Physiological adaptation of *Escherichia coli* after transfer onto refrigerated ground meat and other solid matrices: a molecular approach

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Abstract

Bacteria on meat are subjected to specific living conditions that differ drastically from typical laboratory procedures in synthetic media. This study was undertaken to determine the behavior of bacteria when transferred from a rich-liquid medium to solid matrices, as is the case during microbial process validation. *Escherichia coli* cultured in Brain-Heart Infusion (BHI) broth to different growth phases were inoculated in ground beef (GB) and stored at 5°C for 12 days or spread onto BHI agar and cooked meat medium (CMM), and incubated at 37°C for several hours. We monitored cell densities and the expression of σ factors and genes under their control over time. The initial growth phase of the inoculum influenced growth resumption after transfer onto BHI agar and CMM. Whatever the solid matrix, bacteria adapted to their new environment and did not perceive stress immediately after inoculation. During this period, the σ^E and σ^H regulons were not activated and *rpoD* mRNA levels adjusted quickly. The *rpoS* and *gadA* mRNA levels did not increase after inoculation on solid surfaces and displayed normal growth-dependent modifications. After transfer onto GB, *dnaK* and *groEL* gene expression was affected more by the low temperature than by the composition of a meat environment.

Keywords: *Escherichia coli*; liquid/solid transfer; bacterial stress response; meat; sigma factors; gene expression

1. Introduction

Environmental factors such as temperature, gaseous atmosphere, pH or salt concentration affect bacterial growth, activity and resistance during meat storage or processing (Borch et al., 1996) and can modify bacterial gene expression patterns to a certain extent (Allen et al., 2008). Considering the ability of bacteria to adapt to hostile living conditions, the efficacy of antimicrobial systems must be tested using experimental conditions as close as possible to the ones encountered by bacteria in food matrices. So far, gene expression profiles of microorganisms have been evaluated mainly in typical laboratory systems because of the ease of obtaining a large number of clean cells (Tucker et al., 2002; Polissi et al., 2003; Allen et al., 2008). Inocula are also prepared in nutrient-rich liquid culture media with high mixing rates, high initial cell densities and a lack of competitors. However, bacteria in meat are attached to the food surface and exposed to very different conditions, suggesting that the extrapolation of data obtained from laboratory broth culture to solid food matrices could be largely misleading (Tamplin et al., 2005).

Life on food has often been compared to a biofilm. In natural biofilms, however, bacteria are exposed to nutrient limitation and other stresses likely to result in reduced growth (Hengge-Aronis, 2002; Collet et al., 2008), which is far from the situation encountered in a nutrient rich perishable food like meat or cheese. Biofilms are sessile three-dimensional communities of bacteria that can occur on different surfaces in contact with fluids (Schembri et al., 2003). Bacteria living in biofilms are usually regarded as similar to stationary phase cells in terms of their reduced metabolic activities (Beloin et al., 2004; Xu et al., 2001) and induction of σ^S activity (Lacour and Landini, 2004; Patten et al., 2004; Ren et al., 2004; Domka et al., 2007).

These transcriptional factors, involved in the general stress response, control many of the molecular changes that occur when growth slows down and confer increased stress resistance to immobilized and stationary phase bacteria (Loewen and Hengge-Aronis, 1994; Dodd and Aldsworth, 2002). The resistance conferred by the activation of the σ^S regulon could be of great interest for bacterial survival in meat environments. 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, similar to what is observed with biofilms.

Following the expression and activity of the different transcriptional σ factors will be a good method to assess the physiological states of bacteria growing on meat and to identify the specific molecular modulations triggered during adaptation to meat environments. Members of the σ factor family (encoded by *rpo* genes) bind RNA polymerase to allow the transcription of particular genes so that their expression and activity can change in response to the bacterial growth phase and environmental factors (Abee and Wouters, 1999). The bacterial genome is mainly transcribed by σ^D under optimal growth conditions whereas alternative factors (σ^S, σ^H and σ^E), with different promoter specificities, trigger the expression of different regulons under stressful conditions (Chung et al., 2006). For example, σ^H is active during exposure to high temperatures, inducing production of heat shock proteins (HSPs), whereas σ^E participates in extreme heat and extra-cytoplasmic stress responses (Hayden and Ades, 2008). Measurements of the expression of σ factors, and of specific genes under their control, will also help us to determine whether bacteria perceive stressful conditions or simply adapt when transferred to a new environment, such as meat.

Thus, to better understand the behavior of bacteria transferred to meat, the gene expression and transcriptional activities of σ^D, σ^E, σ^H, σ^S were evaluated in meat inoculated with planktonic

Escherichia coli cells prior to and during subsequent cold storage. The expression profiles of the different σ factors and the genes under their transcriptional control, was thus used to compare adaptation and molecular adjustments triggered by growth conditions in the meat matrix. Ultimately, the results highlight the proper conditions for inoculum preparation when conducting challenge studies and process validation.

2. Materials and methods

2.1. Bacterial strains and general culture conditions

Stock cultures of *E. coli* K12 (MG1655) were stored at -80°C in Brain-Heart Infusion broth (BHI; BD Biosciences, Canada) supplemented with 20% (v/v) glycerol as a cryoprotectant. This laboratory strain was chosen because of the extensive knowledge on its genomic regulation and the molecular tools available for its study. For each experiment, 10 ml of BHI broth was inoculated with a thawed bacterial stock culture and incubated at 37°C for 24 h. Cells were then consecutively sub-cultured (1%) into 50 ml and 200 ml BHI broth. All sub-cultures were incubated in vented erlenmeyer flasks (Duo Cap, VWR International, USA) at 37°C with minimal agitation (150 rpm) to avoid cell stratification. The final 200-ml cell suspensions were grown to an OD_{600} of 0.1, 0.5 or 0.9, corresponding to the lag, exponential and stationary phases of bacterial growth, respectively (data not shown). Planktonic cells were then inoculated onto solid matrices (ground beef, BHI agar or Cooked Meat Medium) to establish *E. coli* growth kinetics and gene expression profiles. For each experiment, aliquots of BHI pre-cultures with the desired OD600 were also collected to determine gene expression levels before inoculation.

111 *2.2. Inoculation of ground beef and cold storage conditions*

112

113 Ground beef (GB) was prepared aseptically from *semimembranosus* Angus AAA beef
114 muscle as previously described (Greer and Jones, 1991; Saucier and Greer, 2001). In this
115 condition, aerobic mesophilic counts prior to inoculation were below detection level (< 10 cfu/g
116 on standard Plate Count Agar). Frozen aliquots of 25 g were thawed overnight at 5°C and
117 inoculated with 100 µl of exponential (OD_{600} 0.5) or stationary (OD_{600} 0.9) phase *E. coli* cultures
118 prepared as described above, to reach a final concentration of Log 7 CFU/g. This final
119 concentration was optimised in a preliminary study where serial 10-fold dilutions of exponential
120 BHI pre-cultures were inoculated onto GB to determine the detection limits of bacterial gene
121 expression by RT-PCR in meat (Cui et al., 2003; De Wet et al., 2008). Even though a
122 concentration of Log 5 CFU/g was sufficient to detect, by RT-PCR, the 16s and 23s rRNA of *E.*
123 *coli* inoculated in meat after one day of storage at 5°C, higher cell concentrations improved
124 bacterial RNA recovery and limited the amount of undesirable eukaryotic RNA, as determined by
125 the amplification of the bovine β-actin transcript (data not shown). Because it was impractical to
126 obtain a concentrated enough cell suspension to inoculate the meat at Log 7 CFU/g with an OD_{600}
127 0.1 *E. coli* pre-culture, only OD_{600} 0.5 and 0.9 cultures were used to prepare the meat inoculum.
128 After 1 h and 1, 3, 6 and 12 days of incubation at 5°C, the inoculated meat samples were
129 suspended in 225 ml of pre-cooled peptone water (Difco Laboratories, USA), transferred to
130 stomacher filter bags and homogenized for 2 min at 230 rpm with a stomacher lab blender
131 (Stomacher 400 circulator, Seward Ltd, UK). The homogenates were centrifuged at 5°C and 100
132 x g for 10 min and 40 ml of the supernatants were filtered through Steriflip® units (20 µm nylon
133 net) to remove meat particles (Millipore, USA). A recovery of 90% of the inoculated bacteria was

obtained by this procedure. One ml of the filtrates was used for cell enumeration and the remaining volume was centrifuged (6500 x g for 10 min at 5°C) to collect bacteria for RNA extraction. For cell counts, appropriate ten-fold serial dilutions of the bacterial suspension were spread on BHI agar and incubated at 37°C for 24 h. Cell pellets collected by centrifugation for RNA preparation were treated with RNAprotect® Bacteria Reagent (Qiagen Inc, Canada) according to the manufacturer's instructions before storage at –80°C. Aliquots of BHI pre-cultures used to prepare the inoculum were treated similarly.

2.3. Growth of bacteria on BHI agar plates and Cooked Meat Medium pellets

Planktonic *E. coli* grown in BHI broth to an OD_{600} of 0.1, 0.5 and 0.9 as described above were also transferred onto BHI agar plates or Cooked Meat Medium (CMM) after dilution in peptone water (0.1% w/v) to obtain cell suspensions of 10^7 CFU/ml. A total of 5×10^6 cells were evenly spread on each of four BHI agar plates pre-warmed to 37°C. After 0.5, 1, 2, 4 and 6 h of incubation, a plate was used for cell enumeration and the three others for total RNA extraction. Bacteria were harvested by washing the agar surface 3 times with 1 ml of peptone water for cell enumeration or RNAprotect® bacteria Reagent for RNA extraction. A sterile folded Pasteur pipette was used to spread and detach the cells from the agar surface.

Cooked Meat Medium was prepared according to the manufacturer's instructions (Oxoid; Fisher scientific, Canada). All flasks containing the suspended meat pellets were pre-warmed at 37°C before inoculation at 10^7 CFU/ml. Inoculated media were first incubated for 5 min at 37°C, 150 rpm to allow uniform attachment of bacteria to the CMM pellets. The liquid phase was

156 removed with a sterile strainer and the CMM pellets were returned to 37°C for 0, 0.5, 1, 2, 4, 6
157 and 8 h. Cooked Meat Medium pellets (20 g) were then transferred to stomacher filter bags with
158 180 ml of peptone water and homogenized with a stomacher lab blender as described for the GB
159 experiment. A portion of 20 ml of the homogenate was filtered through a Steriflip® unit. One ml
160 of the filtrate was used for cell enumeration and the remaining volume was centrifuged at 7000 x
161 g for 2 min before mixing the residual pellets with RNAprotect® reagent prior to freezing. All
162 cell counts were performed as described in section *2.2* and RNAprotect® reagent was removed
163 by a short centrifugation just before freezing the cell pellets as recommended by the manufacturer
164 (Qiagen).

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166 *2.5. Total RNA extraction and RT-PCR experiments*

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168 Frozen cell pellets were resuspended in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA,
169 pH 8.0) with 1 mg/ml of lysozyme (SIGMA Aldrich, Canada) and 20 µl of Proteinase K (Qiagen
170 Inc, Canada). Enzymatic lysis was performed at room temperature for 5 to 20 min, depending on
171 the volume of the cell pellet. Total RNA was extracted with RNeasy Mini or Midi Kits (Qiagen
172 Inc., Canada) following the manufacturer's protocols. For cells harvested from GB, the lysis
173 buffer was supplemented with 100 mM NaCl and 100 µl of 10% SDS (Cui et al., 2003), and a
174 phenol/chloroform extraction was performed before transfer to RNeasy columns. RNA
175 concentrations and the OD_{260}/OD_{280} ratios were measured using a NanoDrop ND 1000 (Thermo
176 Scientific, USA). The RNA samples were incubated at 37°C for 30 min with DNAse (2 U
177 DNAse/10 µg RNA; Ambion, Toronto, Ontario, Canada) before phenol/chloroform purification

178 and NaCl/ethanol precipitation. The quality of total RNA samples was evaluated by
179 electrophoresis on a 1% agarose gel containing ethidium bromide.
180 Total RNA (500 ng) was reverse transcribed with the SuperScript II enzyme and random
181 hexamers (Invitrogen, Toronto, Ontario, Canada). Target cDNAs were then amplified by PCR
182 using specific primers (Table 1). The reactions were performed for 28-30 cycles at 56/58°C using
183 Taq DNA polymerase (Invitrogen), except for 16s rRNA whose amplification required only 20
184 cycles. PCR products were electrophoretically separated on a 2% agarose gel and quantified by
185 densitometry after scanning with a ChemiDOC^{TM} XRS (BIO-RAD, Canada). For each sample,
186 the mRNA levels of transcription factors (*rpoD, E, H* and *S*) and genes under their specific
187 control (*uspA*, *rseB*, *dnaK* and *gadA*, respectively) were normalized to 16s rRNA levels, and
188 expressed as a ratio of target gene over the 16s gene. GroEL/16s ratio was also evaluated for
189 bacteria growing on meat. For all genes, the three biological replicates of each culture were
190 amplified in the same PCR run and the amplicons were separated simultaneously on the same
191 agarose gel (*i.e*., all samples at one OD_{600} for one solid matrix on one gel).
192 Particular attention was paid to the selection of genes from the different σ factor regulons
193 for RT-PCR evaluations, to get an indirect estimate of σ factor activity and to determine how
194 environmental changes were perceived. In order to evaluate σ^H and σ^E transcriptional activities,
195 *dnaK* and/or *groEL*, and a gene involved in the extra-cytoplasmic stress response, *rseB*, were
196 chosen, respectively. Cuny et al. (2007) observed an increase of *dnaK* and *groEL* expression
197 when cells were transferred from liquid to agar LB plates. The universal stress protein A (*uspA*)
198 belongs to a restricted category of genes whose transcription is exclusively controlled by σ^D and,
199 as a consequence, is repressed in several sub-lethal stresses (Kvint et al., 2003). Similarly,
200 glutamate decarboxylase A (*gadA*) was chosen from the numerous genes controlled by σ^S

because its expression increases with many stresses including the entry into stationary growth, low environmental pH and osmotic shock (De Biase et al., 1999; Patten et al., 2004).

2.6. Statistical analysis

The Statview 5.0 software (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis. Three biological replicates were done for all experiments and all numeric values were reported as means ± SEM. Homogeneity of the variance between groups was assessed by a Bartlett test. Time-course changes of bacterial growth and relative mRNA levels were evaluated using a one-way ANOVA and the means were compared using a Student-Newman-Keuls test.

3. Results

3.1. Transcription and activity of sigma factors after transfer to GB and during cold storage

Bacteria grown in BHI broth to the exponential and stationary growth phases were transferred to GB and stored at 5°C. Changes in the transcription of σ factors (σ^D, σ^E, σ^H and σ^S) and of genes under their specific regulation (*uspA*, *rseB*, *dnaK*/*groEL* and *gadA*) were studied to determine if a bacterial stress response was initiated by the meat environment. As expected, no significant growth was observed after 12 days of cold storage. During this period, fewer changes were observed in the expression profiles of stationary phase bacteria than exponential phase ones (Fig. 1), suggesting that bacteria in stationary phase adapted readily to the new meat environment. After transfer from BHI broth to refrigerated meat, the mRNA levels of the

224 "housekeeping" transcriptional factor, *rpoD,* increased significantly in exponential phase bacteria
225 (P = 0.0005; Fig. 1a) but not in stationary phase cells (P = 0.1; data not shown). This appeared to
226 be normal adjustment of σ^D activity in exponential bacteria since *uspA* mRNA levels were not
227 affected (P = 0.6; Fig. 1a).
228 Transferring the cells from BHI broth to cold stored meat did not cause significant change
229 in the *rpoS* mRNA levels during the storage period for inocula grown to an OD_{600} of 0.5 and 0.9
230 (P = 0.3 and 0.1, respectively). Furthermore, σ^S activity, evaluated by *gadA* transcription, was
231 unchanged for stationary phase cells (P = 0.8; data not shown) and decreased only after 12 days
232 of storage at 5°C for exponential phase bacteria (P = 0.03; Fig. 1b). Considering that an acid
233 stress can induce both σ^S activation and *gadA* transcription in entero-hemorrhagic *E. coli* (Olesen
234 and Jespersen, 2010), our data indicate that no molecular adjustment for environmental pH
235 adaptation was necessary during the transfer of *E. coli* from BHI broths (pH 6.9 and 6.3 at OD_{600}
236 0.5 and 0.9 respectively) to a meat environment (pH 5.5).
237 Significant changes in *rpoE* transcription were observed in exponential (P = 0.049; Fig 1c)
238 and stationary (P = 0.01; Fig 1d) phase bacteria without modification of *rseB* transcription (P >
239 0.1). However, time-course changes in *rpoE* mRNA levels differed according to the initial
240 physiological state. The lack of change in *rseB* transcription suggested that the activation of the
241 envelope stress response was limited after transferring bacteria to a meat environment.
242 The heat-shock regulons also appeared to be more sensitive to the new environmental
243 conditions on the refrigerated meat surface. Despite the absence of significant variations in *rpoH*
244 mRNA levels (P > 0.1), the activity of σ^H, evaluated by the transcription of two heat shock genes,
245 changed significantly during cold storage, suggesting an important role for the RpoH regulon in
246 cell growth adaptation under these conditions. In exponential growth phase bacteria (Fig. 2a),

247 *dnaK* and *groEL* mRNA levels were unchanged 1 h after inoculation but increased significantly
248 after 1 day of storage at 5°C. A similar increase in *dnaK* and *groEL* transcription for stationary
249 phase bacteria occurred 3 days after inoculation (Fig. 2b). Thus, σ^H regulons were activated
250 earlier in exponential phase bacteria, suggesting again that stationary phase bacteria were more
251 resistant or better adapted for growth on meat surfaces at 5°C.
252
253 *3.2. Growth parameters and gene expression profiles in bacteria transferred to synthetic*
254 *matrices*
255
256 The same gene expression study as that performed in GB was carried out using BHI agar
257 plates and CMM pellets inoculated with *E. coli* incubated at its optimal growth temperature
258 (37°C). Planktonic bacteria grown to different cell densities (OD_{600} 0.1, 0.5 and 0.9) were
259 inoculated onto BHI agar plates and sterile CMM pellets. After transfer to solid media, cell
260 growth was evaluated for up to 8 h (Table 2). After transfer, bacterial growth typically resumes
261 after a lag period whose duration varies with the initial growth phase of the inoculum. When
262 bacteria from a culture with an OD_{600} of 0.1 were transferred onto BHI agar plates, less than 30
263 min was necessary for growth to resume whereas significant increases in cell concentration did
264 not occur until more than 60 min after transfer for cultures with an OD_{600} of 0.5 and 0.9 ($P <$
265 0.05). For cells adhering to cooked meat pellets, active growth resumed within 1 h, regardless of
266 the OD of the inoculum. Growth resumed faster for a culture with an OD_{600} of 0.1 transferred to
267 BHI agar (< 30 min) but took longer when transferred to CMM (30-60 min), and even longer for
268 cultures with OD_{600} of 0.5 and 0.9 when transferred to BHI agar (1-2 h). Hence, both the initial

269 physiological state of the bacteria and the composition of the new environment influenced growth
270 after the cells were transferred from liquid to solid surfaces.
271 To determine the molecular adjustments triggered by environmental changes, the
272 transcription of σ^D, σ^E, σ^H and σ^S regulons were evaluated prior to and after inoculation on solid
273 surfaces. Significant changes in mRNA levels during liquid to solid transition and subsequent
274 growth on BHI agar plates or CMM pellets were detected by ANOVA (Table 3). The
275 transcription level of the σ^D ("*housekeeping*") regulon varied more extensively after transfer to
276 CMM than it did when transferred to BHI agar. Modifications were only observed for lag phase
277 bacteria transferred onto BHI agar plates whereas transcription of *rpoD* was significantly
278 modified under all experimental conditions using CMM pellets (P = 0.002, 0.004 and 0.025 for
279 lag, exponential and stationary phase bacteria, respectively). The activity of σ^D, estimated using
280 *uspA* mRNA levels, remained unchanged only for exponential phase bacteria on CMM (P =
281 0.43). However, most of these changes appeared to be growth-dependent and demonstrated only
282 the normal adaptation of bacteria to a new environment rather than a true stress response (Fig. 3).
283 The transfer of lag phase bacteria from liquid to CMM pellets (Fig. 3a) was followed by a
284 decrease in *rpoD* and *uspA* mRNA levels (two-fold lower after 30 min of growth than for the
285 planktonic state). For the other physiological states (Fig 3b-c), expression of *rpoD* and *uspA* was
286 lower before bacterial growth resumed on CMM pellets and increased significantly after 4 hours
287 of active growth on the solid surface. The entry into stationary phase of bacteria attached to
288 CMM pellets was also followed by a drop in *uspA* mRNA levels without any change in *rpoD*
289 transcription (between 4 and 6 h), illustrating a normal reduction in σ^D regulon activity during
290 this growth phase.

291 The expression of the σ^S regulon was also highly modified during growth on BHI agar and
292 CMM pellets (Table 3). A decrease in *rpoS* transcription was always observed in the first hour
293 after inoculation on either solid matrix. This reduction occurred 30 min after inoculation on both
294 solid surfaces for all physiological states, except for exponential phase bacteria spread on BHI
295 agar, in which *rpoS* mRNA levels decreased only after 1 h of growth on solid medium (not
296 shown). No rapid induction of σ^S activity was detected immediately after inoculation on solid
297 media and *gadA* mRNA levels only displayed growth-dependent regulation in BHI and CMM
298 experiments, as displayed in a classic growth curve (Patten et al., 2004). For bacteria inoculated
299 on CMM, the *gadA* mRNA levels were equivalent (lag phase bacteria; Fig 3d) or lower
300 (exponential and stationary phase bacteria, Fig 3e-f) than the planktonic levels 30 min after the
301 liquid to solid transition, and only increased when attached cells entered the stationary growth
302 phase. The highest *gadA* mRNA levels were observed after 4 h and 6 h of growth on BHI agar
303 (not shown) and after 6 to 8 h on CMM pellets, after cells had adapted and resumed growth in
304 their new environment.
305 When the growth temperature remained at the optimal 37°C during the transfer from liquid
306 to solid medium, only a small number of changes occurred in heat shock regulons (Table 3).
307 More variation occurred in the transcription of σ^H and σ^E regulons after transfer onto BHI agar
308 than transfer to CMM. Significant changes for *dnaK* transcription in exponential phase bacteria
309 ($P = 0.03$), and for *rseB* in exponential and stationary phase cultures ($P = 0.008$ and 0.02,
310 respectively), were observed after transfer onto BHI agar. For exponential phase bacteria
311 transferred to BHI agar, *rseB* and *dnaK* mRNA levels were similar to those of planktonic cells
312 from the time of inoculation until 6 h post-incubation on the agar surface (Fig. 4a). For stationary
313 phase bacteria (Fig. 4b), lower levels of *rseB* were measured 30 min after inoculation, whereas

314 *dnaK* mRNA levels were not significantly different (P = 0.96). Hence, changes in gene
315 expression were observed at a later stage during growth on BHI agar, not early after transfer as
316 would be expected for a typical stress response. The adaptation to new growth conditions
317 generated the greatest number of significant changes in σ factor expression profiles in lag phase
318 bacteria, compared to other physiological states, independent of the type of solid surface.
319 Furthermore, as illustrated by the transcription levels of *rpoE*, *rpoH*, *rpoS* and *uspA* mRNA in
320 cells growing on BHI agar (Fig. 4c), most of these molecular changes were detected in the first
321 30 min after inoculation and decreased, when compared to planktonic mRNA levels.
322
323 **4. Discussion**
324
325 Cells exposed to unfavorable growth conditions are expected to react with a profound
326 change in σ factor gene expression and activity, depending on the severity of the perceived stress
327 (Chung et al., 2006). We found it valuable to simultaneously follow, over time, the expression of
328 different σ factors and genes under their control in bacteria transferred to a new environment.
329 This allowed us to determine their physiological state and discriminate between changes due to
330 stress and changes due to normal adaptation. When transferred from BHI broth to BHI agar, the
331 bacteria were required to adapt only to the physical change of the growth media (liquid *vs*. solid,
332 nutrient diffusability, oxygenation), whereas cells transferred to CMM pellets or GB were also
333 exposed to changes in the composition of the growth medium. The results of the transfer from
334 BHI broth to BHI agar or CMM pellets indicated that increases in *rpoS* and *gadA* mRNA levels
335 were only observed when growth on solid media resumed and not immediately after transfer to
336 the solid surface, as expected in a typical stress response. These data indicated that neither σ^S

337 transcription nor σ^S activity changed during adaptation to the new environmental conditions
338 following cell attachment. Only growth-dependent changes were observed for these two genes, as
339 reported with a classical growth curve. These results agree with those previously observed for
340 *Salmonella enterica serovar Typhimurium* and *Escherichia coli* O157:H7 subject to different
341 environmental conditions. After inoculation of *S. enterica serovar Typhimurium* into skim milk,
342 *rpoS* expression coordinated with stationary growth and was not affected by the stress perceived
343 during the transfer from Luria Bertani broth to skim milk (Thompson et al., 1999). Different food
344 environments also failed to induce σ^S expression and activity in *E. coli* O157:H7, except tomato
345 and ginger in which the increase of *gadA* mRNA levels was mainly due to acidity (Yokoigawa et
346 al., 2003). Our study demonstrated that, despite changes in life conditions (nutrients, lower pH of
347 fresh meat, refrigeration), *rpoS* mRNA levels did not change significantly between exponential
348 and stationary phase bacteria immobilized in GB after 12 days of storage at 5°C. Under these
349 conditions, *gadA* mRNA levels in exponential bacteria decreased only slightly after 12 days of
350 refrigeration, suggesting reduced resistance to unfavorable conditions can occur after a long
351 period of cold storage.

352 Our results (*rpoS* and *gadA* mRNA levels) indicated that bacteria quickly adapted to new
353 lifestyle conditions and did not perceive true stress when transferred from rich liquid medium to a
354 solid matrix, contrary to what has been previously suggested by Cuny et al. (2007) with *E. coli*
355 K12 MG1655. Using 2D protein electrophoresis, these authors observed that DnaK and GroEL,
356 two protein chaperones whose production is controlled by σ^H, were increased in exponential
357 phase bacteria 10 min after transfer from LB broth to LB agar. In our experiments, increased
358 *dnaK* levels were induced only after 6 h of growth on BHI agar, compared to planktonic cells at
359 an OD_{600} of 0.5. No significant changes in *dnaK* expression were observed for pre-cultures

starting at an OD_{600} of 0.1 and 0.9. This observation suggests that, for the induction of heat shock regulons immediately after transferring exponentially growing cells to a solid surface, the conclusion drawn by Cuny et al. (2007) may have been different if lag or stationary growth phase bacteria or a longer incubation period had been used.

The immobilized way of life in young and mature biofilms also induced the expression of genes related to heat shock, and oxidative and envelope stress response, for example, *ibpA*, *ibpB*, *soxRS* or *rseB* (Beloin et al., 2004; Ren et al., 2004). Domka et al. (2007) reported that cold shock proteins were transiently induced during biofilm formation, suggesting that many genes associated with the stress response are activated after cell immobilization. In our experiments, the attachment of bacteria onto BHI agar and CMM pellets did not involve marked changes in expression of the σ^H and σ^E regulons. On the contrary, our results using GB suggested that bacteria were more affected by cold storage than attachment onto the meat surface. Indeed, the absence of significant changes in *rseB* transcription revealed that the extra-cytoplasmic stress response was not induced in *E. coli* after transfer onto GB. However, incubating *E. coli* in a meat environment at 5°C triggered significant changes in σ^H activity (as measured by *dnaK* and *groEL* mRNA levels) but not σ^H expression. Induction of the σ^H regulon by exposure to low temperatures has been previously reported to enhance the survival of planktonic *E. coli* O157:H7 (Kim et al., 2005). More recently, transcriptome comparisons of *E. coli* O157:H7 incubated at 37°C or 7.5°C for 15 min also revealed cold-dependent increases in transcription of *dnaK*, *clpB* and *ftsH*, three members of the heat shock regulon, without induction of σ^H (Allen et al., 2008). In our study, *dnaK* and *groEL* mRNA levels indicate that the σ^H regulon was activated earlier in exponential phase bacteria than in stationary phase one, confirming that stationary phase bacteria are more resistant and better adapted for growth in more stringent conditions (such as meat stored

383 at 5°C). Even if we developed a powerful tool to study gene expression, the work is technically
384 tedious and bare the limits imposed by the stability of the mRNA molecules under study.
385 In conclusion, our study provides molecular evidence that inoculum preparation is of
386 great importance when studying the molecular changes that occur during bacterial adaptation to
387 new environmental conditions, such as those encountered in foods. Growing cells to inoculate
388 foods is easy but our results demonstrate that a certain adaptation time is required before their
389 physiology approaches that of bacteria naturally contaminating foods. Hence, we recommend that
390 inocula should be prepared in such a way that the cells have time to adapt and grow under the
391 conditions used in the real food system during process validation and challenge studies. Ideally,
392 this means growth to stationary phase, where bacteria are known to be more resistant, or at least
393 incubation in the food of interest before studying the efficacy of antimicrobial compounds or
394 processes. The initial growth phase of bacteria before inoculation onto solid surfaces and the
395 composition (e.g., nutrient content) of the new environment influence the growth parameters and
396 the σ factor expression profiles. Whatever the nature of the solid medium (BHI agar, CMM
397 pellets, GB) and the environmental conditions encountered, σ factor expression and activity
398 indicated that the transition from liquid to solid medium was not perceived as a stress under our
399 experimental conditions, contrary to what was observed by Cuny et al. (2007) with LB medium,
400 but rather triggered rapid adaptation to the new conditions. Data from BHI and CMM
401 experiments suggested that a general adaptation response occurred for bacteria immobilized on a
402 new substrate, similar to what has been observed after subculture in laboratory growth media that
403 provides the classical bacterial growth curve.
404

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519 **Fig. 4. a**) Evolution of mRNA expression profiles in lag phase *E. coli* inoculated on BHI agar.
520 The different electrophoresis gels correspond to one of the three biological replicates. **b-c**)
521 Modifications of RpoE regulons in exponential and stationary phase bacteria after transfer on
522 BHI agar. Gels on the top right corner show one of the three PCR results for *rseB*. Significant
523 variations in normalized mRNA levels were analyzed by ANOVA (P-values in top left corner).
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526

527 Table 1
528 Sequences of specific primers used for RT-PCR experiments. H.T: hybridization temperature; bp:
529 base pair

Genes	Primers (F: forward; R: reverse)	H.T.	Size	Reference *
16s rRNA	**F:** ATG ACC AGC CAC ACT GGA AC	58°C	151 bp	Beloin et al., 2004
	R: CTT CCT CCC CGC TGA AAG TA			
rpoD	**F:** TTC GTA CGC AAG AAC GTC TG	58°C	105 bp	
	R: AGG TAT CGC TGG TTT CGT TG			
rpoE	**F:** GTC GTC CAC CTT CCA GTG AT	56°C	134 bp	
	R: AAT AGT TCG GAA AAC TAT CT			
rpoH	**F:** CAG TTG GCA ACC TGG ATT CC	58°C	101 bp	Carruthers et al., 2009
	R: GCC ATG GTA ATG CAG CTT TTC			
rpoS	**F:** GGA CGC GAC TCA GCT TTA CC	58°C	101 bp	Caspeta et al., 2008
	R: CGA CAT CTC CAC GCA GTG C			
uspA	**F:** AAT GCA GGC TAC CCA ATC AC	58°C	162 bp	
	R: GGT GTT GAT CAG CTG ACG TG			
rseB	**F:** TCT CTG CGT TAT CGA CAT GC	56°C	127 bp	
	R: GTT CAA GTC CCG GTT CAA AA			
dnaK	**F:** ACG GTC TGG ACA AAG GCA CT	58°C	101 bp	Caspeta et al., 2008
	R: GCC GTC AAC TTC GTC GAT TT			
gadA	**F:** GGT GAT GCG CAT TAT GTG TC	58°C	100 bp	
	R: CGG GTG ATC GCT GAG ATA TT			
groEL	**F:** GTG GGT ATC AAA GTT GCA CTGCGT	58°C	183 bp	Carruthers et al., 2009
	R: TTT GGT TGG GTC CAG GAT ACC CAT			

530 * Primers without reference have been designed using the primer3 interface (Rozen
531 and Skaletsky, 2000).
532

Table 2
Comparison of growth parameters on BHI agar and CMM

Medium :	BHI Agar (LOG_{10} CFU/ml)			Cooked Meat Medium (LOG_{10} CFU/g)		
Bacteria :	OD_{600} 0.1	OD_{600} 0.5	OD_{600} 0.9	OD_{600} 0.1	OD_{600} 0.5	OD_{600} 0.9
Incubation				*6.15±0.25**	*6.65±0.08**	*6.68±0.12**
0 h	6.61±0.04[f]	6.61±0.07[d]	6.74±0.02[d]	4.53±0.11[e]	4.07±0.14[f]	4.10±0.08[f]
0.5 h	6.89±0.04[e]	6.17±0.19[d]	6.47±0.14[d]	4.82±0.14[de]	4.29±0.03[f]	4.28±0.14[f]
1 h	7.40±0.01[d]	6.72±0.19[d]	6.78±0.11[d]	5.33±0.38[d]	4.71±0.05[e]	4.69±0.03[e]
2 h	8.27±0.01[c]	7.59±0.16[c]	7.45±0.04[c]	6.29±0.25[c]	5.59±0.11[d]	5.71±0.07[d]
4 h	9.57±0.08[b]	9.18±0.09[b]	9.33±0.07[b]	8.23±0.06[b]	7.65±0.37[c]	7.50±0.19[c]
6 h	10.05±0.06[a]	9.99±0.13[a]	10.08±0.09[a]	9.21±0.11[a]	8.85±0.14[b]	9.06±0.18[b]
8 h	NE[§]	NE	NE	9.51±0.12[a]	9.25±0.14[a]	9.46±0.12[a]
ANOVA	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001
Lag time [#]	0 – 30 min	1 – 2h	1 – 2h	30 min - 1h	30 min - 1h	30 min – 1h

In each column, cell concentrations with the same letter do not differ significantly (P > 0.05).

* Concentrations in LOG CFU/ml for the liquid fraction inoculating the CMM pellets. After the inoculation period (5 min at 37°C), the liquid phase was removed and concentrations on CMM pellets were given as LOG CFU /g.

[#] Time needed to observe the first significant changes in cell number after inoculation.

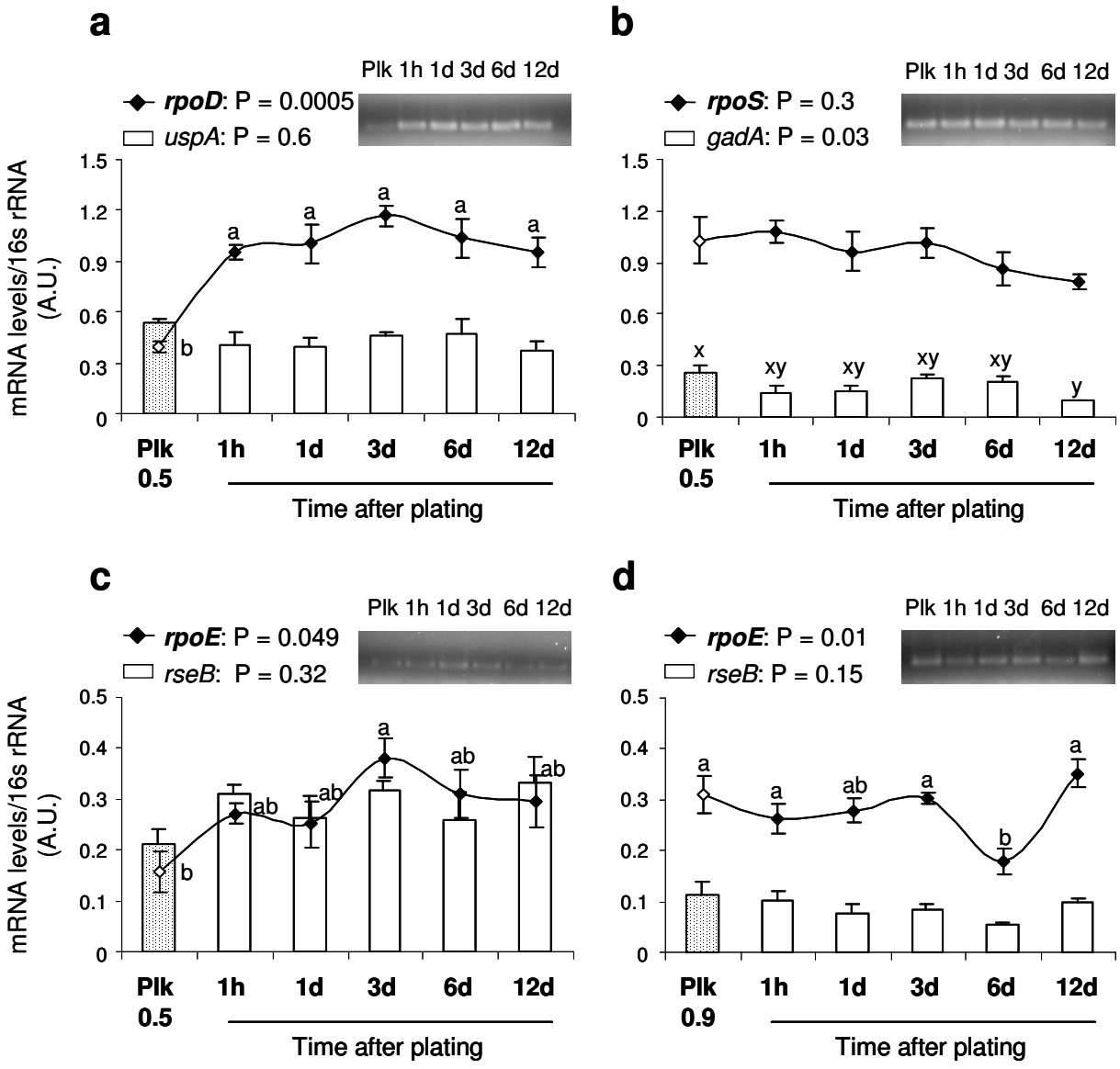
[§] NE: not evaluated.

543 Table 3
544 Summary of significant time-dependant changes for relative mRNA levels in bacteria growing on
545 BHI agar plates or CMM pellets

	Brain Heart Infusion			**Cooked Meat medium**		
Phases / Genes	Lag (OD_{600} 0.1)	Exponential (OD_{600} 0.5)	Stationary (OD_{600} 0.9)	Lag (OD_{600} 0.1)	Exponential (OD_{600} 0.5)	Stationary (OD_{600} 0.9)
RpoD **(σ^D)**	*	NS	NS	**	**	*
↳ *uspA*	*	NS	NS	*	NS	**
RpoE **(σ^E)**	***	NS	NS	NS	NS	NS
↳ *rseB*	NS	**	*	NS	NS	NS
RpoH **(σ^H)**	**	NS	NS	NS	NS	*
↳ *dnaK*	NS	*	NS	*	NS	NS
RpoS **(σ^S)**	***	*	*	*	NS	**
↳ *gadA*	***	***	***	***	***	**

546 One-way ANOVA: * ($P < 0.05$), ** ($0.01 < P < 0.001$), *** ($P < 0.001$); NS: not significant.

547



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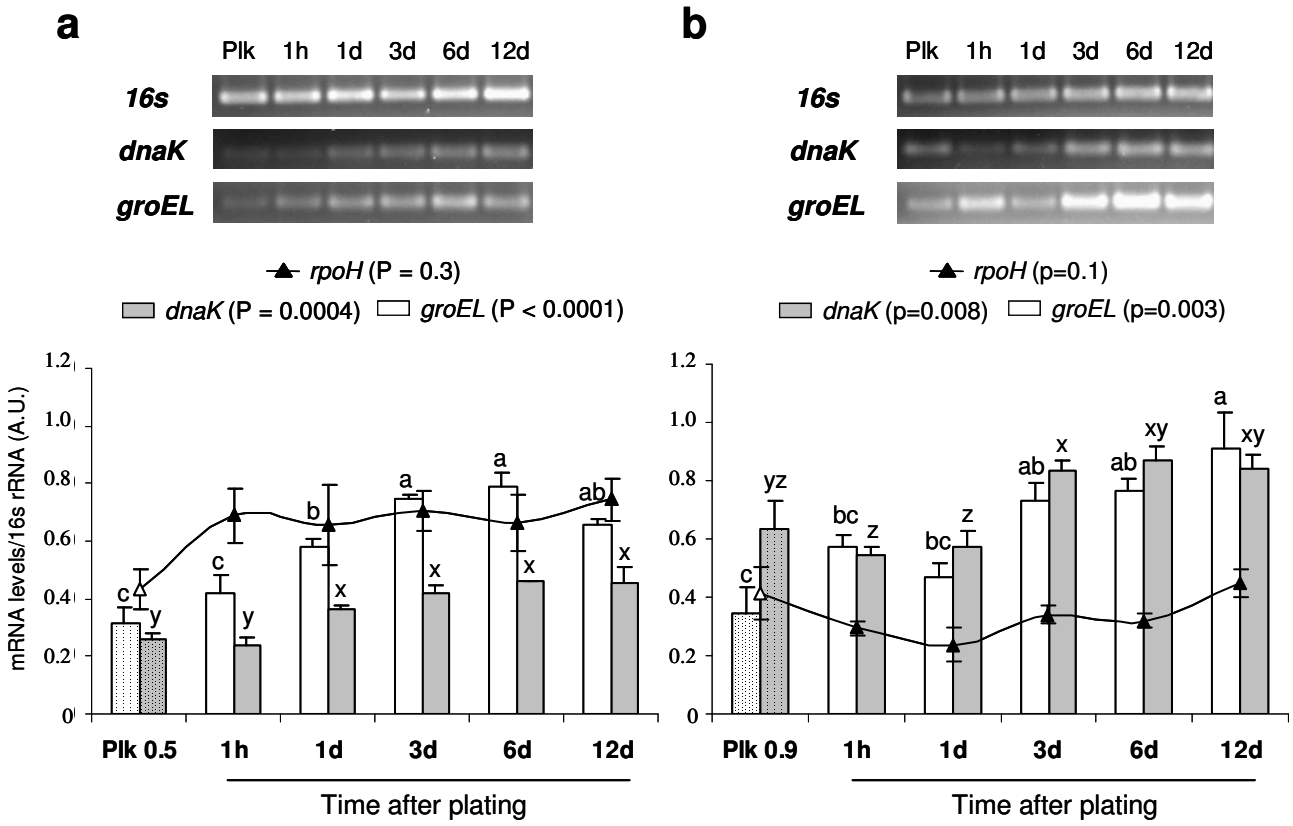

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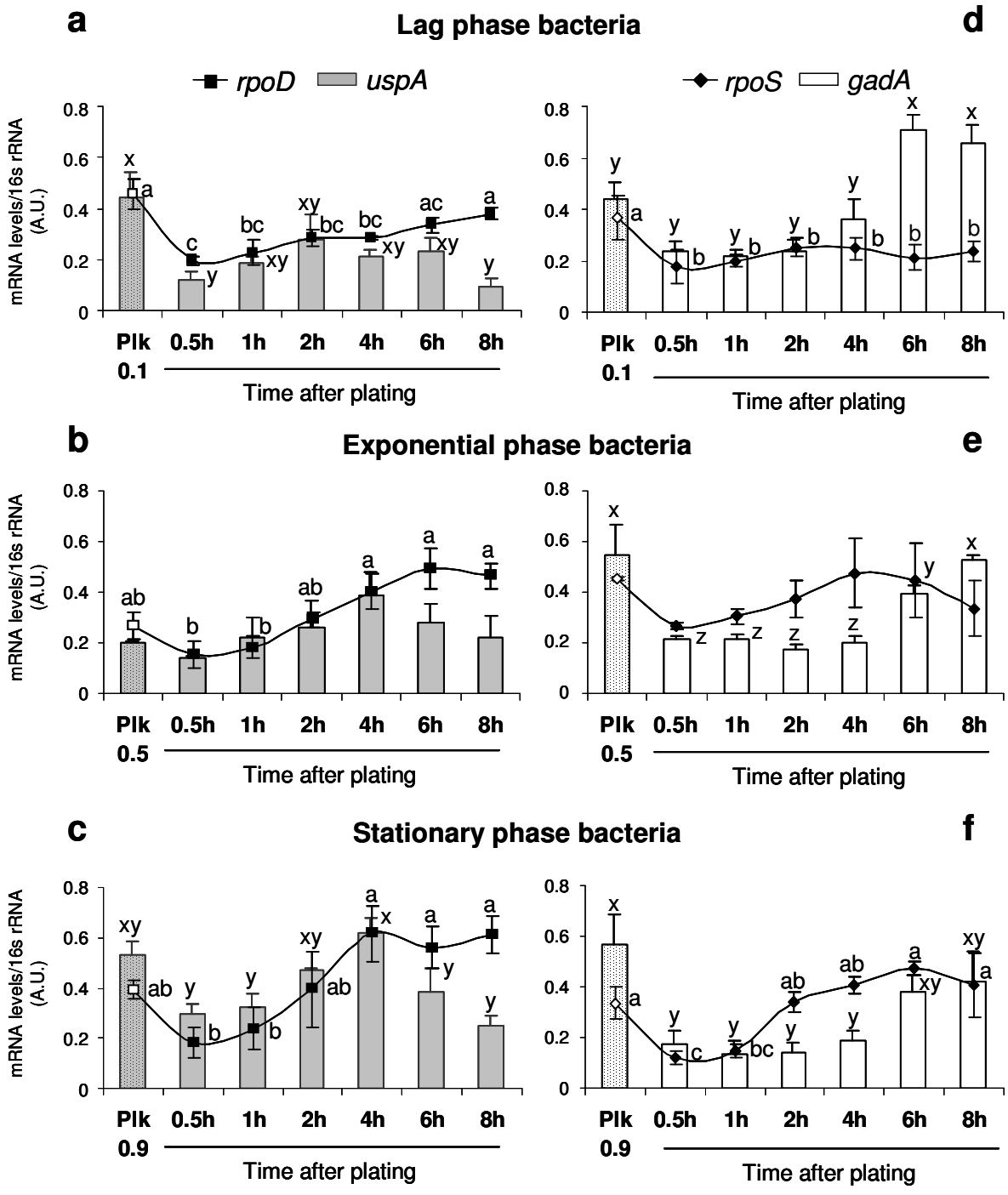

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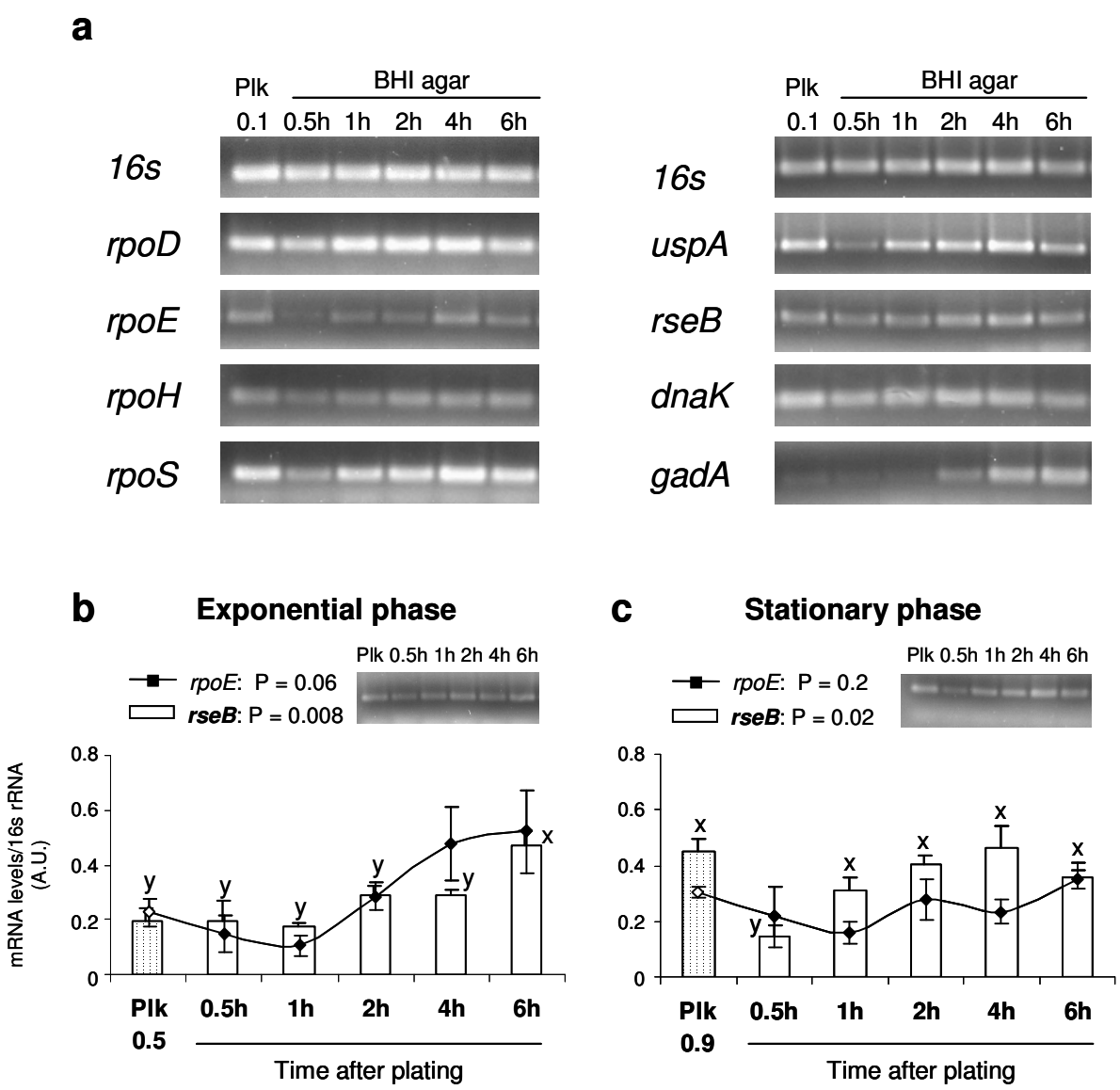

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