ESCHERICHIA COLI SENTENCED TO DEATH BY HEAT STRESS: REACHING OUT FOR THE LAST FUNCTIONAL GENES

A. Guernec[1,2], P. Robichaud-Rincon[1,2] and L. Saucier[1,2]

[1] Department of Animal Science, Faculty of Agriculture and Food Sciences, Université Laval, Québec city, Canada.

[2] Institute of Nutraceuticals and Functional foods, Université Laval, Québec city, Canada

Abstract – *Escherichia coli* K12 was used as a model to investigate physiological and molecular changes during cell adaptation and survival to cooking temperatures used in food industries. Bacteria grown to stationary phase in BHI broth were heated at 58°C or 60°C until a process lethality value (F_{70}^{10}) of 2 or 3 was reached, or until an internal core temperature of 71°C was attained. Growth and cell integrity were evaluated after heating. Transcriptional modifications were analyzed by microarrays and expression of heat-shock genes was quantified by qPCR. Only cells heated at 58°C *F*=2 were still able to grow in liquid or on solid BHI after treatment. However, their transcriptome did not differ from those of bacteria heated at 58°C *F*=3 (P-FDR > 0.01). Transcriptomic data obtained at 71°C were significantly different from the others. The expression of *dnaK* and *groEL* was significantly lower at 71°C than at 58°C and 60°C (P < 0.0001). Furthermore, despite similar cell viability and integrity post-treatment, 132 and 8 genes were differentially expressed at 58°C and 60°C *F*=3, respectively (P-FDR < 0.01) when compared to 71°C. These eight genes, whose expression was up-regulated at 71°C, may be considered as good biomarkers to test antimicrobial efficiency of heat-stress.

Key Words – Bacterial stress response, Heat treatment, gene expression, microarray.

I. INTRODUCTION

Heat treatments, like pasteurization or cooking, have long been used in the industry to limit the total number of microorganisms in foods. Exposure to high temperatures causes important physiological alterations, like protein or membrane degradation, which will finally result in a total inactivation of the organisms [1]. However, recent studies report that some strains of *Escherichia coli* are still able to grow at 65.2°C or can survive in ground beef after cooking to the recommended internal temperature of 71°C [2,3]. In addition, cells subjected to sub-lethal heat treatment become more resistant to further incubation at elevated temperatures [4].

Unfortunately, the effectiveness of antimicrobial systems used in foods is traditionally evaluated *a posteriori* by enumeration of surviving cells grown on agar medium expressed as counts of colony forming units (CFU) or by enrichment procedures. This method only takes into account cells that are readily culturable under laboratory conditions. Those that are too stressed or injured to grow and form distinct colonies are therefore underestimated. However, the failure to reproduce on agar plates does not necessarily indicate that stressed or injured cells are metabolically inactive. Considering cell abilities to adapt and resist in hostile life conditions, new means to assess the efficacy of antimicrobial systems must be developed. Nutrient starvation, high pressure, pH or temperature down- and up-shifts can generate viable but non-culturable cells [5].

To overcome the limitations of cell counts, alternatives based on fluorescent staining or ion fluxes measurements [6,7] have been tested but molecular approaches remain the most powerful techniques to discriminate between live and dead cells after heat shock [8]. Influence of sublethal temperatures has been well documented in *E. coli* and more than 30 proteins are implicated in the heat stress response [4]. Molecular chaperones, like DnaK and GroEL, are involved in protein folding and repair and their accumulation or persistence may be used as an indicator of bacterial physiology under heat stress. Global transcriptome analysis has already been performed in *E. coli* after heat shock but none above 50°C. Our study was conducted to determine both viability and transcriptional changes at temperatures relevant to meat processing. Identification of molecular biomarkers still expressed when cells are no longer able to adapt was also investigated by microarray experiments.

II. MATERIALS AND METHODS

A. Bacterial culture and heating conditions

Escherichia coli K12 MG1655 cells were inoculated (1% v/v) in 200 mL BHI and incubated at 37°C until the stationary growth phase was reached (OD_{600} = 0.9). The cultures were heated in a circulating water bath along with another flask of BHI carrying a type T thermocouple connected to a MultipaQ21 data logger (Datapaq Inc., Wilmington, MA, USA). The temperature of the BHI broth was measured every minute throughout heating. Partial process lethality values (*F*) were calculated according to the table published by Martin *et al.* [9] for heat inactivation of *Enterococcus faecalis* (T ref: 70°C; z-value: 10°C). Four different heat treatments were carried out. Cells were heated at 58°C until computation of partial *F*-values was equal to 2 or 3 (*F* = 2 or 3), at 60°C until *F* = 3 or until an internal core temperature of 71°C was reached. Five biological replicates of heated and control cell suspensions were done. After heating, bacterial cultures were cooled in an ice water bath until the temperature dropped back to no less than 37°C to avoid cold shock.

B. Assessment of growth and cell integrity after treatments

Just after cooling to 37°C, a fraction of the heated suspensions were collected for cell enumeration and integrity tests. Five aliquots of 200 µl each were spread onto five BHI agar plates for a total of 1 mL. Plates were incubated at 37°C for at least 48 h. Heated cell suspensions were inoculated (1% v/v) in 200 mL of fresh pre-warmed BHI broth and incubated at 37°C under constant agitation to assess recuperation and growth. Bacteria were also stained with syto-9 and propidium ionide (LIVE/DEAD Baclight bacterial viability kits; Molecular Probes) to differentiate intact cells from those with membrane damage.

C. Real-time PCR and microarray analysis

The remaining volume of control (37°C) and heated cell suspensions were centrifuged at 7000 x *g* for 2 min. Cell pellets were treated with RNAprotect® bacteria reagent (Qiagen) and stored at -80°C. Frozen cells were lysed in TRIS-EDTA buffer containing both lysozyme and proteinase K. Total RNA extraction was carried out with the RNeasy Midi kit according to the manufacturer's instructions (Qiagen). After DNAse treatment, RNA integrity number (RIN) and concentration were determined with a 2100 bioanalyzer (Agilent Technologies).

Total RNA was reverse transcribed with Superscript-II enzyme and random hexamers (Invitrogen). The cDNA encoding for *dnaK* and *groEL* were amplified with a LightCycler 480 (Roche diagnostic) and quantified by SYBR green incorporation. Quantification was normalized using three house-keeping genes (*16s*, *23s* rRNA, *lptA*) and the normalization factors were calculated with the GeNorm program (PrimerDesign Ltd). The impact of the different temperatures on the transcriptome of the bacteria was investigated with *E. coli* gene expression microarrays (8x15K slides; Agilent technologies) using a two-color design. After reverse transcription with aminoallyl d-UTP, cDNA from control and heated cells were labeled with Alexa fluor 555 and 647, respectively. Labeled cDNA from the five cultures at 37°C were pooled. Equal amounts (300 ng each) of this reference sample and of labeled cDNA from one of the heated groups were mixed and hybridized on each array. Procedures were performed as described in the gene expression hybridization kit (Agilent Technologies). Slides were scanned immediately after the washing step using PowerScanner™ (TECAN group Ltd.) and the images were processed with the Array-Pro© *ANALYZER* software (Media Cybernetics).

D. Statistical analysis

RT-PCR results were analyzed with Statview 5.0 software (SAS Institute). Changes in *dnaK* and *groEL* expression were tested by ANOVA and the Student-Newman-Keuls test for mean comparison. Microarray data were analyzed using R and Limma package. Only data superior to background values and with homogeneous fluorescent signals were considered. After a Loess normalization of the fluorescent data, differences between heat treatments were tested by ANOVA. P-values were corrected by Benjamini-Yekutieli FDR adjustment to minimize false-positives and a significance level of 1% was chosen to identify differentially expressed genes (P-FDR < 0.01). A hierarchical ascendant classification of genes differentially expressed between groups (P < 0.0001) was also performed.

III. RESULTS AND DISCUSSION

As the time needed to kill 90% of *E. coli* K12 above 57°C did not exceed 2 min (*D*-value; in liquid egg and tryptic soy broth [10,11]), the heat treatments used in this study would not generate survivors. However, cells heated at 58°C for 40 min (F = 2) recover their ability to grow on BHI agar plates (Log 1.06 CFU/mL after 48 h at 37°C) or fresh BHI broth (Table 1). Fluorescent staining of bacteria revealed a large, but similar, proportion of damaged cells regardless of temperature or the process lethality, even at 71°C which is considered to be a proper cooking temperature. Total RNA collected from the cells heated at different temperatures also showed similar RIN (data not shown) even if is more difficult to purify high amounts of RNA with less severe treatment that requires a longer time to heat (2-fold higher after 16 min at 71°C than after 40 or 53 min at 58°C for similar quantity of cells lysed).

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

Heat treatment			Post-treatment growth		L/D
Heat (°C)	Time (min)	F_{70}^{10} (min)	BHI agar (Log CFU/mL)	BHI broth Enrichment	Intact cells (%)
58	40	2.0	1.06	+	0.8
58	53	3.0	BDL	-	0.5
60	32	3.0	BDL	-	0.6
71	16	9.8	BDL	-	0.6

L/D: LIVE/DEAD Baclight; BDL : below detection level.

The whole genome analysis indicates that none of the genes were differentially expressed between treatments at 58°C F = 2 and F = 3 (P-FDR > 0.01; Table 2). This result suggests similar strategies to adapt to these extreme conditions even if differences in growth were reported. The absence of differences between 58°C and 60°C F = 3 was expected since their process lethality values were equal.

Table 2. Number of genes differentially expressed between treatments (P-FDR < 0.01).

Comparisons	K12 genes	Down	Up
58°C F = 2 *vs.* 58°C F = 3	0	0	0
58°C F = 2 *vs.* 60°C F = 3	8	0	8
58°C F = 2 *vs.* 71°C	279	75	204
58°C F = 3 *vs.* 60°C F = 3	0	0	0
58°C F = 3 *vs.* 71°C	132	64	68
60°C F = 3 *vs.* 71°C	8	8	0

Down or up-regulated compared to the 2nd treatment cited.

Regarding their transcriptome, cells at 71°C were different from cells heated to 58°C at F =2 than from cells at F = 3 (Fig. 1). Furthermore, 279, 132 and 8 genes were differentially expressed at 71°C compared to 58°C F = 2, F = 3 and 60°C F = 3, respectively (P-FDR < 0.01; Table 2).

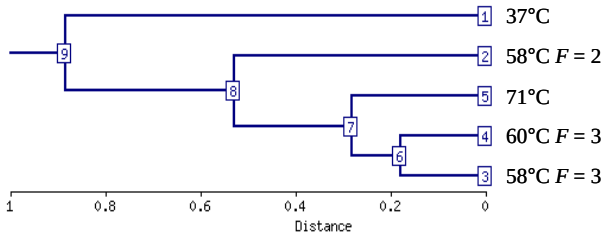

Fig. 1. Hierarchical clustering of heat treatments using the average distance method (P < 0.0001; NIA Array)

The eight genes (Table 3) up-regulated when heat stressed cells were no longer able to survive, may take part as the last molecular signals expressed before death. Following the expression of these biomarkers will be valuable to determine the efficiency of heat treatments used in foods.

Table 3. Genes up-regulated in *E. coli* heated at 71°C.

Symbol	Functions	FC
yedE	Unknown	3.7
cysB	Regulation of cystein biosynthesis	3.6
aroA	Synthesis of aromatic amino-acids	3.5
citE	Energy metabolism	3.2
glyS	ARNt synthesis	3.1
ydiA	Unknown	2.9
glnB	Regulation of glutamine synthase	2.6
hemA	Glutamyl tRNA reductase	2.5

FC: Fold Change = (71°C / 60°C PV3).

Even if cell integrity and RNA quality suggested that the magnitude of the different treatments were equivalent, heat-shock gene expression differs significantly between groups (P < 0.0001; Fig. 2). Both mRNA levels of *dnaK* and *groEL* at 37°C increased by 161 and 183-fold at 58°C F = 2, respectively. Significant differences were observed after a temperature up-shift from 58°C to 60°C suggesting that a maximal level had been reached which then decreased with the severity of the heat treatment. Furthermore, the expression of the two chaperones was significantly lower at 71°C compared to the other treatment. This may indicate that RNA stability of *dnaK* and *groEL* is challenged above 60°C and would result in a less effective protection of the bacteria. By comparison,

cell enumeration alone failed to reveal such physiological differences between heated cells.

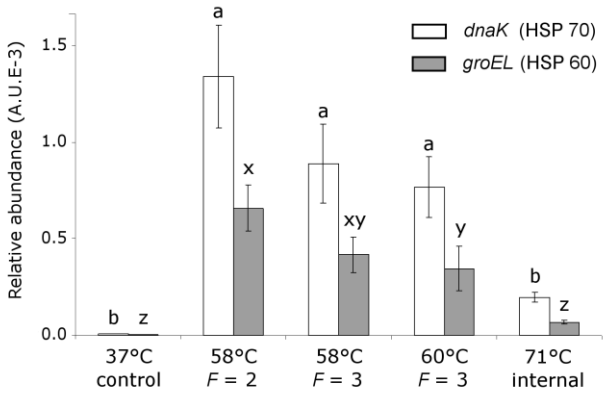

Fig. 2. Heat-shock gene expression in control and heated cells. Means with the same letters (a-b or x-z for *dnaK* and *groEL*, respectively) do not differ (P > 0.05).

IV. CONCLUSION

Molecular analysis reveals more detailed variations in the physiological status of bacteria stressed or injured by heating than cell enumeration. Even if cell growth and integrity are drastically affected above 58°C, variation in gene expression could still be measured suggesting that bacteria remain metabolically active to fight the adverse effects of heat treatment. The comparison of the transcriptome of *E. coli* at these temperatures revealed eight potential biomarkers that were expressed when cells were no longer able to adapt and grow (71°C). The decrease of *dnaK* and *groEL* gene expression between 60°C and 71°C indicated that bacterial stress response is declining or that RNA stability surpasses the ability of the cell to survive. Hence, future experiments will focus on gene promoter activity to determine if the detected RNA molecules (e.g., *dnaK*, *groEL*) are still biologically active or if they are detected simply because of their intrinsic chemical heat stability.

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