUTP (Sigma-Aldrich) for 3 h at 42°C. RNA were then hydrolyzed with 10 μ l of 1 M NaOH and 10 μ l of 0.5 M EDTA at 65°C for 15 min before the reaction was neutralized by adding 10 μ l of 1 M HCl. A QIAquick PCR purification kit (Qiagen Inc.) was used to remove unincorporated nucleotides. Purification was performed according to the manufacturer's procedure by using specific buffers for washing (5 mM KPO_4 [pH 8.5], 80% absolute ethanol) and elution (4 mM KPO_4 , pH 8.5) steps. The amine-modified cDNA obtained from control and heated cells was covalently coupled to the succinimidyl esters of Alexa Fluor 555 and 647 reactive dyes, respectively (Molecular Probes). The cDNA was concentrated using a Vacufuge Plus vacuum concentrator (Eppendorf, Mississauga, Ontario, Canada) and diluted in 4.5 μ l of 0.1 M Na_2CO_3 at pH 9. Reactive dyes were incubated with cDNA samples in the

TABLE 1 Impact of the different heat treatments on cell viability and integrity

Heat treatment ^a			Growth recovery ^b		Integrity of intact cells (%) ^c	RNA	
Temp (°C)	Time (min)	F_{70}^{10} (min)	BHI agar (log CFU/ml)	BHI broth		RIN ^d	Extraction yield ^e (μg RNA/log CFU)
58	40	2.0	1.06	+	0.8	2.3	5.2
58	53	3.0	BDL	—	0.5	2.4	5.1
60	32	3.0	BDL	—	0.6	2.1	4.1
71	16	9.8	BDL	—	0.6	2.3	10.6

^a F_{70}^{10} , calculation of partial process lethality values measured every minute throughout the heating period. The duration of the heating period reflects the time required to reach the final process lethality values desired.

^b Growth on BHI agar and fresh BHI broth was performed immediately after the cooling period at 37°C following each heat treatment. BDL, below detection level; +, growth observed; —, growth not observed.

^c The integrity of the cell membrane was evaluated using a LIVE/DEAD BacLight bacterial viability kit system (Molecular Probes).

^d RIN, RNA integrity number. RIN values for control bacteria maintained at 37°C were superior to 9.0.

^e Values obtained after purification using an RNeasy column and DNase treatment.

dark for 1 h at room temperature. Residual dyes were then separated from the labeled cDNA using QIAquick PCR purification columns. Concentrations of all purified cDNA and efficiencies of each labeling reaction (pmol dye/μg cDNA) were determined using a NanoDrop ND-1000 spectrophotometer and the microarray measurement program.

cDNA was hybridized to an *E. coli* gene expression microarray (Agilent Technologies Inc.) (8 by 15 K slides) according to the two-color microarray-based prokaryote analysis protocol (Agilent Technologies Inc.). Each microarray was composed of 15,208 probes representing the complete genome of four *E. coli* strains: K-12-MG1655, O157:H7 VT2-Sakai, CFT073, and EDL933. Alexa Fluor 555-labeled cDNA obtained from five independent control cultures (grown at 37°C to an OD₆₀₀ of 0.9) were pooled and used as a common reference for all hybridizations. Equal amounts (300 ng each) of this pooled control cDNA and of labeled cDNA from one of the heated groups were mixed with blocking agent in the hybridization buffer (gene expression hybridization kit; Agilent Technologies Inc.) and were loaded onto each array (8 arrays/slide). The biological replicates of the different treatments were randomly distributed on three microarray slides. The microarray slides were incubated in a hybridization chamber with constant vertical rotation for 17 h at 65°C. The slides were then removed from the chamber and immersed for 1 min in GE wash buffer 1 at room temperature and GE wash buffer 2 maintained at 37°C (Agilent Technologies Inc.). The slides were scanned immediately after the washing step using a PowerScanner (Tecan Group Ltd., Switzerland), and feature extractions were processed using Array-Pro 6.3 ANALYZER software (MediaCybernetics Inc.).

Quantification of gene expression by real-time RT-PCR. Real-time reverse transcriptase PCR (RT-PCR) was used both to validate microarray results and to estimate heat shock gene expression in control and heated bacteria. Total RNA of each sample (500 ng) was reverse transcribed with the Superscript II enzyme and random hexamers (Invitrogen). The cDNA of *groEL* (HSP-60) and *dnaK* (HSP-70) and of genes selected according to microarray data (*aroA*, *asd*, *citE*, *cysB*, *fabG*, *glnB*, *glyS*, *hemA*, *lptA*, *oppB*, *rof*, *rseB*, *rssB*, *ydiA*, *yedE*, and *zur*) were then amplified with a LightCycler 480 system (Roche Diagnostics, Quebec, Canada) and quantified by SYBR green incorporation (LightCycler 480 SYBR green I Master). Most of the specific primers (see Table S1 in the supplemental material) were designed using the Primer3 interface (30) unless otherwise specified. The target genes were first amplified and purified after electrophoretic separation on a 1.5% agarose gel. The purified PCR products were quantified using a NanoDrop spectrophotometer and used to establish standard curves for each gene. These five-point standard curves, consisting of 10-fold dilutions of the purified PCR products from 1 pg to 0.1 fg, were used to determine the PCR efficiency of each primer pair (between 80% and 100%) and allowed real-time quantification of the transcription level of the different genes in all samples. The cycling conditions consisted of a denaturing step at 95°C for 10 min followed by 40 cycles of amplification (denaturation at 95°C for 10 s; annealing at 58°C for 10 s; extension at

72°C for 20 s). Finally, a melting curve program was carried out from 70°C to 95°C with a heating rate of 0.1°C/s, showing a single product with a specific melting temperature for each gene and sample evaluated. Quantification was normalized using two housekeeping genes (16S and 23S rRNA) and a gene not affected by heat treatment, according to the microarray results (*lptA*). The normalization factors were calculated from the expression values of these three genes with the GeNorm program (PrimerDesign Ltd., Southampton, United Kingdom) as described by Vandesompele et al. (31).

Statistical analysis. Microarray data were analyzed using R software and the Limma package (part of Bioconductor). Gene expression results were expressed as LOWESS normalized data obtained from log₂ of processed red signal (heated)/processed green signal (control at 37°C). Only raw data greater than background values and with homogeneous fluorescent signals were considered. Signal heterogeneity was estimated as follows: $H = 1 / (\text{trimmed mean of raw intensity} - \text{median of raw intensity}) / [0.5 \times (\text{trimmed mean of raw intensity} + \text{median of raw intensity})]$. H was calculated for green and red fluorescence, and the spots were excluded from the analysis if H red or H green was greater than 0.2. After LOWESS normalization, the differences between the four heat treatments were tested by analysis of variance (ANOVA) (P value < 0.0001 and P value for the false discovery rate [P-FDR] after Benjamini-Yekutieli adjustment < 0.01) followed by an *a priori* test (six contrasts). A hierarchical ascendant classification of genes differentially expressed between groups (P < 0.0001) was also performed. RT-PCR results were analyzed with Statview 5.0 software (SAS Institute Inc., Cary, NC). Normalized gene expression levels were expressed as means $\pm$ standard errors of the means (SEM) ($n \geq 4$). Homogeneity of the variance between groups was assessed by a Bartlett test. Changes in gene expression between groups were tested by ANOVA, and the means were compared using a Student-Newman-Keuls test.

Microarray data accession number. Microarray data were deposited in the NCBI Gene Expression Omnibus database (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE40557.

RESULTS

Impact of the heating processes on bacteria viability and integrity. The different heat treatments applied to bacteria were compared according to their ability to inactivate bacterial growth and the degree of damage they caused to the cell membrane (Table 1). Cells heated at 58°C for $F_0 = 2$ recovered their ability to grow on BHI agar plates (1.06 log CFU/ml after 48 h of incubation at 37°C) and fresh BHI broth, whereas no growth recovery occurred when the three other treatments, involving higher temperatures and longer heating periods, were used. Fluorescent staining (LIVE/DEAD BacLight bacterial viability kit; Molecular Probes) of bacteria revealed very large, but similar, proportions of damaged cells,

FIG 1 Hierarchical clustering of genes differentially expressed ($P < 0.0001$) between heat treatments. (A) Dendrogram of the hierarchical ascendant classification. T1, 58°C and $F^\circ = 2$; T2, 58°C and $F^\circ = 3$; T3, 60°C and $F^\circ = 3$; T4, 71°C core. (B) Gene expression patterns of the six clusters. The black line on each graph corresponds to the mean of the expression profiles of the genes composing the cluster. The total numbers of ORFs and of specific K-12 genes in each cluster are indicated in parentheses above the graphs. Fold change = heat treatment/control at 37°C.

whatever the treatment. Hence, this viability assessment kit was not useful for discriminating the levels of severity of the four heat treatments applied. Total RNA collected from the different heated cells also showed similar RIN values (Table 1). However, we noticed that it was more difficult to purify large amounts of RNA from cells submitted to the less severe treatment than to purify the RNA from cells heated for a shorter period at higher temperatures. For similar amounts of lysed cells, the quantity of RNA obtained

from bacteria heated at 71°C was double that obtained from each of the other three heat treatments. As a consequence, several RNA extractions were performed for the later treatments to obtain the quantities required for the transcriptomic experiments.

Gene expression patterns induced by severe heat treatment. A global transcriptome analysis was conducted to determine the effect of prolonged heating at high temperatures on *E. coli*. The labeled cDNA from control bacteria grown at 37°C and that from

TABLE 2 Number of genes differentially expressed between treatments

Comparison	No. of differentially expressed genes				
	$P < 0.0001$		$P\text{-FDR} < 0.01$		
	All genomes ^a	K-12 strain ^b	K-12 strain	Upregulation ^c	Downregulation ^c
58°C $F^o = 2$ vs 58°C $F^o = 3$	33	7	0	0	0
58°C $F^o = 2$ vs 60°C $F^o = 3$	186	73	8	0	8
58°C $F^o = 2$ vs 71°C	589	343	279	75	204
58°C $F^o = 3$ vs 60°C $F^o = 3$	33	15	0	0	0
58°C $F^o = 3$ vs 71°C	425	237	132	64	68
60°C $F^o = 3$ vs 71°C	110	69	8	8	0

^a Among the 11,494 ORFs encoding for the complete genomes of *E. coli* K-12, O157:H7, CFT073, and EDL933.
^b Among the 4,435 ORFs of *E. coli* K-12 MG1655.
^c Up- or downregulated in the second treatment cited.

cells subjected to one of the different heat treatments were mixed for each hybridization to microarrays covering the complete genome of four strains of *E. coli*. The transcription data for all genes were expressed as log₂ ratios between treatment and control values. The filter criteria (signals/backgrounds > 1, red and green signal heterogeneity < 0.2) used in the first steps of the data analysis led to the removal of 13.7% of the 11,494 open reading frames (ORFs) present on the microarrays. Of the remaining 9,915 ORFs, only 900 genes were differentially expressed between the four heating conditions ($P < 0.0001$) and 54.2% of them belonged to the K-12 strain genome. A hierarchical classification of the expression data corresponding to these genes was performed to gain insight into the behavior of *E. coli* when temperature and/or the process lethality values increase (Fig. 1). An accurate representation of the genomic response triggered by the different treatments was obtained by clustering the genes with the most similar expression profiles into a minimum of six distinct groups (Fig. 1A). Clusters 1, 2, and 3 (C1, C2, and C3) are mainly composed of K-12 genes (more than 60%) and represent about 30%, 22%, and 23% of the differentially expressed ORFs, respectively ($P < 0.0001$), whereas smaller numbers of genes (less than 10%) were contained in the three other clusters (C4, C5, and C6) (Fig. 1B).

The expression of genes from clusters C1 and C3 was reduced as the severity of the heat treatment increased, whereas the genes from clusters C2 and C6 were upregulated under the same conditions. Gene expression profiles of both clusters C4 and C5 were characterized by a peak in bacteria heated until reaching a lethality value of 3. Although each cluster contained a large proportion of “hypothetical” open reading frames, several distinctions can be made between these groups by considering the functions of the genes that compose them (see Table S2 in the supplemental material). Thus, clusters C1 and C3 were characterized by a higher proportion of genes involved in transcription (*rseB*, *rssB*, *melR*, *greB*, *iclR*, *zur*), regulatory functions (*rpoD*, *envR*, *glcC*, *narQ*, *perR*, *rhaR*, *torR*, *rnhA*, *fur*) and cellular processes (for adaptation to atypical conditions, *cspF*, *cspG*, *cspH*, and *lpxP*; for chemotaxis and motility, *flgC*, *flgM*, *fliC*, *fliL*, and *fliM*) than clusters C2 and C6. These last two clusters were composed of genes implicated in different biosynthesis processes (amino acids, *aroA*, *cysB*, *metA*, *metR*, *hisC*, *hisG*, *serC*, and *asd*; proteins, *glyS* and *selD*; cofactors and prosthetic groups, *hemA*). Clusters C2 and C6 had the highest proportions (18% and 33%, respectively) of genes involved in energy metabolism (electron transport, *nuoC*, *nuoE*, *nuoF*, *nuoG*, *nuoI*, *nuoJ*, and *nuoN* and *pntA*; fermentation, *citE*, *citF*, and *pta*).

Gene expression after the different heat treatments. To isolate specific genes expressed when cells are about to die, the microarray data were analyzed at a more stringent statistical level than that used for establishing the expression clusters to limit the number of false positives ($P\text{-FDR} < 0.01$ instead of $P < 0.0001$). Hence, the list of genes differentially expressed between treatments was drastically reduced when P values were corrected by a Benjamini-Yekutieli FDR adjustment with a significance level of 1% ($P\text{-FDR} < 0.01$; Table 2). At this significance level, the whole-genome analysis indicated that no genes were differentially expressed between bacteria treated at 58°C with $F^o = 2$ and 3 ($P\text{-FDR} > 0.01$) even if the cells heated using these conditions displayed different growth performances posttreatment (growth recovery for 58°C at $F^o = 2$ but not at $F^o = 3$). The absence of differences between 58°C and 60°C at $F^o = 3$ was expected since the process lethality values of these treatments were equal. As previously illustrated by the dendrogram of the Hierarchical Ascendant Classification of gene expression (Fig. 1), bacteria heated at 71°C were different from those subjected to the other three conditions. Thus, 279 and 132 genes were differentially expressed at 71°C compared to 58°C $F^o = 2$ and 58°C $F^o = 3$ ($P\text{-FDR} < 0.01$; Table 2). These genes, which were up- or downregulated at 71°C, were involved in a large number of cellular processes as illustrated in Tables 3 and 4.

Remarkably, eight genes from cluster C2 (*aroA*, *citE*, *cysB*, *glnB*, *glyS*, *hemA*, *yedE*, and *ydiA*) were upregulated at 71°C compared to 60°C $F^o = 3$ (Table 3), whereas no genes were downregulated between these two extreme conditions (Table 4). The significant increases of *aroA*, *citE*, and *glyS* expression between 60°C with $F^o = 3$ and 71°C were confirmed by real-time PCR ($P < 0.0004$; Fig. 2). However, no significant changes were observed by quantitative PCR (Q-PCR) for *cysB*, *glnB*, *yedE*, and *ydiA* ($P > 0.05$; data not shown) and the expression of *hemA* decreased significantly, in contrast to what was reported in the microarray analysis ($P = 0.007$; Fig. 2). The PCR expression profiles of *oppB* and *asd* were also significantly increased as shown by comparisons of bacteria heated at 60°C with $F^o = 3$ and 71°C ($P = 0.0002$ and $P < 0.0001$, respectively), whereas their corresponding microarray results under these conditions indicated only a tendency ($P\text{-FDR} = 0.0193$; Table 3) and no variation, respectively. Hence, *aroA*, *citE*, *glyS*, *oppB*, and *asd* were all upregulated at 71°C compared to 60°C $F^o = 3$ and may, therefore, take part in the final active biological functions of the cells before death.

The expression of genes from clusters 1, 3, 4, and 5 was also investigated by Q-PCR to validate the microarray data (Fig. 2).

TABLE 3 ORFs significantly upregulated at 71°C compared to 58°C and 60°C

		58°C F^o = 2 vs 71°C		58°C F^o = 3 vs 71°C		60°C F^o = 3 vs 71°C	
Gene	Description and physiological function ^a	P-FDR	FC	P-FDR	FC	P-FDR	FC
Amino acid biosynthesis							
<i>aroA</i>	5-Enolpyruvylshikimate-3-phosphate synthetase	0.0056	2.1	0.0006	3.1	0.0037	3.5
<i>asd</i>	Aspartate semialdehyde dehydrogenase	0.0001	5.9	0.0003	4.4	ns	
<i>aspC</i>	Aspartate aminotransferase, PLP dependent	0.0012	3.6	0.0058	3.2	ns	
<i>cysA</i>	Sulfate/thiosulfate transporter subunit	0.0048	6.1	ns		ns	
<i>cysB</i>	O-Acetyl-L-serine-binding transcriptional regulator	0.0005	3.5	0.0076	2.7	0.0064	3.6
<i>cysW</i>	Sulfate/thiosulfate transporter subunit	0.0054	4.4	0.0058	5.3	ns	
<i>gdhA</i>	Glutamate dehydrogenase, NADP-specific	0.0024	2.5	0.0181	2.5	ns	
<i>hisG</i>	ATP phosphoribosyl transferase	0.0028	2.8	0.0006	4.1	ns	
<i>ilvD</i>	Dihydroxyacid dehydratase	ns		0.0038	3.2	ns	
<i>metR</i>	Homocysteine-binding transcriptional activator	ns		0.0059	4.7	ns	
<i>serC</i>	3-Phosphoserine aminotransferase	0.0029	3.1	ns		ns	
Other biosynthesis							
<i>dcd</i>	2'-Deoxycytidine 5'-triphosphate deaminase	0.0074	1.8	ns		ns	
<i>hemA</i>	Glutamyl tRNA reductase	0.0008	2.4	0.0012	2.6	0.0079	2.5
<i>narU</i>	Nitrate/nitrite transporter	0.0007	2.6	ns		ns	
<i>panB</i>	3-Methyl-2-oxobutanoate hydroxymethyl transferase	0.0004	2.7	0.0006	2.8	ns	
<i>pyrC</i>	Dihydro-oroatase	0.0015	3.7	0.0020	4.2	ns	
Energy metabolism							
<i>atpB</i>	F0 sector of membrane-bound ATP synthase	0.0015	2.8	0.0019	3.0	ns	
<i>citE</i>	Citrate lyase, citryl-ACP lyase subunit	0.0010	2.9	0.0003	4.5	0.0077	3.2
<i>hyaB</i>	Hydrogenase I, large subunit	0.0075	4.9	ns		ns	
<i>maeA</i>	Malate dehydrogenase	0.0083	2.2	0.0058	2.8	ns	
<i>mrp</i>	Antiporter inner membrane protein	0.0007	2.8	0.0012	2.9	0.0188	2.6
<i>nuoC</i>	NADH; ubiquinone oxydoreductase chain C, D	0.0049	3.1	0.0030	4.7	ns	
<i>nuoF</i>	NADH; ubiquinone oxydoreductase chain F	0.0105	3.2	0.0061	4.2	ns	
<i>nuoG</i>	NADH: ubiquinone oxydoreductase chain G	0.0002	3.6	0.0076	2.5	ns	
<i>nuoI</i>	NADH; ubiquinone oxydoreductase chain I	0.0011	4.1	0.0035	3.9	ns	
<i>nuoJ</i>	NADH; ubiquinone oxydoreductase, unit J	0.0005	3.2	0.0021	3.0	ns	
<i>nuoN</i>	NADH; ubiquinone oxydoreductase, unit N	0.0002	4.2	0.0152	2.5	ns	
<i>pta</i>	Phosphate acetyltransferase	0.0018	4.6	0.0030	5.0	ns	
<i>speA</i>	Biosynthetic arginine decarboxylase PLP binding	0.0003	2.8	0.0089	2.1	ns	
Fatty acid and phospholipid metabolism							
<i>cls</i>	Cardiolipin synthase I	0.0060	1.7	0.0004	2.5	ns	
<i>fabB</i>	3-Oxoacyl-[acyl carrier protein] synthase I	0.0023	2.5	0.0057	2.5	ns	
<i>fabG</i>	3-Oxoacyl-[acyl carrier protein] reductase	0.0075	2.3	ns		ns	
<i>tesA</i>	Acetyl-CoA thioesterase I	ns		0.0035	2.9	ns	
<i>tesB</i>	Acetyl-CoA thioesterase II	ns		0.0058	2.0	ns	
Cell envelope (structure, biosynthesis, etc.)							
<i>murG</i>	N-Acetylglucosaminyl transferase	0.0015	2.6	0.0063	2.3	ns	
<i>skp</i>	Periplasmic chaperone	0.0006	3.9	ns		ns	
<i>slt</i>	Lytic murein transglycosylase, soluble	0.0011	2.4	0.0016	2.6	ns	
<i>tolC</i>	Transport channel	0.0007	3.2	0.0132	2.4	ns	
<i>wzxE</i>	O-antigen translocase	ns		0.0034	2.4	ns	
Transcription							
<i>rnb</i>	RNase II	0.0048	2.6	0.0016	3.6	ns	
<i>rpoS</i>	RNA polymerase, sigma S (sigma 38) factor	0.0065	2.4	ns		ns	
Protein synthesis							
<i>glyS</i>	Glycine tRNA synthetase, beta subunit	0.0002	3.6	0.0011	3.1	0.0077	3.1
<i>selB</i>	Selenocysteinyl-tRNA-specific translation factor	ns		0.0068	3.0	ns	
<i>selD</i>	Selenophosphate synthase	0.0075	2.3	ns		ns	
<i>sufS</i>	Selenocysteine lyase, PLP dependent	0.0083	2.6	0.0033	3.4	ns	

(Continued on following page)

TABLE 3 (Continued)

		58°C F^o = 2 vs 71°C		58°C F^o = 3 vs 71°C		60°C F^o = 3 vs 71°C	
Gene	Description and physiological function ^a	P-FDR	FC	P-FDR	FC	P-FDR	FC
Transport and binding proteins							
<i>lysP</i>	Lysine transporter	0.0057	2.5	ns	6.5	ns	
<i>oppB</i>	Oligopeptide transporter subunit	0.0004	4.6	0.0003	2.8	0.0193	3.6
<i>sapB</i>	Antimicrobial peptide transporter subunit	0.0081	2.1	0.0019	2.7	ns	
<i>trkA</i>	NAD-binding component of Trk transporter	0.0018	2.3	0.0016		ns	
Cellular processes (motility, chemotaxis, adaptation, etc.)							
<i>hdeD</i>	Acid resistance membrane protein	0.0003	2.9	0.0008	2.9	ns	
<i>mdoH</i>	Glucan biosynthesis; glycosyl transferase	0.0052	2.5	0.0041	3.1	ns	
<i>ftsI</i>	Transpeptidase for peptidoglycan synthesis	0.0071	2.8	0.0077	3.2	ns	
Regulatory functions							
<i>crp</i>	DNA-binding transcriptional dual regulator	0.0005	3.1	0.0088	2.3	ns	
<i>dhaL</i>	Dihydroxyacetone kinase, C-terminal domain	0.0072	3.1	ns		ns	
<i>dhaM</i>	Predicted dihydroxyacetone-specific PTS enzymes	0.0074	2.3	0.0130	2.4	ns	
<i>glnB</i>	Regulatory protein PII for glutamine synthase	0.0012	2.3	ns		0.0057	2.6
<i>paaX</i>	Repressor of phenylacetic acid degradation	0.0016	2.9	ns		ns	
<i>relA</i>	ppGpp synthetase I/GTP pyrophosphokinase	0.0063	1.8	ns		ns	
<i>secM</i>	Regulator of <i>secA</i> translation	ns		0.0039	3.0	ns	
Unknown functions							
<i>yedE</i>	Predicted inner membrane protein	0.0004	3.9	0.0009	4.2	0.0091	3.7
<i>ydiA</i>	Conserved protein	0.0011	2.5	0.0004	3.4	0.0063	2.9

^a The physiological functions listed in column 2 correspond to primary annotations of the J. Craig Venter Institute Comprehensive Microbial Resource (JCVI/CMR; 50). FC, fold change = 71°C/(58°C F^o = 2 or 58°C F^o = 3 or 60°C F^o = 3); PLP, proteolipid protein; PTS, phosphotransferase system; ns, not significant (P-FDR > 0.01). Data in italics are not significantly different but expressed a trend.

The *rof* gene displayed the same expression pattern as that suggested by the microarray results and *rseB* expression was effectively decreased as shown by comparisons of 58°C F^o = 2 and 58°C F^o = 3 to 71°C. Similarly, the *zur*, *rssB*, and *fabG* PCR results agreed with the microarray analysis results. However, even if the expression patterns of the different genes tested were similar in the PCR and the microarray experiments, the scale of the changes obtained from PCR data was globally lower than those measured by microarray analysis.

Evaluation of heat shock gene expression in control and heated cells. DnaK (HSP70) and GroEL (HSP60) are the two major components of the main chaperone systems activated in *E. coli* when cells adapt to an increase in temperature. Unfortunately, their corresponding genes, as well as those encoding most of the heat shock genes (*dnaJ*, *groES*, *ibpA*, *ibpB*, *grpE*, *clpB*, *hslJ*, *hslO*, *hslR*), were ignored in our microarray analysis due to the very high intensity of their red fluorescent signal (heat treatments), which generated a red heterogeneity value superior to the chosen threshold ($H > 0.2$). Despite this, the transcription levels of *dnaK* and *groEL* were also evaluated by real-time PCR in cells grown at 37°C and for each experimental condition (Fig. 3). Although cell integrity and RNA quality data suggested that the different treatments caused equivalent magnitudes of damage, except at 58°C with F^o = 2 (Table 1), the levels of heat shock gene expression measured by RT-PCR differed significantly between groups ($P < 0.0001$). Transcription of *dnaK* and *groEL* was increased by 161- and 183-fold, respectively, in bacteria at 58°C F^o = 2 compared to the control at 37°C. No significant differences were observed for *dnaK* mRNA levels between cells heated at 58°C with F^o = 2 and at 60°C

with F^o = 3, whereas a significant reduction was detected for *groEL*. Similar mRNA levels were observed for the two genes when cells were heated at the same process lethality value (58°C F^o = 3 compared to 60°C F^o = 3). From 60°C to 71°C, the expression of the two chaperones decreased significantly and the *dnaK* and *groEL* mRNA levels returned to a basal level at 71°C, similar to that observed in control bacteria at 37°C (Fig. 3). This may indicate that RNA stability or transcription of *dnaK* and *groEL* is challenged above 60°C, possibly as a result of the exhaustion of the bacterial stress response and resistance to high temperature exposure.

DISCUSSION

Heat stress responses in bacteria have been well documented for sublethal conditions but not as thoroughly for temperatures used in the food industry to control bacteria and to prevent cellular adaptation and survival or growth. In order to investigate the impact of severe and lethal heat treatments on the survival and transcriptomic response of bacteria, *E. coli* K-12 were either treated at 58°C to 60°C until a process lethality value of F^o = 2 or 3 was reached or at a core temperature of 71°C, which is recommended for thorough cooking of meat equivalent to a pasteurization, not a sterilization. Limited recovery was observed only for cells treated at 58°C with a F^o = 2, indicating that the effects of the treatments studied ranged from severe to fatal for *E. coli* K-12.

Impact of heat treatments relevant for the food and meat industries. Since the time needed to kill 90% of *E. coli* K-12 (D values) in liquid egg and tryptic soy broth above 57°C did not exceed 2 min (2, 4), the four heat treatments used in this study

TABLE 4 ORFs significantly downregulated at 71°C compared to 58 and 60°C^a

		58°C <i>F</i> ^o = 2 vs 71°C		58°C <i>F</i> ^o = 3 vs 71°C		60°C <i>F</i> ^o = 3 vs 71°C	
Gene	Description and physiological function(s)	P-FDR	FC	P-FDR	FC	P-FDR	FC
Energy metabolism							
<i>fsaB</i>	Fructose-6-phosphate aldolase 2	0.0029	0.2	ns		ns	
<i>hyfG</i>	Hydrogenase 4, subunit	0.0031	0.3	ns		ns	
<i>puuP</i>	Putrescine importer	0.0017	0.2	0.0026	0.2	ns	
<i>sdhC</i>	Succinate dehydrogenase	0.0031	0.2	ns		ns	
<i>talA</i>	Transaldolase A	0.0015	0.3	0.0053	0.4	ns	
Other biosynthesis							
<i>nrdE</i>	RNase diphosphate reductase II alpha	0.0027	0.3	ns		ns	
<i>nrdF</i>	RNase diphosphate reductase II beta	0.0022	0.5	ns		ns	
Cell envelope (structure, biosynthesis, etc.)							
<i>csgC</i>	Predicted curli production protein	0.0038	0.4	ns		ns	
<i>csgG</i>	Outer membrane lipoprotein	0.0072	0.5	0.0006	0.3	ns	
<i>lpxT</i>	Undecaprenyl pyrophosphate phosphatase	ns		0.0067	0.3	ns	
<i>ompL</i>	Predicted outer membrane porin L	0.0062	0.4	ns		ns	
Cellular processes (motility, chemotaxis, adaptation, etc.)							
<i>fliL</i>	Flagellar biosynthesis protein	0.0047	0.4	ns		ns	
<i>fliM</i>	Flagellar motor component	0.0055	0.4	ns		ns	
<i>fliQ</i>	Flagellar biosynthesis protein	0.0052	0.4	0.0063	0.4	ns	
<i>fliR</i>	Flagellar export pore protein	0.0093	0.4	0.0070	0.4	ns	
Regulatory functions							
<i>gmr</i>	Modulator of RNase II stability	0.0017	0.3	0.0035	0.3	ns	
<i>iclR</i>	DNA-binding transcriptional repressor	0.0004	0.2	0.0137	0.3	ns	
<i>perR</i>	DNA-binding transcriptional regulator	0.0002	0.2	0.0017	0.3	ns	
<i>rpoD</i>	RNA polymerase, sigma 70 (sigma D) factor	0.0065	0.2	ns		ns	
<i>rseB</i>	Anti-sigma factor	0.0028	0.1	0.0096	0.2	ns	
<i>rssB</i>	Response regulator of <i>rpoS</i>	0.0012	0.2	ns		ns	
<i>torR</i>	DNA-binding response regulator	0.0022	0.2	0.0026	0.2	ns	
<i>zur</i>	DNA-binding transcriptional repressor, Zn(II)	0.0002	0.2	ns		ns	
Transcription							
<i>mtlR</i>	DNA-binding repressor	ns		0.0083	0.2	ns	
<i>rnc</i>	RNase III	0.0005	0.3	ns		ns	
<i>rnhA</i>	RNase HI	0.0002	0.3	0.0032	0.4	ns	
<i>rof</i>	Modulator of Rho-dependent transcription	ns		0.0035	0.3	ns	
DNA metabolism							
<i>dinB</i>	DNA polymerase IV	0.0082	0.4	ns		ns	
<i>recO</i>	Gap repair protein	0.0002	0.2	ns		ns	
Protein fate (secretion, degradation, etc.)							
<i>gspE</i>	General secretory pathway component, cryptic	0.0009	0.4	ns		ns	
<i>gspF</i>	General secretory pathway component, cryptic	0.0088	0.3	ns		ns	
<i>gspI</i>	General secretory pathway component, cryptic	0.0011	0.4	0.0133	0.4	ns	
<i>gspK</i>	General secretory pathway component, cryptic	0.0072	0.5	ns		ns	
<i>hsrA</i>	Homocysteine efflux system	0.0012	0.2	ns		ns	
<i>lipB</i>	Lipoyl-protein ligase	0.0002	0.2	0.0060	0.3	ns	
<i>tadA</i>	tRNA-specific adenosine deaminase	0.0004	0.3	0.0070	0.4	ns	

^a FC, fold change = 71°C/(58°C *F*^o = 2 or 58°C *F*^o = 3 or 60°C *F*^o = 3); ns, not significant (P-FDR > 0.01). Data in italics are not significantly different but expressed a trend.

were not expected to generate survivors. Some of the gene expression profiles obtained after the clustering of microarray data revealed a peak of transcription at 58°C and 60°C *F*^o = 3 (clusters 5 and 4, respectively) and even at the core temperature of 71°C (cluster 6). These results suggest that the transcriptional machinery is somewhat functional in severely heated bacteria which are

no longer able to reproduce and, therefore, that *E. coli* K-12 might react as VBNC cells above 58°C. Our cell enumerations determined *a posteriori* are in agreement with the previous results obtained with *Bacillus subtilis*, a Gram-positive bacteria in which no growth recovery was observed above 57°C and gene expression was maintained after a complete loss of the ability to grow (32).

FIG 2 Validation of microarray data by Q-PCR. Normalized mRNA levels of the selected genes are expressed in ng. Means $\pm$ standard errors with the same letters (a to c) do not differ significantly between groups ($P > 0.05$). The numbers on the histogram bars correspond to the fold change measured between the selected temperature and 71°C. The cluster number corresponding to each gene is given in parentheses.

These observations suggest that analysis of gene expression should be considered a way to evaluate the real efficiency of antimicrobial treatments that is more appropriate than measuring the remaining growth performance of bacteria, which depends on cell recovery from injury.

FIG 3 Heat shock gene expression in control and heated cells. Means $\pm$ standard errors with the same letters (letters a and b and letters x to z for *dnaK* and *groEL*, respectively) do not differ significantly ($P > 0.05$). AU, arbitrary units.

ery from injury. Indeed, the evaluation of heat shock gene expression by PCR and microarray data revealed that the conditions encountered by bacteria heated at 71°C were very different from the other heat treatments (58°C $F^\circ = 3$ and 60°C $F^\circ = 3$), although the lack of growth and recovery suggest that all treatments were effective. Both *dnaK* gene expression and *groEL* gene expression measured by RT-PCR were significantly decreased from 60°C to the basal level at 71°C, indicating that the protection against temperature upshift was no longer effective and bacterial survival was compromised.

The global transcriptomic analysis gave us a good representation of the molecular changes that took place in *E. coli* under our severe heating conditions. The more drastic the heat conditions became, the more σ^D (*rpoD*) expression was reduced. As a consequence, transcription of the genome was ensured at extreme temperature by alternative sigma factors σ^S and σ^E , as illustrated by the decrease in transcription of their repressors, *rssB* and *rseB*. Increasing temperatures also seemed to favor aerobic over anaerobic metabolism since the transcript levels of Fnr and TorR, two activators of anaerobic respiratory gene transcription, decreased as the temperature increased. Concurrently, transcription of NarQ and OxyR, which control the oxidative-response gene tran-

scription (33), was increased, as was that of the *nuo* genes (*nuoC*, -F, -G, -I, -J, and -N) encoding different elements of the NADH ubiquinone oxidoreductase complex. However, based on the decrease in *perR* and *rof* transcription, prevention of oxidative stress induced by respiration or environmental changes appeared to be reduced (34, 35).

Identification of specific genes to evaluate the efficiency of antimicrobial systems. Identifying the genes still expressed just prior to cell death meant working with RNA samples exhibiting a certain degree of degradation. Several recent studies have looked at how much RNA degradation could influence gene expression measurements, but their conclusions remain a matter of debate (36–38). According to Opitz et al. (36), samples with low RNA quality were still valuable for gene expression analysis but their use generated a greater variability between samples and an underestimation of genes with low transcript levels. Since the degradation rates estimated by the RIN was comparable between treatments, sample variability was not an issue in our study and we could establish gene clusters. Furthermore, the validation of microarray results by Q-PCR confirmed that five genes (*aroA*, *asd*, *citE*, *glyS*, and *oppB*) were significantly upregulated in bacteria heated at 71°C compared to those maintained at 60°C for 32 min ($F = 3$). These genes have different biological functions and are crucial for differences in bacterial viability. The glycyl-tRNA synthetase (GlyS or GlyRS) plays a major role in protein biosynthesis, and it is one of the 10 amino-acyl synthetases involved in correct codon-anticodon recognition between amino acids and tRNA (39, 40).

The CitE enzyme corresponds to the β -subunit of the citrate lyase complex that converts citrate to oxaloacetate and acetyl-coenzyme A (acetyl-CoA), a precursor of fatty acid oxidation and biosynthesis (41). In our experiments, the increase in *citE* transcription at extreme temperatures may be due to acidification of the bacterial cytoplasm. This influence of pH on the expression of citrate lyase has been already reported in *Lactococcus lactis* under natural conditions (42). In *L. lactis*, synthesis of the citrate lyase complex and synthesis of the citrate transporter, CitP, were simultaneously induced to prevent the accumulation of lactate and enhance the alkalization of the cytoplasm by increasing citrate metabolism (42).

OppB (oligopeptide transporter subunit B) is required for the activity of the oligopeptide permease, Opp, a binding protein-dependent system which has been well characterized in *Salmonella enterica* serovar Typhimurium and *E. coli* (43). The Opp plays an important role in the recycling of cell wall peptides, and in enteric bacteria it constitutes the main pathway for the uptake and transport of short peptides irrespective of their composition (44).

Finally, the products of the *asd* and *aroA* genes are involved in the biosynthesis of several essential amino acids. The 5-enolpyruvylshikimate 3-phosphate synthase (AroA, also called EPSPS) is the sixth enzyme of the shikimate pathway that leads to the production of all aromatic amino acids (phenylalanine, tryptophan, tyrosine) and most bacterial aromatic compounds, such as folate, vitamin K, and ubiquinone (45, 46). Similarly, aspartate semialdehyde dehydrogenase (Asd) is a key enzyme of the aspartate biosynthetic pathway responsible for the synthesis of threonine, isoleucine, methionine, and lysine as well as of primary metabolites (47). The biosynthesis activities of these two metabolic pathways can be repressed to various degrees by the amino acids produced (negative feedback) but also by regulating the formation of the enzymes at a transcriptional level (45, 48, 49). The derepression of

aroA and *asd* transcription when the severity of heat treatment increased suggests that heated cells required more essential amino acids to either renew the pool of damaged proteins or to overcome amino acid depletion in the growth medium.

Some of these genes have already received considerable attention related to developing new antimicrobial strategies. For example, AroA is the primary target of a well-known herbicide called glyphosate (46) and the synthesis of Asd inhibitors to overcome bacterial resistance is currently being studied (47). Additionally, according to their critical roles in protein recycling, amino acid biosynthesis, and pH homeostasis, the five genes involved in the last biological functions still active when cells are about to die may be good indicators of the efficiency of antimicrobial systems used in the food industry.

Conclusion. Molecular analysis revealed more detailed variations in the physiological status of bacteria stressed or injured by heating than the classical cell growth and recovery posttreatment. Even when cell growth and integrity were drastically affected above 58°C, variations in gene expression could still be measured, suggesting that bacteria remained metabolically active to fight the adverse effects of the heat treatments applied or that intrinsic RNA chemical stability was maintained beyond the ability of the cell to survive. The comparison of the *E. coli* transcriptomes at these temperatures reveals five potential biomarkers (*aroA*, *asd*, *citE*, *glyS*, and *oppB*) that are still expressed at 71°C when cells are no longer able to adapt and grow. The decrease of *dnaK* and *groEL* gene expression from 60°C to 71°C indicated a decline in the bacterial stress response. Future research may include translation and gene promoter activity studies to determine if the detected RNA molecules are still actively synthesized at these extreme temperatures or if they are detected simply as evidence of residual intrinsic chemical heat stability. The influence of the food matrix with respect to the observed transcription pattern is also of interest.

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