**TITLE:** Transcriptomic analysis of *Escherichia coli* MG1655 cells exposed to pulsed electric fields

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## HIGHLIGHTS

-Transcriptomic response of PEF-treated *E. coli* cells at pH 4.0 was evaluated

-PEF treatments activated a response related to cytoplasmic membrane

-Cell requirement for energy and reducing power after a PEF treatment was confirmed

-PEF limited the development of acid shock response at pH 4.0

-Transcriptomic cell response after PEF was much more limited than after heat

-Transcriptomic and phenotypic results showed a lack of PEF-induced cross-resistance


## ABSTRACT

DNA microarrays were used for the first time to study the mechanism of bacterial inactivation by pulsed electric fields (PEF). *Escherichia coli* MG1655 cells were PEF-treated for 50 pulses (0.08 Hz) at 20 kV/cm (2.10 kJ/kg per pulse) in buffer at pH 4.0 (100 µs PEF treatment time). After this PEF treatment, 47 genes demonstrated transcriptional differences. Among the 20 up-regulated genes, we found cytochrome *bo* oxidase genes (*cyoB*, *cyoC*, *cyoD*) and heme O synthase (*cyoE*), succinate dehydrogenase (*sdhCDAB*) operon, and transcriptional repressors of *bet* genes and chromosomal *ars* operon (*betI* and *arsR*, respectively). Gene class testing showed relevance of the tricarboxylic acid (TCA) cycle pathway, confirming cell requirement for energy and reducing power after PEF treatments. Transcriptomics after this PEF treatment were compared with cell transcriptome after a heat treatment using the same experimental setup, revealing a weaker cell response to PEF, which mainly involves components and functions directly associated with the cytoplasmic membrane.

Resistance experiments confirmed results observed by transcriptomics, showing the lack of cross-resistance phenomena by PEF treatments.

*Industrial relevance:* The molecular approach to the description of the mechanism of microbial inactivation by pulsed electric fields (PEF) could result in a more efficient design of preservation process. Confirmation of cytoplasmic membrane as a PEF bacterial target, as well as metabolic routes involved in cell response to PEF, could assist in deciding other technologies to combine with PEF. Furthermore, the lack of PEF-induced cross resistance under non-thermal conditions to other food preservation technologies, such as heat and antimicrobial compounds, could help in maintaining the efficiency of hurdle technologies that rely upon the imposition of multiple stresses. This information will facilitate the knowledge-based enhancement of current intervention methods or the design of new preservation processes involving PEF.

## 1. INTRODUCTION

Bacterial inactivation to guarantee food safety is generally achieved by heat treatments in the food industry. As an alternative, pulsed electric fields (PEF) treatment is a non-thermal process with, in contrast to heat treatments, a potential to inactivate microorganisms with minimal impact on organoleptic and nutritional food properties (Barbosa-Cánovas, Góngora-Nieto, Pothakamury & Swanson, 1999). The design of effective PEF processes to provide safe food with an extended shelf life requires, among other things, the description of the events leading to bacterial death during a PEF treatment.

Electroporation as a consequence of PEF is considered the key event in bacterial death. Most studies suggest that PEF exert its bactericidal action primarily on the cytoplasmic membrane (Aronsson, Rönner & Borch, 2005; García, Gómez, Mañas, Raso & Pagán, 2007; Wouters, Bos & Ueckert, 2001). Permeabilization studies revealed that irreversible pores caused cell death, while reversible pores could indicate the occurrence of sublethal injuries in cytoplasmic membrane of bacterial cells (García et al., 2007; Wouters et al., 2001). Actually, sublethal injuries in this bacterial structure have been described after PEF treatments as a function of treatment pH (García, Gómez, Raso & Pagán, 2005). Repair of sublethal damages in this structure involved lipid synthesis and energy production in *Escherichia coli* cells (García et al., 2007). In addition, damaged *Listeria monocytogenes* cells in their cytoplasmic membranes also required energy production to recover (Somolinos, Espina, Pagán & García, 2010). All this knowledge of the mechanism of bacterial inactivation by PEF has allowed the design of combined processes (Ait-Ouazzou et al., 2011, 2012; Monfort, Sagarzazu, Condón, Raso & Álvarez, 2013) based on hurdle theory (Leistner & Gorris, 1995).

However, in-depth studies are needed in order to better understand bacterial death by PEF treatments.

The "age of omics" has changed the study of microbial physiology by introducing global analysis tools such as comparative genomics and global expression techniques including DNA microarrays (transcriptomics) (Wecke & Mascher, 2011). Study of gene expression by transcriptome analysis, i.e. RNA transcripts present in the cell under a given condition, could allow for discovery of cell resistance mechanisms and/or the regulatory network that coordinate bacterial stress response (Jordan, Hutchings & Mascher, 2008). By comparing gene expression profiling in bacterial cells before and after PEF exposure, sets of genes up-regulated or down-regulated by PEF could be identified and, consequently, the proteins activated by PEF could be inferred. Function of genes/proteins induced under these circumstances would be a reflection of the mechanism of resistance and consequently about the way the microorganisms are inactivated by PEF. Although this approach has been successfully used to describe the mode of action of many antibiotics (Wecke & Mascher, 2011), and the mechanisms of bacterial adaptation and inactivation by heat (Guernec, Robichaud-Rincon & Saucier, 2013; Gunasekera, Csonka & Paliy, 2008) or by high hydrostatic pressure (HHP) (Bowman, Bittencourt & Ross, 2008), to the best of our knowledge transcriptome analysis of bacterial response to PEF has not been reported.

Furthermore, transcriptional profiling has also shown induction of general stress responses and proteins after a given treatment that could be involved in cross-resistance phenomena, such as heat-shock-induced genes *rpoH*, *dnaK*, *dnaJ*, *groEL*, *groES*, or *grpE* (Carruthers & Minion, 2009), or $\sigma^B$-dependent general stress response induced by the cell wall antibiotic vancomycin (Shin et al., 2010). Transcriptome profiling of bacterial cells treated by PEF would allow a better understanding of the mechanism of

104 inactivation and the mechanisms of resistance that cells use to cope with PEF
105 treatments.

106 The objective of this study was to investigate the transcriptomic response of *E.*
107 *coli* MG1655 upon exposure to PEF treatments by microarray hybridization.
108 Furthermore, a comparison with transcriptomic response to lethal heat treatments was
109 performed in order to contribute to the description of the mechanism of bacterial
110 inactivation by PEF. Finally, contribution of transcriptomic response to bacterial stress
111 response after PEF treatments was evaluated.

112

## 2. MATERIAL AND METHODS

### *2.1 Micro-organisms and growth conditions*

The strain used was *Escherichia coli* MG1655. The culture was maintained in a cryovial at -80°C.

Broth subcultures were prepared by inoculating, with one single colony from a plate, a test tube containing 5 mL of sterile Tryptic Soy Broth (Oxoid, Basingstoke, Hampshire, England) with 0.6% Yeast Extract added (Oxoid) (TSBYE). After inoculation, the tubes were incubated overnight at 37°C. With these subcultures, 250 mL Erlenmeyer flasks containing 50 mL of TSBYE were inoculated to a final concentration of $10^4$ colony-forming units (CFU)/mL. These flasks were incubated under agitation (130 rpm) (Selecta, mod. Rotabit, Barcelona, Spain) at 37°C until the stationary growth phase was reached (24 h / 2·× $10^9$ CFU/mL).

### *2.2 PEF treatments*

PEF treatments were carried out using equipment that delivered exponential-decay pulses, as previously described (García, Gómez, Raso, et al., 2005). High electric field pulses were produced by discharging a set of 10 capacitors (6800 pF) (Behlke, C-20C682, Kronberg, Germany) via a thyristor switch (Behlke HTS 160-500SCR) in a batch treatment chamber. The capacitors were charged using a high-voltage dc power supply (FUG, HCK 2500M 35000, Rosenhein, Germany), and a function generator (Tektronix AFG 320, Wilsonville, OR, USA) delivered the on-time signal to the switch. The treatment chamber was made of a cylindrical plastic tube closed with two polished stainless steel electrodes (Raso, Álvarez, Condón & Sala, 2000). The gap between electrodes was 0.25 cm, and the electrode area was 2.01 $cm^2$. The actual voltage and electrical intensity applied were measured with a high voltage probe and a current probe

138 respectively connected to an oscilloscope (Tektronix TDS 3012B). The PEF equipment
139 includes provisions for measuring sample temperature. Immediately after the treatment
140 a thermocouple type K of 0.9 mm diameter, pneumatically activated, enters into the
141 treatment chamber and the temperature is measured in the centre of the chamber.

142 Before the treatment, microorganisms were likewise centrifuged at 6000 × *g* for
143 5 min and resuspended for a final concentration of approximately $2 \times 10^8$ CFU/mL in
144 McIlvaine citrate-phosphate buffer of pH 4.0, and electrical conductivity was adjusted
145 to 2 mS/cm, similar to food products such as apple juice. Next, 0.5 mL of the samples
146 was placed into the treatment chamber with a sterile syringe, as it has been previously
147 described (Raso et al., 2000). Cell suspensions were treated for 50 pulses (1 Hz, pulse
148 width 2 μs) at electric field strengths of 10, 20, 25, 30, and 35 kV/cm, corresponding to
149 specific energies of 0.47, 2.10, 3.35, 4.74, and 8.07 kJ/kg per pulse, respectively.
150 Experiments started at room temperature (22±2ºC). In all experiments the temperature
151 of the samples after treatment was lower than 35 ºC. Samples for transcriptomic assay
152 were subjected to 50 exponential waveform pulses of 20 kV/cm at a repetition rate of
153 0.08 Hz (pulse width 2 μs).

154 Immediately after treatment, 0.5 mL samples were used to extract RNA or 0.1
155 mL samples were used for survivors' enumeration.

156

157 ***2.3 Heat treatments***

158 For the transcriptomic assay, a heat treatment was carried out in an incubator
159 (FX Incubator, Ref ZE/FX, from ZEU-INMUNOTEC) at 48°C with a thermocouple
160 (Ahlborn, mod. Almemo 2450, Holzkirchen, Germany) to monitor the temperature
161 during the heat treatment. Once the temperature had stabilized, 50 μL of a diluted cell
162 suspension was added into a sterile tube containing 450 μL of McIlvaine citrate-

phosphate buffer of pH 4.0. The initial bacterial concentration was approximately $2 \times 10^8$ CFU/mL. After 10 min, 0.5 mL samples were immediately used to extract RNA or 0.1 mL samples were used for survivors' enumeration.

### *2.4 Cross-resistance treatments*

Cells of *E. coli* MG1655 were PEF-treated in McIlvaine citrate-phosphate buffer of pH 4.0 with the conditions selected for transcriptomic assay, as previously explained. Control cells were subjected to: a) 0 min in McIlvaine citrate-phosphate buffer of pH 4.0 and b) 10 min in McIlvaine citrate-phosphate buffer of pH 4.0, the same duration as the PEF treatment.

PEF-treated cells were treated by heat or constituents of essential oils citral (95%; Sigma-Aldrich, Steinheim, Germany), and carvacrol (95%; Sigma-Aldrich). For the 3 types of experiments, the initial bacterial concentration was approximately $1 \times 10^7$ CFU/mL and treatment media was McIlvaine citrate-phosphate buffer of pH 4.0. Citral and carvacrol have been used successfully in combined processes to preserve fruit juices (Espina, Somolinos, Pagán & García-Gonzalo, 2010) or liquid whole egg (Espina, Monfort, Álvarez, García-Gonzalo & Pagán, 2014).

Heat treatment was performed at 52°C for 10 min in an incubator (FX Incubator) as previously explained. Citral and carvacrol are practically immiscible in water, so a vigorous shaking method was used to prepare suspensions (Friedman, Henika & Mandrell, 2002). Citral and carvacrol were added at final concentrations of 300 and 100 μL/L, respectively, to treatment media before addition of cell suspensions, and were maintained at 20°C for 30 min.

### *2.5 Counts of viable cells*

188 The physiological response of the bacterial cells was investigated by
189 enumeration of viable cells.
190 After treatments, samples were diluted in Phosphate Buffered Saline, pH 7.3
191 (PBS; Oxoid). Next, 0.1 mL samples were pour-plated onto Tryptic Soy Agar (Oxoid)
192 with 0.6% Yeast Extract added (Oxoid) (TSAYE).
193 Plates were incubated at 37°C for 24 h. After plate incubation, the colonies were
194 counted with an improved image analyzer automatic counter (Protos; Analytical
195 Measuring Systems, Cambridge, United Kingdom), as it had been previously described
196 (Condón et al., 1996).
197 Both heat and PEF treatment conditions for transcriptomic assay were chosen to
198 obtain a level of bacterial cell inactivation less than 50% in 10 min in order to attain a
199 transcriptomic response in the same amount of time under lethal conditions, but in
200 which most cells are alive.
201
202 ***2.6 Detection of sublethal injury***
203 In order to determine bacterial cell injury, treated samples were also plated on
204 TSAYE with 3% of sodium chloride (Panreac, Barcelona, Spain) added (TSAYE-SC).
205 This was the maximum non-inhibitory sodium chloride concentration for native cells
206 previously determined (data not shown).
207 Selective media plates were incubated for 48 h. Previous experiments showed
208 that longer incubation times did not influence survival counts.
209 The number of sublethally injured cells was estimated by the difference in the
210 number of CFU obtained after plating PEF-treated cells in the nonselective (TSAYE)
211 and the selective (TSAYE-SC) media. The proportion of sublethally injured cells was

212 estimated by the difference in the number of $\log_{10}$ cycles of CFU obtained after plating
213 PEF-treated cells in the same media.
214
215 ***2.7 Statistical analysis***
216 Inactivation was expressed as difference in $\log_{10}$ counts before and after every
217 treatment. The error bars in the figures indicate the mean ± standard deviations from the
218 data obtained from at least 3 independent experiments carried out with different
219 microbial cultures. ANOVA and *t*-tests were performed with GraphPad PRISM®
220 (GraphPad Software, Inc., San Diego, USA) and differences were considered significant
221 if $p \leq 0.05$.
222
223 ***2.8 RNA isolation, labeling and hybridization***
224 RNA samples were obtained from untreated and treated cultures that were
225 prepared as described above. Cultures were immediately pelleted by centrifugation at
226 6000 × g for 5 min. Three biological replicates were performed under identical
227 conditions. RNA was isolated using the RNeasy Kit (Qiagen). The quality of the
228 isolated RNA was examined by Tape Station using the R6K ScreenTape Kit (Agilent).
229 All samples gave RIN (RNA Integrity Number) values of >9.
230 The labeling and hybridization was performed as per the Agilent Two-Color
231 Microarray-Based Prokaryote Analysis Fair Play III Labeling Protocol v. 1.3 by
232 Bioarray SL (www.bioarray.es).
233 Labeled cDNAs were hybridized on Agilent's *E. coli* Microarray Kit 8x15K, ID
234 020097. Hybridization and subsequent washing of the slides were performed according
235 to the manufacturer's instructions.

The microarray slides were scanned using an Agilent microarray scanner (G2505C), and data were processed with Agilent's Feature Extraction software (v. 10.7).

### *2.9 Analysis of Gene Expression*

Data were normalized using *Tquantile* method to allow comparison between the totalities of the arrays. Multiple testing correction was performed using Benjamini and Hochberg correction (Benjamini & Hochberg, 1995). Spots with an adjusted $p$-value < 0.05 were considered statistically significant, and kept false positive rate to less than 5%. Expression levels obtained from 3 independent biological replicates of the treated samples and 4 independent biological replicates of the untreated samples were compared using the Limma package (Smyth, 2004) of the Bioconductor software (Gentleman et al., 2004). A functional grouping of genes was made according to the data from the NCBI (http://www.ncbi.nlm.nih.gov/COG/) using GOStats package of the Bioconductor software.

## 3. RESULTS AND DISCUSSION

### *3.1 Transcriptomic response in PEF-treated cells*

To determine the effect of PEF on the transcriptome *E. coli* MG1655, whose complete genome sequence is available (Blattner et al., 1997), DNA microarrays were used to compare transcript levels within stationary-phase bacterial cells subjected to a mild PEF condition at pH 4.0 with regard to bacterial cells held in the same pH 4.0 media without any additional treatment (control cells).

The transcriptomics analyses were carried out at pH 4.0, since PEF technology has been proposed to pasteurize acidic food, such as fruit juices (Saldaña, Puértolas, Monfort, Raso & Álvarez, 2011; Timmermans et al., 2014). Due to high PEF resistance shown by bacterial spores, the low pH conditions of acidic food would allow control of bacterial spores by inhibiting the germination stage (Raso & Barbosa-Cánovas, 2003). It should be taken into account that an acid shock response might be expected in control cells (Foster, 2004), which should be considered for discussion of the results.

In order to select adequate treatment conditions for the transcriptomic assay, *E. coli* cells were PEF-treated for 50 pulses (pulse width 2 μs, 1 Hz) at an electric field range of 10-35 kV/cm in buffer at pH 4.0 (Fig. 1). Under these treatment conditions, cell inactivation determined in nonselective medium ranged from 0.2 to 2.4 log cycles of the initial population. Differential plating technique with selective and nonselective media allowed for evidencing of a population of alive cells with repairable damages in their cytoplasmic membranes, that are targeted but not inactivated by PEF and consequently might develop a transcriptomic response. Recovery of cells in a selective medium with sodium chloride (Mackey, 2000) showed sublethal injuries in cytoplasmic membranes after PEF treatments higher or equal to 20 kV/cm. Thus, a PEF treatment for 50 pulses at 20 kV/cm inactivated 40 % of the *E. coli* cells and injured an additional

277 40 % of the surviving population. This treatment provided the most adequate
278 conditions, since almost 90 % of cells were targeted, and a big proportion of the treated
279 population could respond to PEF stress. Pulse frequency was reduced in order to extend
280 the total treatment time to 10 min with the aim of allowing cells to respond adequately
281 to the treatment, and of comparing these results with those of heat treatment. Therefore,
282 for transcriptomics studies, frequency was changed from 1 to 0.08 Hz. Modification of
283 frequency did not modify cell resistance to the selected PEF treatment ($p > 0.05$) (data
284 not shown).

285 Statistical analysis of DNA microarrays indicated that, after a PEF treatment
286 (100 μs PEF treatment time, 10 min processing time, pH 4.0), 47 genes demonstrated
287 transcriptional differences with an adjusted $p$-value < 0.05, as compared with control
288 cells (10 min, pH 4.0). The significance and differences in transcript levels for all genes
289 are depicted as a volcano plot (Fig. 2). The upper corners of this graph show genes with
290 both large fold changes and statistical significance. Genes with a negative value of $\log_2$
291 fold change are considered to be repressed, while the transcription of those genes with a
292 positive value was activated by PEF treatment. Of the 47 genes differentially expressed,
293 20 genes were up-regulated, while 27 were down-regulated after PEF treatment as
294 compared to control cells (Fig. 2). Tables 1 and S1 show the 45 most significantly
295 differentially expressed genes, 20 up- and 25 down-regulated, respectively. All genes
296 identified as differentially transcribed, along with their fold change value, are presented
297 in Table S2 of the supplementary material.

298 Living organisms respond to stressful environmental conditions by redirecting
299 protein synthesis to alleviate cell damage (Harcum & Haddadin, 2006). As a
300 consequence, transcription of genes involved in cell response and repair is stimulated,
301 while those not involved in these functions, such as genes with roles in cell division

302 machinery, are usually down-regulated. Up-regulated genes after PEF treatment with
303 annotation included cytochrome *bo* oxidase genes (*cyoB*, *cyoC*, *cyoD*) and heme O
304 synthase (*cyoE*) involved in proper functioning of cytochrome *bo* oxidase, succinate
305 dehydrogenase (*sdhCDAB*) operon, and transcriptional repressors of *bet* genes and
306 chromosomal *ars* operon (*betI* and *arsR*, respectively) (Table 1). Cytochrome *bo*
307 oxidase, one of the 3 major terminal oxidases in the aerobic respiratory chain of *E. coli*,
308 consists of 4 subunits encoded by the *cyoB*, *cyoA*, *cyoC*, and *cyoD* genes, all of which
309 are necessary for a functional enzyme. Cytochrome *bo* oxidase contributes to the
310 generation of a proton motive force (PMF), functioning as a proton pump (Puustinen,
311 Finel, Haltia, Gennis & Wikstrom, 1991). Succinate dehydrogenase plays an important
312 role in cellular metabolism and directly connects the tricarboxylic acid (TCA) cycle
313 with the respiratory electron transport chain (Cecchini, Schroder, Gunsalus &
314 Maklashina, 2002). These results demonstrate that PEF-treated cells activate a response
315 involving components and functions directly associated to cytoplasmic membrane,
316 which is considered a main target of PEF treatments (García et al., 2007; García et al.,
317 2005; Wouters et al., 2001). DNA microarrays, used for the first time to study
318 transcriptome of PEF-treated cells, assisted in a better description of PEF-bacterial
319 targets, identifying the pathways and/or structures that cells would follow to repair the
320 damages caused by the treatment.

321 In order to increase power to detect differential expression and to reduce the
322 interpretive challenge, gene-class testing (GCT) is widely used as an analytical tool
323 (Allison, Cui, Page & Sabripour, 2006). Gene classes are usually based on Gene
324 Ontology (GO) categories (for example, genes that are involved in transporter activities
325 or in response to stimulus). These up-regulated genes as a consequence of the PEF
326 treatment are distributed into 7 functional groups (Fig. S1): aerobic respiration, cellular

327 respiration, energy derivation by oxidation of organic compounds, generation of
328 precursor metabolites and energy, oxidation-reduction process, single-organism
329 metabolic process, and TCA cycle. Among them, TCA cycle is the final GO term for
330 the ontology tree for the up-regulated genes (Fig. 3 and Table 2). The TCA pathway is a
331 catabolic pathway of aerobic respiration and the first step in generating precursors for
332 biosynthesis as it generates energy and reducing power (in the form of NADH, NADPH
333 or $FADH_2$) which is used to build bacterial components or to generate new ATP
334 molecules in the electron transport system. Nevertheless, Ceragioli et al. (2010)
335 described up-regulation of inorganic ion transport and metabolism in *Bacillus cereus*
336 after several disinfectant treatments, whereas energy production and conversion genes
337 were commonly down-regulated. As observed under our conditions, generation of
338 precursor metabolites and energy genes were up-regulated by a PEF treatment. This
339 difference may reside in the nature of the treatments, being PEF a method based on
340 physical effects rather than chemical, as is the case with disinfectants. Bowman et al.
341 (2008) similarly described a suppression of a wide range of energy production and
342 conversion in *L. monocytogenes* cells after a HHP treatment, another non-thermal food
343 preservation technology based on physical effects. Moreover, GCT indicated that HHP
344 caused overexpression of genes associated with DNA repair mechanisms, septal rings,
345 transcription and translation protein complexes, and lipid and peptidoglycan
346 biosynthetic pathways, among others (Bowman et al., 2008), which confirmed that
347 structures such as DNA, ribosomes, and septal rings were involved in the mechanism of
348 inactivation by HHP. Differential trancriptomic response of PEF-treated cells would
349 show a different mechanism of bacterial inactivation by PEF as compared with that by
350 HHP due to the multitarget nature of the latter technology. Therefore, our results would
351 confirm the cell requirement for energy to repair sublethal damages in cytoplasmic

membrane caused by PEF treatments and reducing power needed, probably related to synthesis of new lipids, as described by García et al. (2006), indicating that mainly cell envelopes are affected during the inactivation process. Further transcriptomic studies during the repair of sublethally PEF-injured cells in recovery liquid medium (García, Mañas, Gómez, Raso & Pagán, 2006) would possibly reveal more genes implied in biosynthetic requirements required, such as lipid synthesis. Moreover, comparison of our results with the transcriptome of PEF-treated bacterial cells under different treatment conditions (pH, squared waveform pulses, real food systems, etc) and /or grown in the treatment medium, might reveal other new insights in the mechanism of bacterial inactivation by PEF.

Interpretation of down-regulated genes became more complex because control cells were held at pH 4.0 for 10 min in which transcripts related to acid shock response could have been expressed (Foster, 2004). Under these circumstances, those genes could be underestimated in PEF-treated cells as compared with control cells, appearing as down-regulated. Indeed, comparison of transcriptomic response in PEF-treated cells in relation to control cells showed that down-regulated genes with annotation after PEF treatment included genes coding for ammonium transporter (*amtB*), acid shock protein (*asr*), glutamine-binding protein (*glnH*), nitrogen regulatory proteins (*glnK*, *nac*), sigma E factor (*rpoE*), and a gene involved in superoxide response (*soxS*) (Table S1). Overrepresentation analysis of functional categories (based on gene ontology-GO) of genes differentially expressed after the PEF treatment reveals a rather simple pattern. Genes down-regulated as a consequence of the PEF treatment are distributed into 5 functional groups: glutamine biosynthetic process, glutamine family amino acid biosynthetic process, glutamine family amino acid metabolic process, glutamine metabolic process, and response to abiotic stimulus. Among them, abiotic stimulus and

glutamine biosynthetic process were the final GO terms for the ontology tree for the down-regulated genes (Fig. S2 and S3 and Tables S3 and S4). The former included genes *asr* and *rpoE* (Table S3). These down-regulated genes during PEF treatment have been previously associated with acid shock response (Armalyte, Seputiene, Melefors & Suziedeliene, 2008; Coutts, Thomas, Blakey & Merrick, 2002; Lu et al., 2013). Our hypothesis is that transcriptomic expression of genes related to acid shock response (*amtB, asr, glnH, glnK, glnP, nac*) was more active in control cells (at pH 4.0 for 10 min) than in PEF-treated cells, indicating that PEF led to a weaker acid shock response in comparison with control cells, although both samples were held at pH 4.0 for 10 min. Instead, PEF-treated cells might be focusing their active pathways in responding to the lethal treatment by generating energy and reducing power, as shown above. In addition, our results indicate that acid resistance mechanisms would not be involved in cell repair and/or response to PEF albeit cells were treated under acid conditions.

### *3.2 Comparison of transcriptomics in PEF-treated and heat-treated cells*

PEF treatments have been suggested as an alternative to heat treatments for food preservation (Barbosa-Cánovas et al., 1999). Mechanism of inactivation by heat treatments has been well studied (Gould, 2000), as well as bacterial transcriptome after heat stresses (Carruthers & Minion, 2009; Gunasekera et al., 2008; Harcum & Haddadin, 2006). In order to compare cell responses after PEF and heat treatments under our selected conditions (10 min processing time, pH 4.0), transcriptomics after the PEF treatment were compared with transcriptome in cells after a lethal heat treatment. A heat treatment targeting a similar proportion of bacterial cells than PEF treatment was selected: after 10 min at 48°C heat treatment inactivated 50 % of the *E. coli* cells and injured 55 % of the surviving population (data not shown). In contrast to

PEF treatment, heat treatment caused the differential expression of 1,430 genes, 509 of which were up-regulated (Fig. 4) as compared with only 20 up-regulated genes in PEF-treated cells (Fig. 2). These results show an extensive cell response caused by heat treatments as compared with PEF treatments, and consequently more structures might be targeted by heat. While PEF inactivation seems to be location-specific related exclusively with cytoplasmic membrane, heat causes pleiotropic cellular effects and the ultimate inactivation seems to result from multitarget damage, such as ribosome destabilization, membrane permeabilization, enzyme denaturation, and DNA damage, among others. Other explanations could be that heat action is gradual, while PEF may cause a remarkable cell stress that would not allow a cell response and/or that PEF stress might not allow for part of the transcription due to electrostatic interferences in transcriptional machinery. Transcriptomics of PEF-treated cells would be showing a response to the damage caused in bacterial cells that was leading them to recover the components needed rather than preparing new mechanisms for additional stresses. According to these results, PEF and heat treatments do not share any over-expressed genes (Fig. 5), indicating that probably primary targets of PEF are secondary for heat treatments due to the multitarget nature of this technology.

Many previously identified HS response genes are up-regulated during our heat treatment, including genes encoding HS proteins *clpB, htpGX*, and *ibpAB*, proteases *clpX*, *degP, hslV*, *hflX* and *lon*, and major chaperone subunits *groL*, *groS*, *dnaK*, *dnaJ*, and *grpE*, σ factors *rpoD*, *rpoE*, and *rpoH*, among others (data not shown). This was expected as application of heat resulted in an increase in unfavorable protein interactions such as misfolding and aggregation. Notably, *rpoE* was down-regulated due to the PEF treatment. RpoE transcription factor of *E. coli* regulates the expression of genes whose products are devoted to extracytoplasmic activities. The RpoE regulon is

induced upon misfolding of proteins in the periplasm or the outer membrane (Missiakas, Mayer, Lemaire, Georgopoulos & Raina, 1997) and is essential for bacterial growth at a high temperature (Hiratsu, Amemura, Nashimoto, Shinagawa & Makino, 1995). RpoE drives transcription of a number of genes whose functions revolve around heat shock (HS) and misfolded proteins (Ades, Grigorova & Gross, 2003). This situation would indicate that whereas in a prolonged heat treatment, proteins are denatured, during a PEF treatment proteins would not change their conformational state. Remarkably, García et al. (2006), showed that protein synthesis was not required to repair sublethal injuries in the cytoplasmic membrane of PEF-treated *E. coli* cells. Still, there were commonly down-regulated genes after PEF and heat treatments, such as *amtB, asr, glnH*, *glnK*, *nac*, and *soxS*, among other hypothetical proteins (Table 3) which could be attributed to a weaker response to acidic conditions in comparison with untreated cells, as explained above.

### *3.3 Cross-resistance evaluation after a PEF treatment*

The development of stress tolerance has been generally attributed to the induction of specific sets of proteins (Storz & Hengge-Aronis, 2000). In the particular case of cross-resistance responses, the development of resistance has been linked either to the induction of the general stress responses, attributed to the expression of alternative sigma factor $\sigma^S$ in *E. coli* (Storz & Hengge-Aronis, 2000), or to the existence of an overlap between the responses triggered by different stresses such as synthesis of heat-shock proteins (HSPs), involving chaperones and proteases, which is induced rapidly and transiently upon exposure to high temperature (Abee & Wouters, 1999). Previous studies had demonstrated that general stress response would not play a role in PEF-mediated bacterial cell inactivation: opposite to most results obtained when heat-

or cold-shocked cells were heated or pressure-treated (Cebrián, Sagarzazu, Pagán, Condón & Mañas, 2010; Sagarzazu, Cebrián, Condón, Mackey & Mañas, 2010; Somolinos, García, Mañas, Condón & Pagán, 2008). Somolinos et al. (2008) found that none of the 4 *E. coli* strains tested in their study increased their PEF resistance as a consequence of a heat or a cold shock. In addition, although cross-resistance induced by previous heat treatments has been widely demonstrated (Arroyo, Cebrián, Condón & Pagán, 2012; Cebrián, Raso, Condón & Mañas, 2012; Pagán & Mackey, 2000), influence of a previous PEF treatment on following lethal stresses applied for food preservation, such as heat or antimicrobial compounds, has not been reported in the literature. Only Álvarez and Heinz (2007) reported an increase of ultrasound resistance in previously PEF-treated cells, which was attributable to a membrane reorganization caused by PEF treatment.

In order to evaluate the contribution of transcriptome in PEF-treated cells we studied the stress response after a PEF treatment. Observations from transcriptome of PEF-treated cells regarding presence of "response to stimulus" GO category only for down-regulated genes, which would indicate that PEF treatments should not induce cross-resistance phenomena to other treatments. In order to verify whether this phenomenon might be evidenced under practical conditions, *E. coli* cells were PEF treated under the same conditions of the transcriptome experiments (20 kV/cm, pH 4.0, 50 pulses at 0.08 Hz, 100 μs PEF treatment time, 10 min processing time) and immediately subjected to a following heat, citral, or carvacrol treatment (Fig. 6). Cell inactivation of the PEF-stressed cells was compared with the inactivation of untreated cells, and of control cells incubated for 10 min in buffer at pH 4.0, the same conditions used for control cells in transcriptomic analysis. As expected from transcriptome analysis that revealed no activation of stress response proteins, a previous PEF

477 treatment did not increase the resistance of survivors to heat, citral, or carvacrol.
478 Moreover, PEF-treated cells had a decreased resistance to following inactivating
479 treatments in comparison with unstressed cells. While a heat treatment for 10 min at 52
480 ºC (pH 4.0) inactivated less than 1 $\log_{10}$ cycle on unstressed cells, more than 3 $\log_{10}$
481 cycles of survivors to the PEF treatment were inactivated (Fig. 6). Furthermore, cell
482 resistance to a citral treatment (300 µL/L, 30 min, pH 4.0) decreased in more than 1
483 $\log_{10}$ cycles for PEF-stressed cells in comparison with the resistance of unstressed cells
484 (Fig. 6). As for carvacrol resistance (100 µL/L, 30 min, pH 4.0), no differences ($p <$
485 0.05) were detected among PEF-stressed and unstressed cells (Fig. 6). Sensitization of
486 PEF-stressed cells could be attributed to the lower resistance of PEF injured cells (Fig.
487 1). Briefly, transcriptomic and phenotypic analyses revealed that PEF treatments did not
488 induce a cross-resistance to heat, citral, or carvacrol treatments.
489

## 4. CONCLUSIONS

This study is the first to demonstrate and characterize the whole-genome responses of stationary-phase *E. coli* after a PEF treatment, reflecting physiological response of bacteria to PEF.

After selecting treatments targeting almost 90 % of cells, *E. coli* did not show a large transcriptomic response to the PEF treatment. Of the 47 genes differentially expressed, 20 genes were up-regulated, while 27 were down-regulated. Up-regulated genes after PEF treatment with annotation included *cyoB*, *cyoC*, *cyoD*, *cyoE*, *sdhCDAB* operon, *betI*, and *arsR*. TCA cycle is the final GO term for the ontology tree for the up-regulated genes confirming the cell requirement for energy and reducing power needed to repair sublethal damages in cytoplasmic membrane caused by PEF treatments. In addition, our results indicate that acid shock response was weaker in PEF-treated cells than in control cells, suggesting that acid resistance mechanisms might not involved in *E. coli* MG1655 repair and/or response to PEF.

A stronger transcriptomic response caused by heat treatments, as compared with PEF treatments, indicated that heat might target more structures than PEF.

Finally, lack of induction of cross-resistance to heat, citral, or carvacrol treatments after PEF treatments was demonstrated, which will help in maintaining the efficiency of hurdle technologies that rely upon the imposition of multiple stresses.

We consider that this information will facilitate the knowledge-based enhancement of current interventions or the design of new preservation processes involving PEF.

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## 6. FIGURE CAPTIONS

**Fig. 1.** $Log_{10}$ cycles of inactivation of stationary phase cells of *Escherichia coli* MG1655 after PEF treatments at 10, 20, 25, 30, and 35 kV/cm for 50 pulses in citrate-phosphate buffer of pH 4.0. Survivors were recovered in the non-selective (white bars) and selective media with NaCl (black bars). Data are means±standard deviations (error bars).

**Fig. 2.** Volcano plot of transcriptional differences in *Escherichia coli* MG1655 after PEF treatment at 20 kV/cm for 50 pulses in citrate-phosphate buffer of pH 4.0. Individual differences are plotted as $log_2$ fold change vs. $-log_{10}$ adjusted *p*-value. Points above the line at $p < 0.05$ indicate differential expression at false discovery rate of 5%. The number of genes up- or down- regulated ($p < 0.05$) is indicated.

**Fig. 3.** Gene ontology (GO) tree for up-regulated genes belonging to the TCA cycle GO term (GO:0006099) after PEF treatment of *Escherichia coli* MG1655. GO Biological Process Analysis with *p*-value lower than 0.05.

**Fig. 4.** Volcano plot of transcriptional differences in *Escherichia coli* MG1655 after heat treatment at 48°C for 10 min in citrate-phosphate buffer of pH 4.0. Individual differences are plotted as $log_2$ fold change vs. $-log_{10}$ adjusted *p*-value. Points above the line at $p < 0.05$ indicate differential expression at false discovery rate of 5%. The number of genes up- or down- regulated ($p < 0.05$) is indicated.

546 **Fig. 5.** Comparison of levels of genome-wide expression in *Escherichia coli* MG1655
547 after lethal PEF and heat treatments. The number of differentially expressed genes are
548 shown as a Venn diagram. Numbers of up-regulated genes are shown in bold, and
549 numbers of down-regulated genes are shown in italics.
550
551 **Fig. 6.** $Log_{10}$ cycles of inactivation of stationary phase cells of *Escherichia coli*
552 MG1655 after heat (H; 52°C, 10 min), citral (Cit; 300 µL/L, 30 min), and carvacrol
553 (Car; 100 µL/L, 30 min) treatments. Control cells were kept during 0 (white bars) or 10
554 min (grey bars); or PEF-treated at 20 kV/cm for 50 pulses (0.08 Hz) in citrate-phosphate
555 buffer of pH 4.0 (black bars). For all conditions, treatment medium was citrate-
556 phosphate buffer of pH 4.0. Survivors were recovered in the non-selective media. Initial
557 cell concentration was adjusted to $10^7$ CFU/mL for all the experiments. Data are
558 means±standard deviations (error bars).
559

## 7. SUPPLEMENTARY FIGURE CAPTIONS

**Fig. S1.** Gene ontology (GO) tree for the enriched GO categories after PEF treatment of *Escherichia coli* MG1655. GO Biological Process Analysis with *p*-value lower than 0.05.

**Fig. S2.** Gene ontology (GO) tree for down-regulated genes belonging to the response to abiotic stimulus GO term (GO:0009628) after PEF treatment of *Escherichia coli* MG1655. GO Biological Process Analysis with *p*-value lower than 0.05.

**Fig. S3.** Gene ontology (GO) tree for down-regulated genes belonging to the glutamine biosynthetic process GO term (GO:0006542) after PEF treatment of *Escherichia coli* MG1655. GO Biological Process Analysis with *p*-value lower than 0.05.

## 8. REFERENCES


Abee, T., & Wouters, J.A. (1999). Microbial stress response in minimal processing. *International Journal of Food Microbiology*, 50, 65-91.

Ades, S.E., Grigorova, I.L., & Gross, C.A. (2003). Regulation of the alternative sigma factor $\sigma^E$ during initiation, adaptation, and shutoff of the extracytoplasmic heat shock response in *Escherichia coli*. *Journal of Bacteriology*, 185, 2512-2519.

Ait-Ouazzou, A., Cherrat, L., Espina, L., Lorán, S., Rota, C., & Pagán, R. (2011). The antimicrobial activity of hydrophobic essential oil constituents acting alone or in combined processes of food preservation. *Innovative Food Science & Emerging Technologies*, 12, 320-329.

Ait-Ouazzou, A., Espina, L., Cherrat, L., Hassani, M., Laglaoui, A., Conchello, P., & Pagán, R. (2012). Synergistic combination of essential oils from Morocco and physical treatments for microbial inactivation. *Innovative Food Science & Emerging Technologies,* 16, 283-290.

Álvarez, I., & Heinz, V. (2007). Hurdle technology and the preservation of food by pulsed electric fields. In H.L.M. Lelieveld, S. Notermans, & S.W.H. de Haan (Eds.), *Food preservation by pulsed electric fields: from research to application* (pp. 165-177). Cambridge: CRC Press and Woodhead Publishing Limited.

Allison, D.B., Cui, X., Page, G.P., & Sabripour, M. (2006). Microarray data analysis: from disarray to consolidation and consensus. *Nature Reviews Genetics*, 7, 55-65.

Armalyte, J., Seputiene, V., Melefors, O. & Suziedeliene, E. (2008). An *Escherichia coli asr* mutant has decreased fitness during colonization in a mouse model. *Research in Microbiology*, 159, 486-493.

599 Aronsson, K., Rönner, U., & Borch, E. (2005). Inactivation of *Escherichia coli*,
600 *Listeria innocua* and *Saccharomyces cerevisiae* in relation to membrane
601 permeabilization and subsequent leakage of intracellular compounds due to pulsed
602 electric field processing. *International Journal of Food Microbiology*, 99, 19-32.
603 Arroyo, C., Cebrián, G., Condón, S., & Pagán, R. (2012). Development of
604 resistance in *Cronobacter sakazakii* ATCC 29544 to thermal and nonthermal processes
605 after exposure to stressing environmental conditions. *Journal of Applied Microbiology*,
606 112, 561-570.
607 Barbosa-Cánovas, G.V., Góngora-Nieto, M.M., Pothakamury, U.R., & Swanson,
608 B.G. (1999). *Preservation of foods with pulsed electric fields*. San Diego, USA:
609 Academic Press Ltd.
610 Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate - a
611 practical and powerful approach to multiple testing. *Journal of the Royal Statistical*
612 *Society Series B-Methodological*, 57, 289-300.
613 Blattner, F.R., Plunkett, G. 3rd, Bloch, C.A., Perna, N.T., Burland, V., Riley, M.,
614 Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W.,
615 Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B., Shao, Y. (1997). The complete
616 genome sequence of *Escherichia coli* K-12. *Science*, 277, 1453-1462.
617 Bowman, J.P., Bittencourt, & C.R., Ross, T. (2008). Differential gene expression
618 of *Listeria monocytogenes* during high hydrostatic pressure processing. *Microbiology*,
619 154, 462-475.
620 Carruthers, M.D., & Minion, C. (2009). Transcriptome analysis of *Escherichia*
621 *coli* O157:H7 EDL933 during heat shock. *FEMS Microbiology Letters*, 295, 96-102.

622 Cebrián, G., Raso, J., Condón, S., & Mañas, P. (2012). Acquisition of pulsed
623 electric fields resistance in *Staphylococcus aureus* after exposure to heat and alkaline
624 shocks. *Food Control*, 25, 407-414.
625 Cebrián, G., Sagarzazu, N., Pagán, R., Condón, S., & Mañas, P. (2010).
626 Development of stress resistance in *Staphylococcus aureus* after exposure to sublethal
627 environmental conditions. *International Journal of Food Microbiology*, 140, 26-33.
628 Cecchini, G., Schroder, I., Gunsalus, R.P., & Maklashina, E. (2002). Succinate
629 dehydrogenase and fumarate reductase from *Escherichia coli*. *Biochimica et Biophysica*
630 *Acta*, 1553, 140-157.
631 Ceragioli, M., Mols, M., Moezelaar, R., Ghelardi, E., Senesi, S., & Abee, T.
632 (2010). Comparative transcriptomic and phenotypic analysis of the responses of
633 *Bacillus cereus* to various disinfectant treatments. *Applied and Environmental*
634 *Microbiology*, 76, 3352-3360.
635 Coutts, G., Thomas, G., Blakey, D., & Merrick, M. (2002). Membrane
636 sequestration of the signal transduction protein GlnK by the ammonium transporter
637 AmtB. *EMBO Journal,* 21, 536-545.
638 Espina, L., Somolinos, M., Pagán, R. & García-Gonzalo, D. (2010) Effect of
639 citral on the thermal inactivation of *Escherichia coli* O157:H7 in citrate phosphate
640 buffer and apple juice. *Journal of Food Protection*, 73, 2189-2196.
641 Espina, L., Monfort, S., Álvarez, I., García-Gonzalo, D. & Pagán, R. (2014).
642 Combination of pulsed electric fields, mild heat and essential oils as an alternative to the
643 ultrapasteurization of liquid whole egg. *International Journal of Food Microbiology*,
644 189, 119–125.
645 Foster, J.W. (2004). *Escherichia coli* acid resistance: tales of an amateur
646 acidophile. *Nature Reviews Microbiology*, 2, 898-907.

Friedman, M., Henika, P.R., & Mandrell, R.E. (2002). Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli, Listeria monocytogenes*, and *Salmonella enterica*. *Journal of Food Protection*, 65, 1545-1560.

García, D., Gómez, N., Mañas, P., Raso, J., & Pagán, R. (2007). Pulsed electric fields cause bacterial envelopes permeabilization depending on the treatment intensity, the treatment medium pH and the microorganism investigated. *International Journal of Food Microbiology*, 113, 219-227.

García, D., Gómez, N., Mañas, P., Condón, S., Raso, J., & Pagán, R. (2005). Occurrence of sublethal injury after pulsed electric fields depending on the micro-organism, the treatment medium pH and the intensity of the treatment investigated. *Journal of Applied Microbiology*, 99, 94-104.

García, D., Gómez, N., Raso, J., & Pagán, R. (2005). Bacterial resistance after pulsed electric fields depending on the treatment medium pH. *Innovative Food Science & Emerging Technologies*, 6, 388-395.

García, D., Mañas, P., Gómez, N., Raso, J., & Pagán, R. (2006). Biosynthetic requirements for the repair of sublethal membrane damage in *Escherichia coli* cells after pulsed electric fields. *Journal of Applied Microbiology*, 100, 428-435.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y., & Zhang, J. (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biology,* 5, R80.

Gould, G.W. (2000). Preservation: past, present and future. *British Medical Bulletin,* 56, 84-96.

672 Guernec, A., Robichaud-Rincon, P., & Saucier, L. (2013). Whole-genome
673 transcriptional analysis of *Escherichia coli* during heat inactivation processes related to
674 industrial cooking. *Applied and Environmental Microbiology*, 79, 4940-4950.
675 Gunasekera, T.S., Csonka, L.N., & Paliy, O. (2008). Genome-wide
676 transcriptional responses of *Escherichia coli* K-12 to continuous osmotic and heat
677 stresses. *Journal of Bacteriology*, 190, 3712-3720.
678 Harcum, S.W., & Haddadin, F.T. (2006). Global transcriptome response of
679 recombinant *Escherichia coli* to heat-shock and dual heat-shock recombinant protein
680 induction. *Journal of Industrial Microbiology & Biotechnology,* 33, 801-814.
681 Hiratsu, K., Amemura, M., Nashimoto, H., Shinagawa, H., & Makino, K.
682 (1995). The *rpoE* gene of *Escherichia coli*, which encodes $\sigma^E$, is essential for bacterial
683 growth at high temperature. *Journal of Bacteriology*, 177, 2918-2922.
684 Jordan, S., Hutchings, M.I., & Mascher, T. (2008). Cell envelope stress response
685 in Gram-positive bacteria. *FEMS Microbiology Reviews*, 32, 107-146.
686 Leistner, L., & Gorris, L.G.M. (1995). Food preservation by hurdle technology.
687 *Trends in Food Science & Technology*, 6, 41-46.
688 Lu, P.L., Ma, D., Chen, Y.L., Guo, Y.Y., Chen, G.Q., Deng, H.T., & Shi, Y.G.
689 (2013). L-glutamine provides acid resistance for *Escherichia coli* through enzymatic
690 release of ammonia. *Cell Research*, 23, 635-644.
691 Mackey, B.M. (2000). Injured bacteria. In B.M. Lund, T.C. Baird-Parker, &
692 G.W. Gould (Eds.), *The microbiological safety and quality of food* (pp.315-341).
693 Gaithersburg, Maryland: Aspen Publisher Inc.
694 Missiakas, D., Mayer, M.P., Lemaire, M., Georgopoulos, C., & Raina, S. (1997).
695 Modulation of the *Escherichia coli* $\sigma^E$ (RpoE) heat-shock transcription-factor activity
696 by the RseA, RseB and RseC proteins. *Molecular Microbiology*, 24, 355-371.

Monfort, S., Sagarzazu, N., Condón, S., Raso, J., & Álvarez, I. (2013). Liquid whole egg ultrapasteurization by combination of PEF, heat, and additives. *Food and Bioprocess Technology*, 6, 2070-2080.

Pagán, R., & Mackey, B. (2000). Relationship between membrane damage and cell death in pressure-treated *Escherichia coli* cells: differences between exponential- and stationary-phase cells and variation among strains. *Applied and Environmental Microbiology*, 66, 2829-2834.

Puustinen, A., Finel, M., Haltia, T., Gennis, R.B., & Wikstrom, M. (1991). Properties of the two terminal oxidases of *Escherichia coli*. *Biochemistry,* 30, 3936-3942.

Raso, J., Álvarez, I., Condón, S., & Sala, F.J. (2000). Predicting inactivation of *Salmonella senftenberg* by pulsed electric fields. *Innovative Food Science & Emerging Technologies*, 1, 21-30.

Raso, J., & Barbosa-Cánovas, G.V. (2003). Nonthermal preservation of foods using combined processing techniques. *Critical Reviews in Food Science and Nutrition*, 43, 265-285.

Sagarzazu, N., Cebrián, G., Condón, S., Mackey, B., & Mañas, P. (2010). High hydrostatic pressure resistance of *Campylobacter jejuni* after different sublethal stresses. *Journal of Applied Microbiology*, 109, 146-155.

Saldaña, G., Puértolas, E., Monfort, S., Raso, J., & Álvarez, I. (2011). Defining treatment conditions for pulsed electric field pasteurization of apple juice. *International Journal of Food Microbiology*, 151, 29-35.

Shin, J.H., Kim, J., Kim, S.M., Kim, S., Lee, J.C., Ahn, J.M., & Cho, J.Y. (2010). $\sigma^B$-dependent protein induction in *Listeria monocytogenes* during vancomycin stress. *FEMS Microbiology Letters*, 308, 94-100.

722 Smyth, G.K. (2004). Linear models and empirical bayes methods for assessing
723 differential expression in microarray experiments. *Statistical Applications in Genetics*
724 *and Molecular Biology*, 3, Article3.
725 Somolinos, M., Espina, L., Pagán, R., & García, D. (2010). *sigB* absence
726 decreased *Listeria monocytogenes* EGD-e heat resistance but not its Pulsed Electric
727 Fields resistance. *International Journal of Food Microbiology*, 141, 32-38.
728 Somolinos, M., García, D., Mañas, P., Condón, S., & Pagán, R. (2008). Effect of
729 environmental factors and cell physiological state on Pulsed Electric Fields resistance
730 and repair capacity of various strains of *Escherichia coli*. *International Journal of Food*
731 *Microbiology*, 124, 260-267.
732 Storz, G., & Hengge-Aronis, R. (2000). *Bacterial stress responses*. Washington
733 DC: ASM Press.
734 Timmermans, R.A.H., Nierop Groot, M.N., Nederhoff, A.L., van Boekel,
735 M.A.J.S., Matser, A.M., & Mastwijk, H.C. (2014). Pulsed electric field processing of
736 different fruit juices: Impact of pH and temperature on inactivation of spoilage and
737 pathogenic micro-organisms. *International Journal of Food Microbiology*, 173, 105-
738 111.
739 Wecke, T., & Mascher, T. (2011). Antibiotic research in the age of omics: from
740 expression profiles to interspecies communication. *Journal of Antimicrobial*
741 *Chemotherapy*, 66, 2689-2704.
742 Wouters, P.C., Bos, A.P., & Ueckert, J. (2001). Membrane permeabilization in
743 relation to inactivation kinetics of *Lactobacillus* species due to pulsed electric fields.
744 *Applied and Environmental Microbiology*, 67, 3092-3101.
745

**Table 1.** Up-regulated genes after PEF treatment based on adjusted *p*-value of microarray data.

| *Gene* | Systematic Name | logFC | Description |
|---|---|---|---|
| *arsR* | b3501 | 0.92 | Transcriptional repressor of chromosomal *ars* operon |
| *betI* | b0313 | 1.10 | Probably transcriptional repressor of *bet* genes |
| *cyoB* | b0431 | 1.72 | Cytochrome o ubiquinol oxidase subunit I |
| *cyoC* | b0430 | 1.59 | Cytochrome o ubiquinol oxidase subunit III |
| *cyoD* | b0429 | 1.45 | Cytochrome o ubiquinol oxidase subunit IV |
| *cyoE* | b0428 | 1.45 | Protoheme IX farnesyltransferase |
| ECs0483 | ECs0483 | 1.40 | Cytochrome o ubiquinol oxidase subunit |
| *insX* | ABE-0285026 | 1.09 | Predicted IS protein |
| *msyB* | b1051 | 1.17 | Acidic protein suppresses mutants lacking function of protein export |
| *nudE* | b3397 | 1.19 | orf, hypothetical protein |
| *phoH* | b1020 | 1.47 | PhoB-dependent, ATP-binding Pho regulon Component; may be helicase; induced by P starvation |
| *sdhA* | b0723 | 1.25 | Succinate dehydrogenase, flavoprotein subunit |
| *sdhB* | b0724 | 1.18 | Succinate dehydrogenase, iron sulfur protein |
| *sdhC* | b0721 | 1.81 | Succinate dehydrogenase, cytochrome b556 |
| *sdhD* | b0722 | 1.64 | Succinate dehydrogenase, hydrophobic subunit |
| *tatA* | b3836 | 0.83 | orf, hypothetical protein |
| *yafQ* | b0225 | 0.78 | orf, hypothetical protein |
| *ycgZ* | c_1607 | 1.22 | Hypothetical protein YcgZ |
| *yidF* | c_4598 | 0.84 | Hypothetical protein YidF |
|  | c_1605 | 0.91 | Hypothetical protein |

**Table 2.** Up-regulated genes belonging to the TCA cycle GO term (GO:0006099) after PEF treatment of *Escherichia coli* MG1655. GO Biological Process Analysis with *p*-value lower than 0.05 (Hypergeometric Test).

| *Gene* | Systematic Name | logFC | Description |
|---|---|---|---|
| *sdhA* | b0723 | 1.25 | Succinate dehydrogenase, flavoprotein subunit |
| *sdhB* | b0724 | 1.18 | Succinate dehydrogenase, iron sulfur protein |
| *sdhC* | b0721 | 1.81 | Succinate dehydrogenase, cytochrome b556 |
| *sdhD* | b0722 | 1.64 | Succinate dehydrogenase, hydrophobic subunit |

**Table 3.** Common regulated genes after PEF and heat treatments of *Escherichia coli* MG1655.

| *Gene* | Systematic Name | logFC PEF | logFC Heat | Description |
|---|---|---|---|---|
| *amtB* | b0451 | -4.42 | -4.33 | Probable ammonium transporter |
| *asr* | b1597 | -7.18 | -5.91 | Acid shock protein |
| *astC* | b1748 | -2.77 | -3.12 | Acetylornithine delta-aminotransferase |
| ECs2304 | ECs2304 | -2.65 | -3.26 | Hypothetical protein |
| *glnH* | b0811 | -2.19 | -1.24 | Periplasmic glutamine-binding protein; permease |
| *glnK* | b0450 | -5.52 | -4.73 | Nitrogen regulatory protein P-II 2 |
| *glnP* | b0810 | -1.26 | -2.06 | Glutamine high-affinity transport system; membrane component |
| *nac* | b1988 | -2.69 | -2.56 | Nitrogen assimilation control protein |
| *rumA* | b2785 | -1.20 | -2.32 | Putative enzyme |
| *soxS* | b4062 | -1.63 | -3.47 | Regulation of superoxide response regulon |
| *ydgD* | b1598 | -1.72 | -2.73 | orf, hypothetical protein |
| *yfgG* | b2504 | -1.47 | -2.30 | orf, hypothetical protein |
| | c_0569 | -4.81 | -4.53 | Hypothetical protein |
| | c_0897 | -2.83 | -2.02 | Hypothetical protein |
| | c_1989 | -6.97 | -5.82 | Putative acid shock protein |
| | c_2148 | -2.79 | -3.19 | Succinylornithine transaminase |
| | c_2149 | -0.92 | -1.35 | Hypothetical protein |
| | c_3023 | -1.54 | -1.66 | Hypothetical protein |

**FIGURE 1**

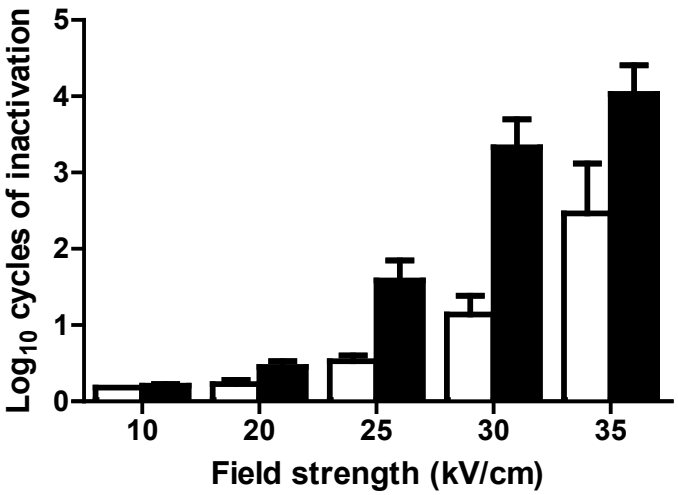

Log10 cycles of inactivation
0
1
2
3
4
5
10
20
25
30
35
Field strength (kV/cm)

**FIGURE 2**

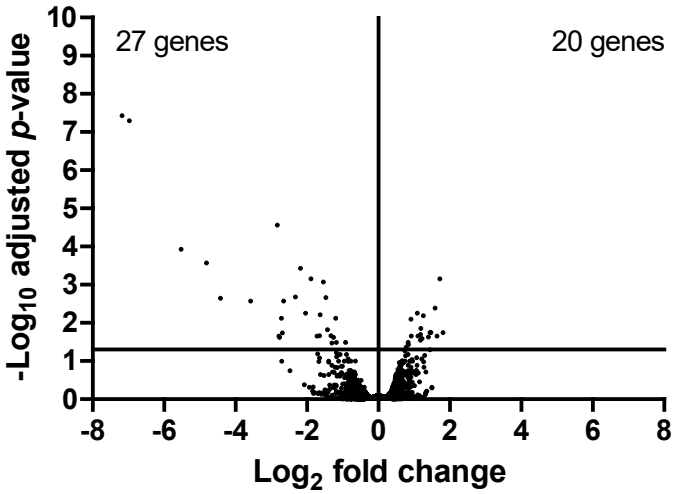

27 genes
20 genes
-Log10 adjusted p-value
Log2 fold change
10
9
8
7
6
5
4
3
2
1
0
-8
-6
-4
-2
0
2
4
6
8

**FIGURE 3**

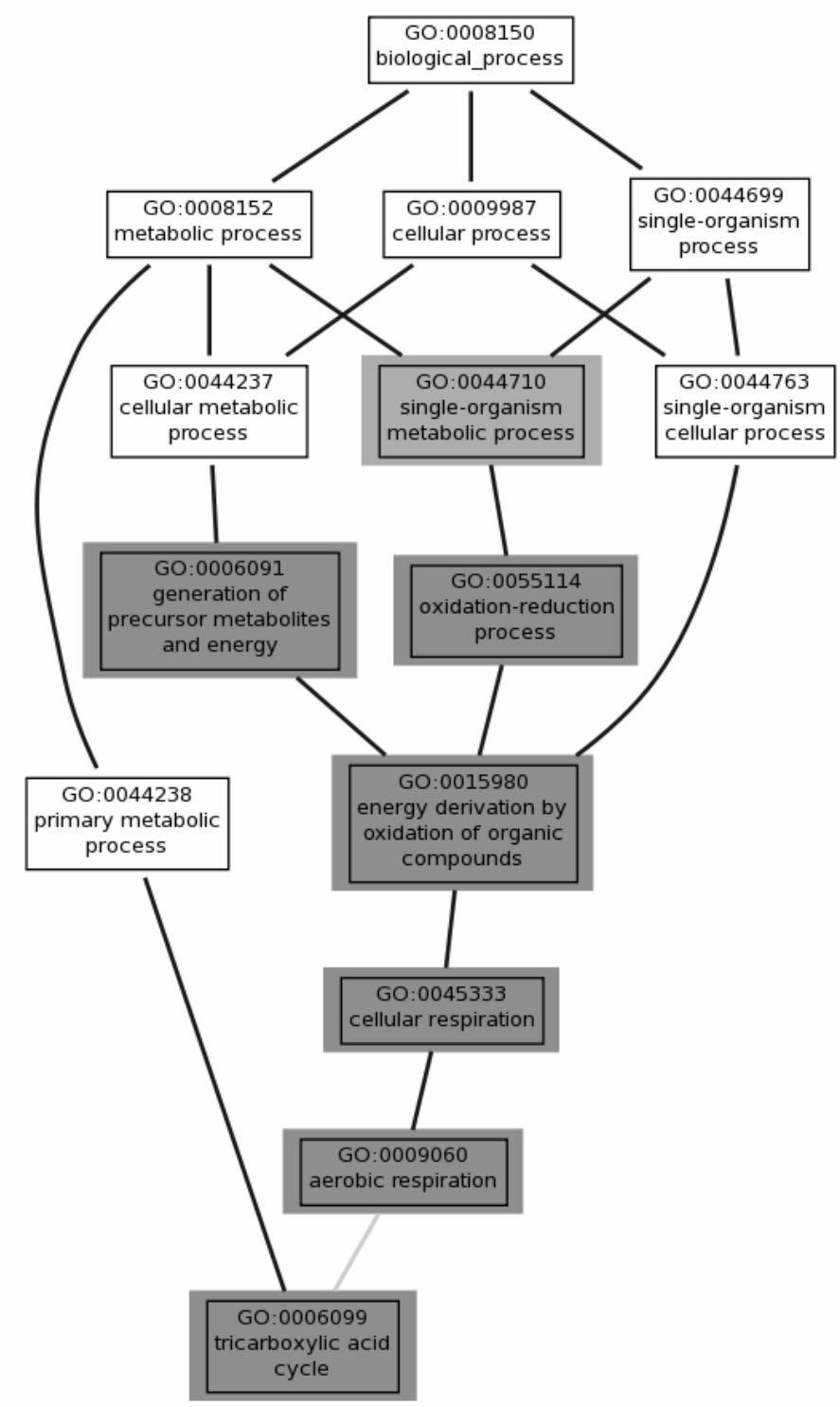

GO:0008150
biological_process
GO:0008152
metabolic process
GO:0009987
cellular process
GO:0044699
single-organism
process
GO:0044237
cellular metabolic
process
GO:0044710
single-organism
metabolic process
GO:0044763
single-organism
cellular process
GO:0006091
generation of
precursor metabolites
and energy
GO:0055114
oxidation-reduction
process
GO:0044238
primary metabolic
process
GO:0015980
energy derivation by
oxidation of organic
compounds
GO:0045333
cellular respiration
GO:0009060
aerobic respiration
GO:0006099
tricarboxylic acid
cycle

**FIGURE 4**

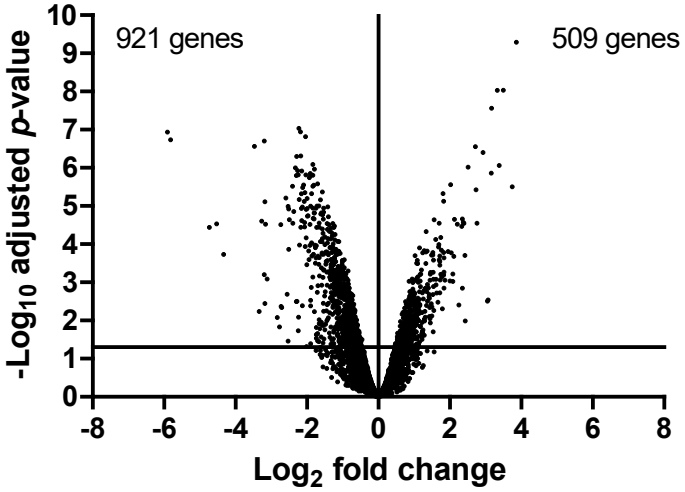

921 genes
509 genes
-Log10 adjusted p-value
Log2 fold change
10
9
8
7
6
5
4
3
2
1
0
-8
-6
-4
-2
0
2
4
6
8

**FIGURE 5**

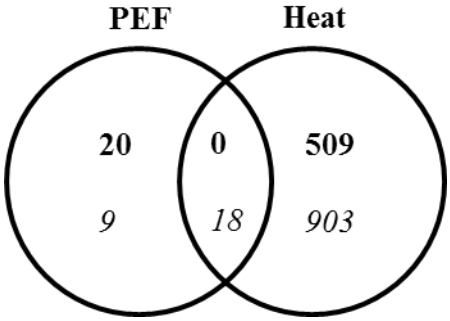
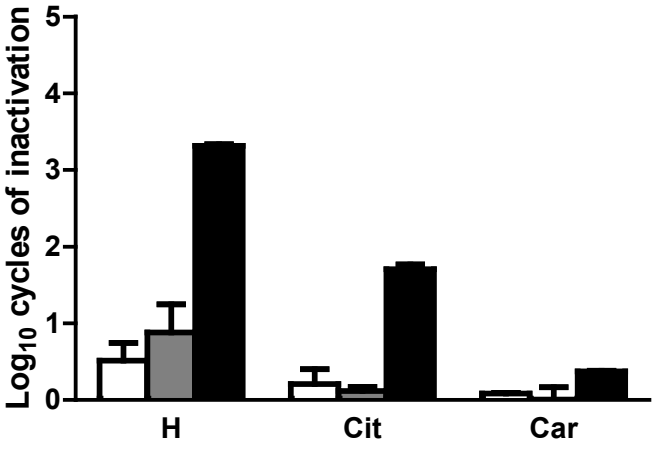


**FIGURE 6**



# FIGURE S1

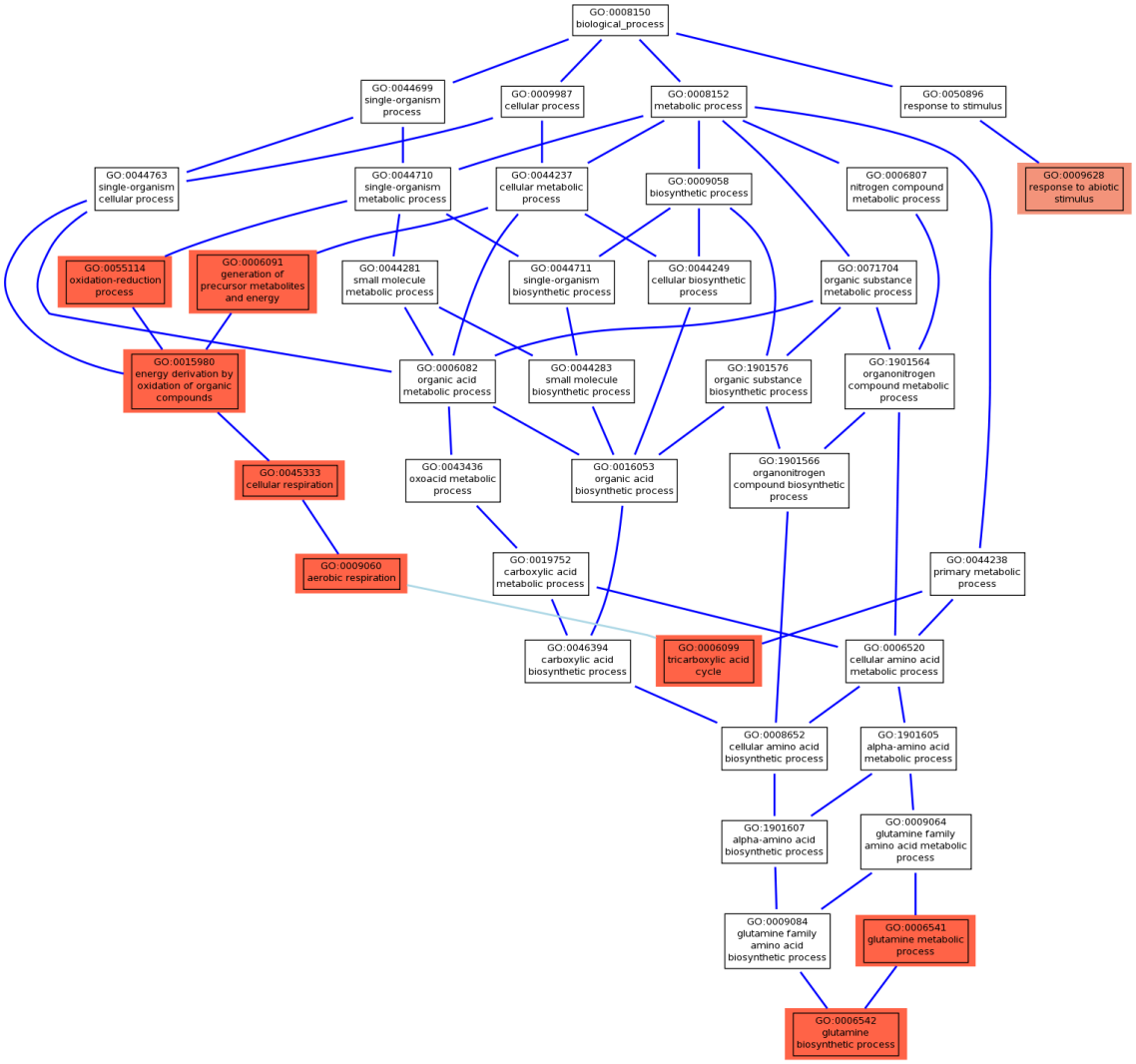

GO:0008150
biological_process
GO:0044699
single-organism
process
GO:0009987
cellular process
GO:0008152
metabolic process
GO:0050896
response to stimulus
GO:0044763
single-organism
cellular process
GO:0044710
single-organism
metabolic process
GO:0044237
cellular metabolic
process
GO:0009058
biosynthetic process
GO:0006807
nitrogen compound
metabolic process
GO:0009628
response to abiotic
stimulus
GO:0055114
oxidation-reduction
process
GO:0006091
generation of
precursor metabolites
and energy
GO:0044281
small molecule
metabolic process
GO:0044711
single-organism
biosynthetic process
GO:0044249
cellular biosynthetic
process
GO:0071704
organic substance
metabolic process
GO:0015980
energy derivation by
oxidation of organic
compounds
GO:0006082
organic acid
metabolic process
GO:0044283
small molecule
biosynthetic process
GO:1901576
organic substance
biosynthetic process
GO:1901564
organonitrogen
compound metabolic
process
GO:0045333
cellular respiration
GO:0043436
oxoacid metabolic
process
GO:0016053
organic acid
biosynthetic process
GO:1901566
organonitrogen
compound biosynthetic
process
GO:0009060
aerobic respiration
GO:0019752
carboxylic acid
metabolic process
GO:0044238
primary metabolic
process
GO:0046394
carboxylic acid
biosynthetic process
GO:0006099
tricarboxylic acid
cycle
GO:0006520
cellular amino acid
metabolic process
GO:0008652
cellular amino acid
biosynthetic process
GO:1901605
alpha-amino acid
metabolic process
GO:1901607
alpha-amino acid
biosynthetic process
GO:0009064
glutamine family
amino acid metabolic
process
GO:0009084
glutamine family
amino acid
biosynthetic process
GO:0006541
glutamine metabolic
process
GO:0006542
glutamine
biosynthetic process

**FIGURE S2**

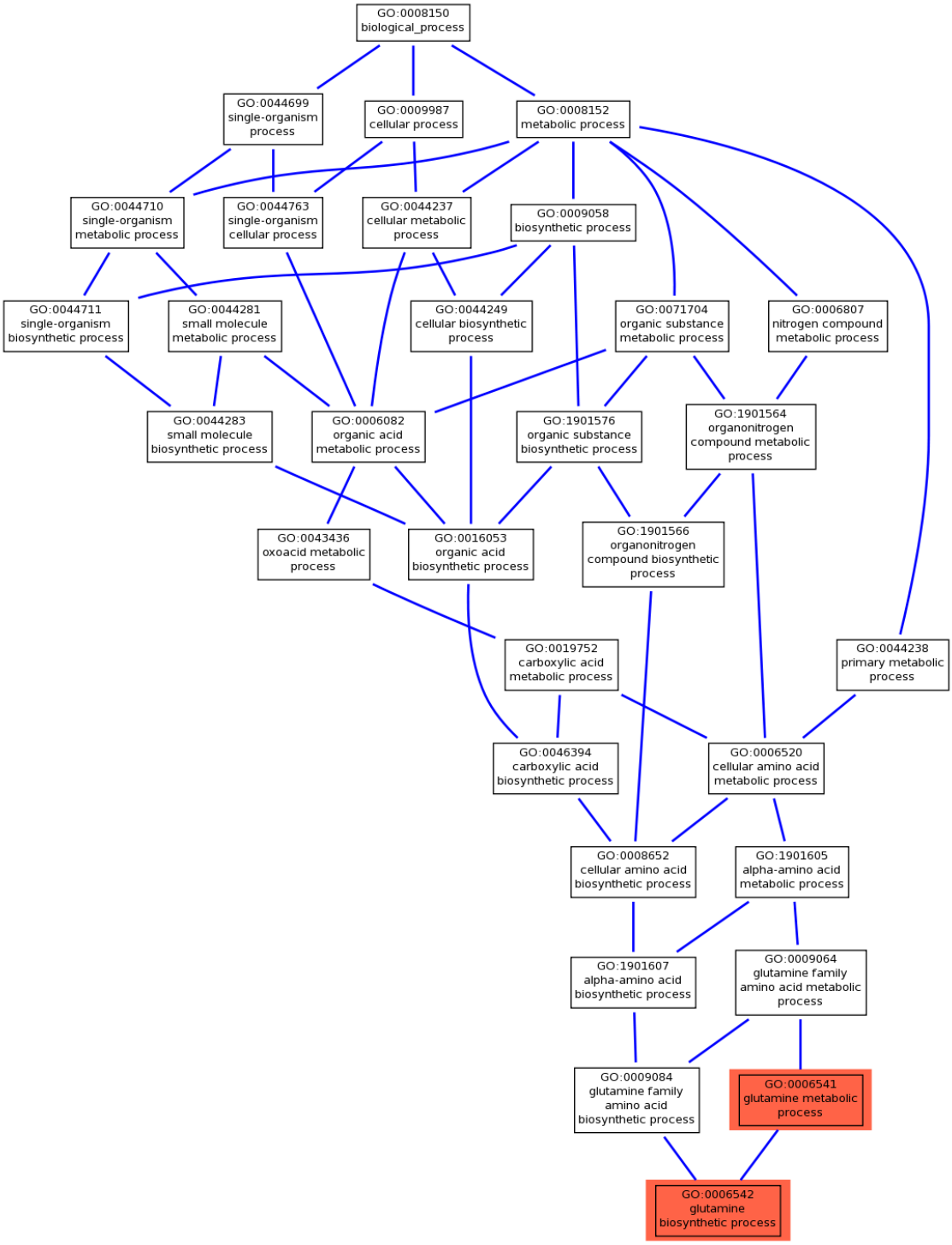

GO:0008150
biological_process
GO:0044699
single-organism
process
GO:0009987
cellular process
GO:0008152
metabolic process
GO:0044710
single-organism
metabolic process
GO:0044763
single-organism
cellular process
GO:0044237
cellular metabolic
process
GO:0009058
biosynthetic process
GO:0044711
single-organism
biosynthetic process
GO:0044281
small molecule
metabolic process
GO:0044249
cellular biosynthetic
process
GO:0071704
organic substance
metabolic process
GO:0006807
nitrogen compound
metabolic process
GO:0044283
small molecule
biosynthetic process
GO:0006082
organic acid
metabolic process
GO:1901576
organic substance
biosynthetic process
GO:1901564
organonitrogen
compound metabolic
process
GO:0043436
oxoacid metabolic
process
GO:0016053
organic acid
biosynthetic process
GO:1901566
organonitrogen
compound biosynthetic
process
GO:0019752
carboxylic acid
metabolic process
GO:0044238
primary metabolic
process
GO:0046394
carboxylic acid
biosynthetic process
GO:0006520
cellular amino acid
metabolic process
GO:0008652
cellular amino acid
biosynthetic process
GO:1901605
alpha-amino acid
metabolic process
GO:1901607
alpha-amino acid
biosynthetic process
GO:0009064
glutamine family
amino acid metabolic
process
GO:0009084
glutamine family
amino acid
biosynthetic process
GO:0006541
glutamine metabolic
process
GO:0006542
glutamine
biosynthetic process

**FIGURE S3**

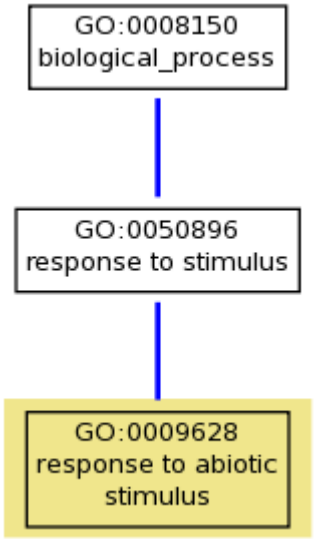

GO:0008150
biological_process
GO:0050896
response to stimulus
GO:0009628
response to abiotic
stimulus

**Table S1.** Top 25 down-regulated genes after PEF treatment based on adjusted *p*-value of microarray data.

| *Gene* | Systematic Name | logFC | Description |
|---|---|---|---|
| *amtB* | b0451 | -4.42 | Probable ammonium transporter |
| *asr* | b1597 | -7.18 | Acid shock protein |
| *astC* | b1748 | -2.77 | Acetylornithine delta-aminotransferase |
| ECs2304 | ECs2304 | -2.65 | Hypothetical protein |
| *glnH* | b0811 | -2.19 | Periplasmic glutamine-binding protein; permease |
| *glnK* | b0450 | -5.52 | Nitrogen regulatory protein P-II 2 |
| *glnP* | b0810 | -1.26 | Glutamine high-affinity transport system; membrane component |
| *nac* | b1988 | -2.69 | Nitrogen assimilation control protein |
| *rnlA* | b2630 | -1.30 | Putative cell division protein |
| *rpoE* | b2573 | -1.33 | RNA polymerase, sigma-E factor; heat shock and oxidative stress |
| *rumA* | b2785 | -1.20 | Putative enzyme |
| *soxS* | b4062 | -1.63 | Regulation of superoxide response regulon |
| *spy* | b1743 | -3.57 | Periplasmic protein related to spheroblast formation |
| *ydgD* | b1598 | -1.72 | orf, hypothetical protein |
| *yfgG* | b2504 | -1.47 | orf, hypothetical protein |
| *yncJ* | c_1860 | -2.04 | Hypothetical protein YncJ precursor |
| Z2283 | Z2283 | -2.32 | orf, hypothetical protein |
| | b3913 | -1.65 | orf, hypothetical protein |
| | b3914 | -1.89 | orf, hypothetical protein |
| | c_0569 | -4.81 | Hypothetical protein |
| | c_0897 | -2.83 | Hypothetical protein |
| | c_1989 | -6.97 | Putative acid shock protein |
| | c_2142 | -2.72 | Hypothetical protein |
| | c_2148 | -2.79 | Succinylornithine transaminase |
| | c_3023 | -1.54 | Hypothetical protein |

**Table S2.** Differentially expressed genes after PEF treatment based on adjusted *p*-value of microarray data.

| *Gene* | Systematic Name | logFC | Description |
|---|---|---|---|
| *amtB* | b0451 | -4.42 | Probable ammonium transporter |
| *arsR* | b3501 | 0.92 | Transcriptional repressor of chromosomal *ars* operon |
| *asr* | b1597 | -7.18 | Acid shock protein |
| *astC* | b1748 | -2.77 | Acetylornithine delta-aminotransferase |
| *betI* | b0313 | 1.10 | Probably transcriptional repressor of *bet* genes |
| *cyoB* | b0431 | 1.72 | Cytochrome o ubiquinol oxidase subunit I |
| *cyoC* | b0430 | 1.59 | Cytochrome o ubiquinol oxidase subunit III |
| *cyoD* | b0429 | 1.45 | Cytochrome o ubiquinol oxidase subunit IV |
| *cyoE* | b0428 | 1.45 | Protoheme IX farnesyltransferase |
| ECs0483 | ECs0483 | 1.40 | Cytochrome o ubiquinol oxidase subunit IV |
| ECs2304 | ECs2304 | -2.65 | Hypothetical protein |
| *glnH* | b0811 | -2.19 | Periplasmic glutamine-binding protein; permease |
| *glnK* | b0450 | -5.52 | Nitrogen regulatory protein P-II 2 |
| *glnP* | b0810 | -1.26 | Glutamine high-affinity transport system; membrane component |
| *insX* | ABE-0285026 | 1.09 | Predicted IS protein |
| *msyB* | b1051 | 1.17 | Acidic protein suppresses mutants lacking function of protein export |
| *nac* | b1988 | -2.69 | Nitrogen assimilation control protein |
| *nudE* | b3397 | 1.19 | orf, hypothetical protein |
| *phoH* | b1020 | 1.47 | PhoB-dependent, ATP-binding Pho regulon component; may be helicase; induced by P starvation |
| *rnlA* | b2630 | -1.30 | Putative cell division protein |
| *rpoE* | b2573 | -1.33 | RNA polymerase, sigma-E factor; heat shock and oxidative stress |
| *rumA* | b2785 | -1.20 | Putative enzyme |
| *sdhA* | b0723 | 1.25 | Succinate dehydrogenase, flavoprotein subunit |
| *sdhB* | b0724 | 1.18 | Succinate dehydrogenase, iron sulfur protein |
| *sdhC* | b0721 | 1.81 | Succinate dehydrogenase, cytochrome b556 |
| *sdhD* | b0722 | 1.64 | Succinate dehydrogenase, hydrophobic subunit |
| *soxS* | b4062 | -1.63 | Regulation of superoxide response regulon |
| *spy* | b1743 | -3.57 | Periplasmic protein related to spheroblast formation |
| *tatA* | b3836 | 0.83 | orf, hypothetical protein |
| *yafQ* | b0225 | 0.78 | orf, hypothetical protein |
| *ycgZ* | c_1607 | 1.22 | Hypothetical protein YcgZ |
| *ydgD* | b1598 | -1.72 | orf, hypothetical protein |
| *yfgG* | b2504 | -1.47 | orf, hypothetical protein |
| *yidF* | c_4598 | 0.84 | Hypothetical protein YidF |
| *yncJ* | c_1860 | -2.04 | Hypothetical protein YncJ precursor |
| *ynfM* | b1596 | -1.17 | Putative transport protein |
| Z2283 | Z2283 | -2.32 | orf, hypothetical protein |

| | | |
|---|---|---|
| b3913 | -1.65 | orf, hypothetical protein |
| b3914 | -1.89 | orf, hypothetical protein |
| c_0569 | -4.81 | Hypothetical protein |
| c_0897 | -2.83 | Hypothetical protein |
| c_1605 | 0.91 | Hypothetical protein |
| c_1989 | -6.97 | Putative acid shock protein |
| c_2142 | -2.72 | Hypothetical protein |
| c_2148 | -2.79 | Succinylornithine transaminase |
| c_2149 | -0.92 | Hypothetical protein |
| c_3023 | -1.54 | Hypothetical protein |

**Table S3.** Down-regulated genes belonging to the response to abiotic stimulus GO term (GO:0009628) after PEF treatment of *Escherichia coli* MG1655. GO Biological Process Analysis with *p*-value lower than 0.05 (Hypergeometric Test).

| *Gene* | Systematic Name | logFC | Description |
|---|---|---|---|
| *asr* | b1597 | -7.18 | Acid shock protein |
| *rpoE* | b2573 | -1.33 | RNA polymerase, sigma-E factor; heat shock and oxidative stress |

**Table S4.** Down-regulated genes belonging to the glutamine biosynthetic process GO term (GO:0006542) after PEF treatment of *Escherichia coli* MG1655. GO Biological Process Analysis with *p*-value lower than 0.05 (Hypergeometric Test).

| *Gene* | Systematic Name | logFC | Description |
|---|---|---|---|
| *glnH* | b0811 | -2.19 | Periplasmic glutamine-binding protein; permease |
| *glnP* | b0810 | -1.26 | Glutamine high-affinity transport system; membrane component |