Physiological state of bacteria growing in meat during cold storage: a molecular approach

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Abstract— **Bacteria growing on meat are subjected to specific life conditions (nutrient, pH, salt, temperature) which differ drastically from typical laboratory procedures on synthetic media, so physiological and molecular adjustments are expected. This study was conducted to determine the behaviour of bacteria when transferred from a rich-liquid medium to a solid food matrix as it is the case during microbial process validation. Planktonic *Escherichia coli* cultured in Brain Heart Infusion (BHI) to exponential and stationary growth phases were inoculated onto aseptically prepared ground beef meat and stored at 5°C for 12 days. No growth was observed during storage and time-course expression of sigma factors (encoded by *rpoD, E, H, S*) and of genes under their transcriptional control was established by RT-PCR. Overall, gene expression profiles were more stable in stationary phase bacteria. After transfer onto refrigerated meat, *rpoD* mRNA levels increased significantly in exponential phase bacteria (P = 0.0005) but not in stationary phase ones (P = 0.1). The *rpoS* mRNA levels were not significantly changed whatever the physiological state of the inoculum (P > 0.1) and a decrease of *gadA* transcription, controlled by σ^S, were only observed after 12 days of cold storage in exponential phase bacteria (P = 0.03). Surprisingly, activation of σ^H was only observed after more than 1 day of storage. Although the sigma factors expression profiles indicate that bacteria quickly adapt, cells do not seem to perceive a real stress after transfer from a rich medium to the cold meat environment.**

Keywords— ***Escherichia coli*, meat, sigma factors.**

I. INTRODUCTION

Conditions such as temperature, pH or salt content affect bacterial growth, activity and resistance during meat storage or processing and modify bacterial gene expression pattern to various extend [1]. Considering the ability of bacteria to adapt to hostile life conditions, the efficacy of antimicrobial systems must be tested in experimental models as close as possible to real conditions encountered by bacteria in food matrices. But so far, the expression profiles of micro-organisms in stressful conditions have been evaluated in typical laboratory systems [1-3]. Inocula are also prepared in nutrient-rich liquid media with high mixing rate and high initial cell densities. However, bacteria in ground beef meat (GBM) are attached to food surface and exposed to very different conditions, suggesting that extrapolation from data obtained in laboratory broth culture to solid food matrices could be largely misleading [4].

Life on solid food matrices has often been compared to biofilm where bacteria are exposed to nutrient limitation and other stresses likely to result in reduced growth [1, 5-6]. However, to our knowledge, no studies have yet been conducted to demonstrate that bacteria contaminating meat adopt a physiological state close to planktonic stationary phase cells as it is the case in biofilm formation where σ^S induction is observed [7-9]. This transcriptional factor, involved in the general stress response, controls most of the molecular changes when growth slows down and confers to bacteria a higher resistance to stress [10]. In fact, the bacterial genome is mainly transcribed by σ^D in optimal conditions. Alternative factors (σ^H, σ^E and σ^S) trigger the expression of specific regulon under stressful conditions [11]. Sigma H is active during exposure to high temperatures, inducing production of chaperone proteins (e.g., DnaK, GroEL) whereas σ^E participates to extreme heat and extracytoplasmic stress response [12].

To understand the specific molecular changes triggered during adaptation to a meat environment, we determined the expression profiles of sigma factors, and of genes under their control, in planktonic bacteria after their transfer onto refrigerated GBM.

II. MATERIAL AND METHODS

A. Bacterial strain and culture conditions

GBM was prepared aseptically from *semi-membranosus* Angus AAA beef [13]. Frozen Aliquots of 25 g were cooled at 5°C and inoculated with 100 µl of exponential (OD_{600} 0.5) and stationary phase (OD_{600} 0.9) BHI pre-cultures of *Escherichia coli* K12 MG1655 to reach a final concentration of 10^7 CFU/g. This optimal concentration was determined in a preliminary study where serial 10-fold dilutions of exponential pre-cultures were first inoculated onto GBM to determine the detection limits of bacterial gene expression by RT-PCR in meat [14-15]. Even if a cell concentration of 10^5 CFU/g was sufficient to detect 16S and 23S rRNA in meat growing bacteria by RT-PCR after one day of storage at 5°C, higher cell concentrations in the inoculum improves bacterial RNA recovery and limits drastically the amount of undesirable eukaryotic RNA (data not shown). Accordingly, concentrations of 10^7 CFU/g were used for inoculation of exponential and stationary phase bacteria to establish growth kinetics and expression profiles in our experiment.

After 1h and 1, 3, 6 and 12 days of incubation at 5°C, the inoculated meat was homogenized with a stomacher lab blender (Seward Ltd.) in 225 ml peptone water. The homogenate was centrifuged (100 x g, 5°C) for 10 min and 40 ml of the supernatant was filtered through a 20 µm nylon membrane to remove meat particles. One ml of the filtrate was used for enumeration on BHI agar plates. The remaining volume was centrifuged at 5°C and 6500 x g for 10 min. Cell pellets were then treated with RNAprotect bacteria reagent (Qiagen) and stored at -80°C.

B. RT-PCR experiments and statistical analysis

Frozen cell pellets were lysed with lysozyme and proteinase K in pH 8.0 Tris-EDTA containing NaCl and SDS [14]. Cell particles were removed by phenol/chloroform extraction and the aqueous phase obtained was used for total RNA extraction with RNeasy midi kit (Qiagen). After DNAse treatment, RNA concentrations were measured and their integrity was checked with OD_{260}/OD_{280} ratios and agarose gel electrophoresis. Total RNA samples were reverse-transcribed with the Superscript-II enzyme and random hexamers (Superscript Firststrand synthesis system for PCR kit; Invitrogen). Target cDNAs were amplified by PCR, electrophoretically separated and quantified after gel acquisition on Chemi-doc (Biorad). The relative mRNA levels of each transcription factor (*rpoD, E, H*, *S* genes encoding for σ^D, σ^E, σ^H, σ^S) and of genes under their specific control (*upsA, rseB, dnaK* and *groEL, gadA, respectively*) were obtained after normalization against 16S rRNA levels and expressed as ratio in arbitrary unit (AU).

Statistical analysis was performed using the Statview 5.0 software (SAS Institute). All experiments were repeated three times and all values were reported as means ± standard error. Homogeneity of variance between groups was assessed by the Bartlett test. Time-course changes of gene expression were evaluated using ANOVA and Student-Newman-Keuls test for means comparison.

III. RESULTS AND DISCUSSION

No significant growth was observed after 12 days of storage at 5°C similarly to what was previously reported for *E. coli* O157:H7 in GBM at 4°C [16]. During this period, fewer changes were observed in the expression profiles of stationary phase bacteria than exponential phase ones (Table 1) suggesting that stationary phase cells adapted readily to the new lifestyle conditions.

Table 1 Summary for significant time-dependant changes of relative mRNA levels in GBM living bacteria

Genes (protein)	Exponential phase (OD_{600} 0.5)	Stationary phase (OD_{600} 0.9)
RpoD **(σ^D)**	P = 0.0005	NS
↳ *uspA*	NS	NS
RpoE **(σ^E)**	P = 0.05	P = 0.01
↳ *rseB*	NS	NS
RpoH **(σ^H)**	NS	NS
↳ *dnaK; groEL*	P = 0.0004; P < 0.0001	P = 0.008; P = 0.003
RpoS **(σ^S)**	NS	NS
↳ *gadA*	P = 0.03	NS

P: one-way ANOVA P-values; NS : Not Significant.

After transfer from BHI broth to refrigerated meat, the *rpoD* mRNA levels increased significantly in exponential phase bacteria (P = 0.0005) but not in stationary phase ones (P = 0.1). This adjustment may contribute to normal σ^D activity in exponential bacteria since *uspA* mRNA levels were not affected (P = 0.6). Significant changes in *rpoE* transcription were also observed in exponential (P = 0.049) and stationary phase bacteria (P = 0.01). Furthermore, the absence of modification for *rseB* transcription (P > 0.1) suggests that the activation of the envelope stress response is limited after transfer of bacteria into meat environment. These results indicate that no real strong stress was perceived by the bacteria immediately after inoculation onto a solid meat matrix by opposition to what was observed by Cuny et al. [17] with the same bacteria during transfer from Luria Bertani (LB) broth to LB agar plates. Meat is a nutrient rich food matrix. Variations in nutrient availability may account for the observed differences.

RpoS mRNA levels were not modified for inocula grown to exponential and stationary phase (P > 0.1) indicating again an absence of stress in planktonic bacteria when transferred onto GBM. Furthermore, σ^S activity, followed by *gadA* transcription, decreased only after 12 days of storage at 5°C for the exponential phase bacteria (P = 0.03) whereas no changes were detected in stationary phase ones (P = 0.8). Considering that transcription of *rpoS* correlates with the levels of alarmone ppGpp [18], we can postulate that no nutritional deprivation was perceived by the bacteria in its new meat environment.

While stress-dependant modifications of the σ^S regulon were not observed in bacteria after transfer and subsequent growth onto GBM, the heat-shock regulons appeared to be the most reactive to the new environmental conditions on the refrigerated meat surface. The activity of σ^H changed significantly during cold storage, as measured by *dnaK and groEL* transcription (Fig. 1). Surprisingly, *rpoH* expression was not significantly altered in bacteria after inoculation (P > 0.1 whatever the inoculum used; Fig. 1). Both *dnaK* and *groEL* mRNA levels changed significantly during bacterial growth, whatever the original physiological state, suggesting an important role for the RpoH regulon in cell adaptation under

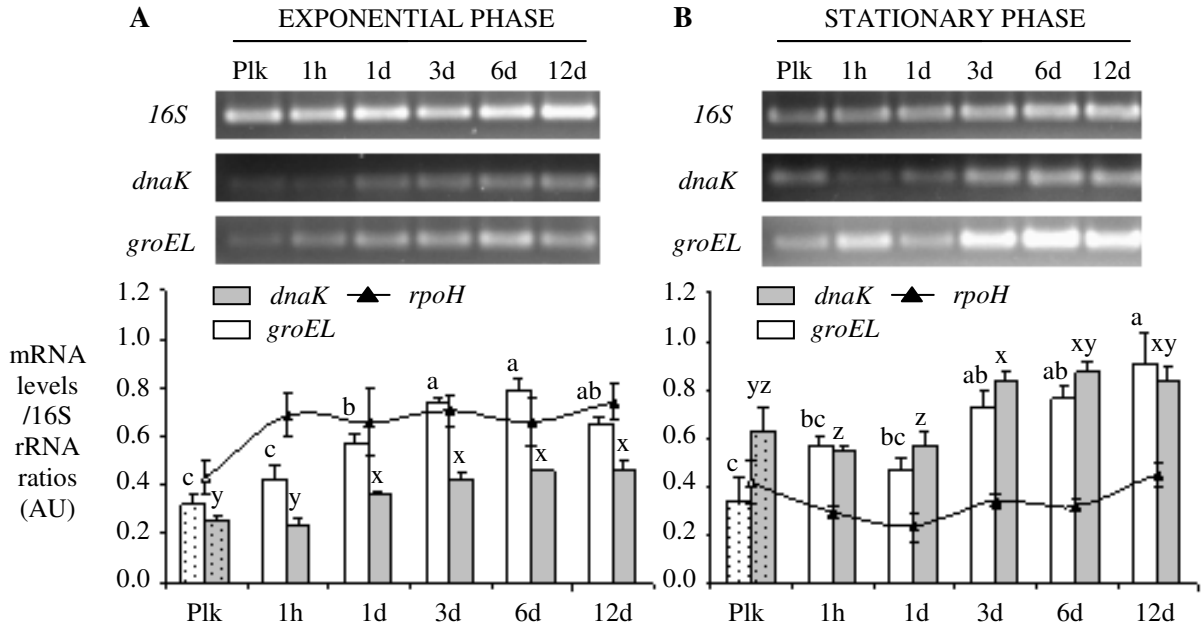

Fig. 1 Variations of *rpoH*, *dnaK* and *groEL* after transfer of exponential (A) and stationary (B) phase bacteria from BHI broth to GBM. Normalized mRNA levels are expressed in arbitrary unit. Means with the same letters (a-c or x-z for *groEL* and *dnaK*, respectively) do not differ significantly (P > 0.05). Gels illustrate PCR results for one of the biological replicates. Plk: planktonic bacteria grown to an OD_{600} of 0.5 or 0.9; d: days after inoculation.

these conditions. In exponential growth phase bacteria, *dnaK* and *groEL* mRNA levels were unchanged 1h after inoculation but increased significantly after 1 day of cold storage. A similar increase in *dnaK* and *groEL* transcription for stationary phase bacteria occurred after 3 days post-inoculation (Fig 1B). Thus, σ^H regulons were activated earlier in exponential phase bacteria than the other sigma factors, confirming that stationary phase bacteria are more resistant or better adapted to various stresses including growth on meat surfaces at 5°C.

IV. CONCLUSION

Our study provides evidences that physiological state of bacteria in the inoculum is of great importance for further examining the molecular changes that occur during bacterial adaptation to a new environment. Furthermore, sigma factor expression and activity indicate that the transition from rich liquid medium to refrigerated GBM is not perceive as a stress, but only triggers adaptation processes in *E. coli*. Knowing that stationary phase bacteria are more resistant to various stresses, it is important to let the cells adapt and grow to that extend in the new meat environment prior to antimicrobial systems/process validation.

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