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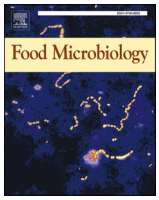

Genotypic and phenotypic characterization of a *Salmonella* Typhimurium strain resistant to pulsed electric fields

S. Guillén [a], L. Nadal [a], N. Halaihel [b], P. Mañas [a], G. Cebrián [a,*]

[a] Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón– IA2, Universidad de Zaragoza-CITA, 50013, Zaragoza, Spain
[b] Departamento I+D+i, Alquizvetek S.L, Zaragoza, 50013, Zaragoza, Spain

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ABSTRACT

Pulsed Electric Fields (PEF) technology is regarded as one of the most interesting alternatives to current food preservation methods, due to its capability to inactivate vegetative microorganisms while leaving the product's organoleptic and nutritional properties mostly unchanged. However, many aspects regarding the mechanisms of bacterial inactivation by PEF are still not fully understood. The aim of this study was to obtain further insight into the mechanisms responsible for the increased resistance to PEF of a *Salmonella* Typhimurium SL1344 variant (SL1344-RS, Sagarzazu et al., 2013), and to quantify the impact that the acquisition of PEF resistance has on other aspects of *S. enterica* physiology, such as growth fitness, biofilm formation ability, virulence and antibiotic resistance. WGS, RNAseq and qRT-PCR assays indicated that the increased PEF resistance of the SL1344-RS variant is due to a higher RpoS activity caused by a mutation in the hnr gene. This increased RpoS activity also results in higher resistance to multiple stresses (acidic, osmotic, oxidative, ethanol and UV-C, but not to heat and HHP), decreased growth rate in M9-Gluconate (but not in TSB-YE or LB-DPY), increased ability to adhere to Caco-2 cells (but no significant change in invasiveness) and enhanced antibiotic resistance (to six out of eight agents). This study significantly contributes to the understanding of the mechanisms of the development of stress resistance in *Salmonellae* and underscores the crucial role played by RpoS in this process. Further studies are needed to determine whether this PEF-resistant variant would represent a higher, equal or lower associated hazard than the parental strain.

1. Introduction

Pulsed Electric Fields (PEF) technology consists in the application of short-duration (1–100 μs) high electric field pulses (10–50 kV/cm) to food products placed between two electrodes (Heinz et al., 2001). This technology has been under research for decades as a potential alternative to thermal treatments for the preservation of different food products, and data obtained to date demonstrate that PEF can inactivate vegetative cells of bacteria and yeasts at temperatures below those used in thermal processing (Álvarez et al., 2006), thus potentially allowing for a high degree of food safety while minimizing quality and nutritional losses.

However, despite decades of effort and despite the substantial body of information available concerning microbial inactivation by PEF, the mechanisms of microbial inactivation and, especially, of resistance acquisition to this technology still remain largely unknown. Thus, although permeabilization of cellular envelopes as a result of the application of PEF (also called "electroporation" or "electropermeabilization"), is believed to be the principal mechanism of microbial inactivation by this technology (Barbosa-Cánovas et al., 1999; Ho and Mittal, 1996; Kinosita et al., 2012; Mañas and Pagán, 2005; Pavlin et al., 2007; Tsong, 1991; Weaver and Chizmadzhev, 1996), the molecular mechanisms of pore formation and resealing in bacteria are almost completely unknown. It is assumed that bacterial membrane electroporation occurs essentially as depicted for artificial membranes, for eukaryotic cells, or in molecular dynamic simulations (El Zakhem et al., 2006; Pavlin et al., 2008; Sözer et al., 2017; Tarek, 2005). However, little is known, for instance, about the manner in which the complex structure of bacterial envelopes exerts an influence on the electroporation phenomenon, or about the potential contribution of other phenomena to inactivation after exposure to PEF, such as oxidative damage (Marcén et al., 2019; Pakhomova et al., 2012).

* Corresponding author.
E-mail address: guiceb@unizar.es (G. Cebrián).

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Similarly, factors determining the differences in resistance to PEF among bacterial species and/or strains, as well as the influence of growth and treatment conditions on bacterial survival, are far from being completely understood. The structure, composition and physical state of membranes and envelopes are presumed to play a major role in bacterial resistance to PEF but results obtained to date are, in many cases, inconclusive. Hence, various authors have hypothesized that the differences in PEF resistance among cells grown and treated at different temperatures would be related to differences in the fluidity of their membranes; results in the literature are nevertheless contradictory (Cebrián et al., 2016a). Similarly, Chueca et al. (2015) demonstrated that PEF-treated cells activate a response involving components and functions directly associated with the cytoplasmic membrane: this indicates that mainly the cell envelope is the element most affected during the inactivation process. This confirms the observed cellular requirement of energy for the repair sublethal damage to the cytoplasmic membrane caused by PEF treatments, probably related to the synthesis of new lipids (García et al., 2005). The role of the outer membrane and of other envelope characteristics, such as surface charge, has also been studied (Arroyo et al., 2010; Golberg et al., 2012), but is poorly understood.

In addition, little is known about the ability of bacterial cells to develop homologous and cross-resistance to PEF; neither is much known about the impact that the development of PEF resistance can exert on other aspects of bacterial physiology. Regarding the first issue, only a few papers have dealt with it (Arroyo et al., 2012; Cebrián et al., 2012), although it seems that the phenomenon of homologous and cross-resistance would be of much less relevance against PEF than in the case of other technologies, such as heat treatments (Cebrián et al., 2016b). The information available regarding the second topic is even more scarce, although a recent study has investigated the impact of PEF on *S. enterica* virulence (Sanz-Puig et al., 2019).

In a previous study, we reported the isolation of a PEF-resistant *Salmonella enterica* ser. Typhimurium SL1344 (SL1344-RS) strain obtained after repeated rounds of PEF treatment and outgrowth of survivors (Sagarzazu et al., 2013). This increased PEF resistance was accompanied by increased resistance to certain other agents including hydrogen peroxide and acidic pH, and was associated with the entry of *S. enterica* cells into stationary growth phase. Altogether, results strongly suggested that the greater PEF-resistance of the variant SL1344-RS could be related to the general stress sigma factor RpoS, since the latter is preferentially expressed in the stationary phase of growth; moreover, RpoS activity leads to an acquisition of tolerance to a variety of stresses (Hengge-Aronis, 1996). However, this hypothesis awaits validation. Thus, our study's aim was to obtain further insight into the mechanisms responsible for the increased stress resistance of *Salmonella* Typhimurium strain SL1344-RS, as well as to quantify the impact that the acquisition of PEF resistance has on other aspects of *S. enterica* physiology, such as growth fitness, biofilm formation ability, virulence, and antibiotic resistance.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Salmonella enterica serovar Typhimurium SL1344 and a PEF-resistant variant of the same strain (SL1344-RS) (Sagarzazu et al., 2013) were used in this study. Strains were maintained frozen at −80 °C in cryovials for long-term preservation. Cultures were grown in tryptic soy broth (Oxoid, Basingstoke, UK) supplemented with 0.6% w/v yeast extract (Oxoid; TSB-YE) in 96-well microtiter plates and incubated at 37 °C under static conditions as described in Guillén et al. (2020).

2.2. Resistance treatments

2.2.1. Sodium chloride resistance treatments

Resistance to osmotic medium was evaluated in TSB-YE supplemented with 30% w/v of sodium chloride (VWR International; NaCl). Treatments were carried out at 37 °C, and the initial concentration was of approximately 10^7 CFU/mL. After the selected contact time of up to 32 h, serial dilutions were prepared and pour-plated for survival counts as described below.

2.2.2. High hydrostatic pressure (HHP) treatments

HHP treatments were carried out in a Stansted Fluid Power S-FL-085-09-W (Harlow, London, England) apparatus (Ramos et al., 2015). The pressure-transmitting fluid was a mixture of propylene glycol and distilled water (50/50, v/v). An automatic device was employed to set pressure and time during the pressurization cycle. Cell suspensions were prepared at a cell concentration of approximately 10^7 CFU/mL in citrate-phosphate McIlvaine buffer of pH 7.0. Samples were packed in plastic bags, which were sealed without headspace and introduced in the treatment chamber. Treatments were applied at 300 MPa for different treatment times up to 30 min, and temperature never exceeded 40 °C.

2.2.3. Ultraviolet C light (UV–C) treatments

UV-C treatments were carried out in microtiter plates under static conditions. Microtiter plates were coated with 1 layer of a microplate sealing film (BREATHseal, Greiner bio-one, Frickenhausen, Germany) and placed at a distance of 22.50 cm from a 32 W UV-C lamp (VL-208G, Vilber, Germany). Radiation intensity was measured by means of a UVX radiometer (UVP, LLC, Upland, CA). Under these experimental conditions, intensity of 0.47 ± 0.02 mW/cm^2 was attained. The treatment medium was citrate-phosphate McIlvaine buffer of pH 7.0, and the initial concentration was of approximately 10^7 CFU/mL. Treatment times of up to 180 s were applied and temperature never exceeded 40 °C.

2.2.4. Recovery after different treatments and survival counting

After treatments, samples were adequately diluted in Buffered Peptone Water (Oxoid; BPW) and plated on the recovery medium, tryptic soy agar with 0.6% w/v yeast extract (Oxoid; TSA-YE). Plates were incubated for 24 h at 37 °C, after which the number of colony-forming units (CFU) per plate was counted.

2.2.5. Survival and modeling curves

Survival curves were obtained by plotting the logarithm of the survival fraction ($\log_{10} N/N_0$) versus treatment time (h for NaCl treatments; min for HHP, and s for UV-C treatments). Since deviations from linearity were frequently observed in survival curves, the Geeraerd inactivation model-fitting tool GInaFiT was used to fit experimental data and to calculate inactivation kinetic parameters (Geeraerd et al., 2005). Equations (1) and (2), respectively, were used to fit those curves which showed shoulder and tailing phenomena.

$$N_t = N_0 \cdot \exp^{-K_{max} \cdot t} \cdot \left[\frac{\exp^{K_{max} \cdot S_l}}{1 + (\exp^{K_{max} \cdot S_l} - 1) \cdot \exp^{-K_{max} \cdot t}} \right] \quad \text{Eq. 1}$$

$$N_t = (N_0 - N_{res}) \cdot \exp^{-K_{max} \cdot t} + N_{res} \quad \text{Eq. 2}$$

In these equations, N_t represents the number of survivors, N_0 the initial count, and t the treatment time.

Survival curves were obtained in triplicate on independent working days and model fitting was performed on each of the three replicates separately.

This model describes the survival curves by means of three parameters: shoulder length (S_l), defined as the time before exponential inactivation begins; inactivation rate (K_{max}), defined as the slope of the exponential portion of the survival curve; and N_{res} which describes residual population density (tail). The traditional decimal reduction time

value (D-value) can be calculated from the K_{max} parameter using Equation (3).

$$D - \text{value} = 2.303/K_{max} \quad \text{Eq. 3}$$

2.3. Maximum growth rate determination assays

Growth fitness characterization assays were carried out in three different media: TSB-YE, Luria-Bertani (LB) broth supplemented with 100 μM 2-2′dipyridyl (DPY), an iron chelator, and minimal medium, M9-broth, supplemented with 20 mM gluconate as the principal carbon source in the intestine (Bleibtreu et al., 2013). Growth rates were calculated in triplicate on independent working days, and were carried out following the protocol described by Guillén et al. (2022). Model fitting was performed on each of the three replicates separately. The growth curves thereby obtained were fitted with the Baranyi and Roberts model (Baranyi and Roberts, 2000):

$$Y_t = Y_0 + \mu^{'}\cdot A_t - \frac{Y_{max} - Y_0}{M}\cdot\ln\left[1 - e^{-M} + \left(e^{-M}\cdot\frac{Y_{max} - Y_0 - \mu^{'}\cdot A_t}{Y_{max} - Y_0}\right)\right] \quad \text{Eq. 4}$$

$$A_t = t - \lambda\cdot\left[1 - \frac{1}{h_0}\cdot\ln\left(1 - e^{-h_0\cdot t} + e^{-h_0\cdot\left(\frac{t}{\lambda}-1\right)}\right)\right] \quad \text{Eq. 5}$$

where Y_t is the $\log_{10}$ of cell concentration at time t (CFU/mL); Y_0 is the $\log_{10}$ of the initial cell concentration (CFU/mL); Y_{max} is the $\log_{10}$ of maximum cell concentration (CFU/mL); $\mu^{'}$ is growth rate ($\log_{10}$/h); λ is the lag phase (h); and M and h_0 are curvature parameters. These latter two parameters, M and h_0, were considered constant values (set at a value of 10) as suggested in Baranyi and Roberts (2000). Curve fitting was carried out using GraphPad PRISM® (GraphPad Software, San Diego, CA, USA) statistical software.

The maximum specific growth rate was calculated from the growth rate, in $\log_{10}$ scale, using Equation (6).

$$\mu_{max} = \mu^{'}\cdot\ln(10) \quad \text{Eq. 6}$$

2.4. Virulence assays

The human colon carcinoma Caco-2 cell line (TC7 clone) was kindly provided by Dr. Edith Brot-Laroche (Université Pierre et Marie Curie-Paris 6, UMR S 872, Les Cordeliers, France) at passage 25 and used in experiments at passage 30–35. Cells were maintained in 75 cm^2 flasks at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were grown in Dulbecco's Modified Eagle's Medium + Gluta-MAX™ (DMEM, Invitrogen, France) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen, France), 1% Minimal Essential Medium with Non-Essential Amino Acids (MEM NEAA 100X, Invitrogen, France), and 1% antibiotics (penicillin/streptomycin, Invitrogen). Once the cells reached 80% confluence, they were dissociated with 0.05% Trypsin-1 mM EDTA (Invitrogen) and seeded at a density of approximately 15,000 cells per well in 96-well tissue culture plates (Nunc, France) containing 200 μL of complete medium per well. Plates were incubated in humidified atmosphere containing 5% CO_2 at 37 °C for 15–17 days to attain fully differentiated cell layers. Culture medium was replaced every two days, and cell confluence was confirmed by optical microscopy. Adhesion and invasion assays were performed following the protocol described in Guillén et al. (2022). Adhesion and invasion rates were calculated as the percentage of adhered or invading bacteria, respectively, in relation to the initially inoculated bacterial population.

2.5. Biofilm formation ability assay

Biofilm formation ability was evaluated in a 96-well microtiter plate as described in Guillén et al. (2022). For each replicate experiment, four wells were inoculated for each strain and negative controls, i.e., four uninoculated wells, were also included. In order to establish meaningful comparisons, the area under the curve (AUC) was calculated as described in Espina et al. (2015). Briefly, the absorbance at 580 nm vs time (up to 72 h, with measurements every 24 h) was plotted for each strain and the AUC values were calculated using GraphPad software following the trapezoid rule, according to which the total area is the sum of all rectangular trapezoids, each defined by two adjacent absorbance values with respect to the area under the curve (in the y axis) and the time between those measurements (in the x axis). The formula we applied was:

$$\text{AUC} = \sum_{i=1}^{n-1}\frac{x_i\cdot(y_i + y_{i+1})}{2} \quad \text{Eq. 7}$$

where x_i is the time between measurements in hours, y_i is the absorbance value at 580 nm for each measurement, and n is the total number of measurements.

2.6. Antibiotic resistance assays

The minimum inhibitory concentration (MIC) of seven antibiotics representative of different classes (ampicillin, cephalexin, chloramphenicol, kanamycin, nalidixic acid, oxytetracycline, and sulfanilamide) against the two *S. enterica* strains was determined by Broth Dilution Susceptibility Tests as described in Guillén et al. (2022). The range of concentrations used to determine the MICs of an antibiotic was 0–512 μg/mL, except for sulfonamides, for which the range was 0–4096 μg/mL. MICs were then determined as the lowest concentration of antibiotic that completely inhibited growth (optical absorbance equal to or lower than non-inoculated wells) of each strain after 24 h of cultivation at 37 °C.

2.7. Whole genome sequencing (WGS) and identification of mutations

Total genomic DNA (gDNA) from the parental *S.* Typhimurium SL1344 strain and the PEF-resistant variant (SL1344-RS) was extracted using a DNeasy Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

The genomes of the resistant variant and of the parental strain under study were sequenced by the STAB VIDA firm (Portugal) in an Illumina Hiseq 4000 platform, using 150bp paired-end reads. The resulting reads were then subjected to a trimming process using the CLC Genomic Workbench version 12.0. The quality of data thereby obtained was determined by Phred quality score for each cycle using the FastQC program (v3.4.1.1) (Andrews, 2010). The high-quality sequencing reads were then mapped (length and similarity fractions of 0.8 each) against the *Salmonella enterica* subsp. *enterica* serovar Typhimurium SL1344 reference genome (Kröger et al., 2012). After mapping, we applied a variant calling algorithm to detect the variants that satisfied the requirements specified by the following filters: minimum frequency = 35%, minimum quality (Phred) = 20, minimum coverage = 20, minimum count = 5, and direction filtering. Detection of insertions and deletions was also performed by means of an InDels detection tool using the following criteria: minimum number of reads = 5 and P-value threshold = 0.0001. Sequences were deposited in the NCBI Sequence Read Archive (BioProject PRJNA814285).

2.8. RNA extraction and cDNA synthesis

RNA of parental *S.* Typhimurium SL1344 strain and its PEF-resistant variant (SL1344-RS) was isolated by phenol-chloroform extraction with a subsequent cleanup procedure using the RNeasy Mini Kit (Qiagen) (Atshan et al., 2012). Cells were pelleted by centrifugation at 8000×*g* for 3 min in a 4 °C refrigerated centrifuge. The pellet was re-suspended in 100 μL of RNase-free water. The tube was vigorously vortexed for 3 min

and 100 μL of acid phenol was added with chloroform (1:1). It was then vortexed for 1 min and incubated at 70 °C for 30 min. The vortex process was repeated periodically every 5 min. Next, the tube was centrifuged at 12,000×*g* for 10 min and 100 μL from the aqueous (top only) phase was transferred into a new tube. 700 μL of lysis buffer were added into the aqueous phase and the subsequent steps were carried out according to the manufacturer protocol of RNeasy Mini Kit (Quiagen). The samples, once purified, were treated with DNase to remove residual DNA using the Rapidout Removal Kit (Thermo Fisher Scientific, Massachusetts, USA), also following manufacturer's instructions, and extracted RNA samples were frozen at −80 °C until complementary DNA (cDNA) synthesis.

The RNA previously isolated was converted to cDNA using the Superscript IV Reverse Transcriptase kit (Invitrogen, Carlsbad, USA) using random hexamer primers following the protocol described by the manufacturer. Once the cDNA was obtained, it was stored at −80 °C until RNAseq and qPCR assays were carried out.

2.9. RNA sequencing (RNA-seq)

RNAseq assays were performed by the STAB VIDA firm (Portugal) in an Illumina Novaseq platform, using 150bp paired-end reads. The library construction of cDNA molecules was carried out using a Ribosomal Depletion Library Preparation Kit. After sequenciation, the high-quality sequencing reads were mapped (length and similarity fractions of 0.8 each) against the *Salmonella enterica* subsp. enterica serovar Typhimurium SL1344 reference genome (GenBank: Accession No. FQ312003.1) (Kröger et al., 2012) and analyzed using CLC Genomics Workbench 12.0.3. Gene expression was normalized by calculating reads per kilobase per million mapped reads (RKPM), given by dividing the total number of reads by the number of mapped reads (in millions) x the length in kilobases (Mortazavi et al., 2008). Differential expression analysis (parental *vs* SL1344-RS) was carried out using a multi-factorial statistical analysis tool based on a negative binomial model that uses a generalized linear model approach influenced by the multi-factorial EdgeR method (Robinson et al., 2010). The differentially expressed genes were filtered using standard conditions (Fold change ≥2 or ≤ −2 and FDR *p*-value ≤0.05) (Raza and Mishra, 2012; van Iterson et al., 2010). Further control of the Family-Wise Error Rate (FWER, false positives) was carried out by applying the Bonferroni correction ($p < 0.05$). RNASeq data presented in this article were deposited in the NCBI Sequence Read Archive (BioProject PRJNA814285).

2.10. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Afterwards, expression of three RpoS-dependent genes (katE, katN and otsB) of SL1344-RS and the parental strain was determined using Quantitative Reverse Transcriptase PCR (qRT-PCR). rpoZ was used as a reference gene for qPCR normalization (Lévi-Meyrueis et al., 2014).

qPCR amplification was performed using the GoTaq qPCR Master Mix (Promega, Madison, USA) and the primers described in Table 1. The qPCR assays were carried out with a CFX Connect Real-Time System (Bio-Rad Laboratories, Hercules, USA) using a protocol of 5 min at 94 °C for GoTaq enzyme activation, followed by 44 cycles at 94 °C for 10 s and 40 s at a temperature of 55 °C for acquisition of annealing, elongation and fluorescence data. A melting curve between 65 °C and 90 °C was obtained after the last amplification cycle, and at a temperature transition rate of 0.5 °C/s. All amplification reactions were run in triplicate.

The mRNA levels for the genes of interest were quantified from the C_t value, which is the PCR cycle number that generated a common signal for each gene in the exponential phase of amplification. To correct for sampling errors, the levels of expression of each gene, as determined from their C_t values, were normalized to the level of rpoZ gene. The relative expression of the genes investigated in each resistant variant was compared to that for parental cells and the fold change in transcription was calculated using $2^{-\Delta\Delta Ct}$ (Pfaffl, 2001).

Table 1
Primer sequences used in Real-Time PCR to quantify the expression of RpoS-dependent genes.

Gene target	Forward primer (5' -to- 3')	Reverse primer (5' -to- 3')
rpoZ	CGAAGAAGGTCTGATTAAC	GACGACCTTCAGCAATA
katE	GGCGTCTGTTCTCTTAT	CTGGAAGTTATGGTAGGG
katN	TGAGTCATCTGGAAATTAT	CGATAAAGTTCCGCTTC
otsB	TTAACCGTATCCCCCGAACTC	CCGCGAGACGGTCTAACAAC

2.11. Statistical analysis

All determinations were carried out in triplicate on different working days. Standard deviations (SD) and statistical analyses (ANOVA and Tukey tests; *p*-value <0.05) were calculated using GraphPad PRISM® statistical software (GraphPad Prism version 8.00 for Windows, GraphPad Software, San Diego, California, USA).

3. Results and discussion

3.1. Genetic characterization of the PEF-resistant variant

First, we performed a WGS analysis of the parental strain SL1344 available in our laboratory. This allowed us to identify four single nucleotide variations (SNVs) in comparison with the reference genome of this same strain in GeneBank (Accession No. FQ312003.1) (Kröger et al., 2012). Those SNVs are listed in Table S1. It should be noted that heterogeneity among the genomes of supposedly identical microbial strains/cultures has already been widely reported and discussed (Dorman and Thomson, 2020). In any case, our results highlight the relevance (even more so, the necessity) of periodically revisiting/re-sequencing the genome of microbial strains stored and used in different laboratories, especially when experimental evolution and/or mechanistic studies are being carried out.

Secondly, WGS analysis of the PEF-resistant variant SL1344-RS was performed to identify the genetic changes responsible for the phenotypic changes observed by Sagarzazu et al. (2013). We then compared the genomes of strains SL1344 and SL1344-RS. As can be observed in Table 2, two SNVs were found.

The first SNV was found in the hnr gene at position 902 bp, resulting in the substitution of a Leucine (Leu) by a Proline (Pro). This protein, Hnr, regulates the turnover of the alternative sigma factor σ^S (RpoS) by promoting its proteolysis (Zhou and Gottesman, 1998), and it also controls which mRNAs are destroyed by stimulating polyadenylation (Carabetta et al., 2009). RpoS is regarded as the master regulator of the general stress response in many Gram-negative bacteria, including *S. enterica* (Battesti et al., 2011; Hengge, 2011; Lago et al., 2017; Österberg et al., 2011). Therefore, any change in *hnr* has the potential of affecting RpoS activity and, consequently, the stress resistance of *Salmonella* Typhimurium cells. Given the fact that this variant displayed a higher resistance to different stresses, Sagarzazu et al. (2013) had already speculated that this might be connected with increased RpoS activity. Conversely, the lack of the stationary-phase inducible sigma factor RpoS has been shown to result in decreased resistance to PEF (Somolinos et al., 2008). In the case of PEF, cell membrane damage appears to be a major contributor to cell death; it has been suggested that proteins synthesized under the control of sigma factor RpoS are responsible, among other functions, for repairing most of the cell membrane damage caused by PEF treatment (Somolinos et al., 2008). This would suggest that the expression of sigma factor RpoS is related to a protective effect of the cell envelope. We verified this hypothesis via transcriptomic and phenotypic assays. as described below.

The second SNV was, apparently, a reversion of a mutation found when comparing the genome of the parental strain, *S.* Typhimurium

Table 2
Mutations identified in the *S.* Typhimurium SL1344-RS strain (as compared to our *S.* Typhimurium SL1344 parental strain) by whole genome sequencing (WGS). All detected mutations were single nucleotide variations (SNV).

Region	Genes	Locus tag	Mutation type	Amino acid change	Description
1805904	*hnr*	SL1344_1684	c.902 T > C	Leu301Pro	hypothetical regulatory protein
3284620	*yggW*	SL1344_3079	c.804 T > C	No change	possible oxygen-independent coproporphyrinogen III oxidase

SL1344, with that of the reference strain in *yggW* gene at position 804 bp, resulting in a silent mutation. Although the function of YggW, also named HemW in *E. coli*, is poorly understood, it probably acts as a heme chaperone (Haskamp et al., 2018). It cannot be excluded that, instead of a reversion, this difference in the *yggW* gene between the parental and the PEF-resistant strain would be due to the fact that this variant had been isolated from a culture of the parental strain that had not harbored that mutation. In any case, since this was a silent mutation, no phenotypic change would be associated with it. Therefore, the phenotypic changes observed in the strain *S.* Typhimurium SL1344-RS would be solely linked to the mutation found in *hnr*.

3.2. Comparative global gene expression analysis (RNAseq)

In order to determine the impact of the observed genetic changes on *Salmonella* physiology and to acquire a deeper insight into the mechanisms leading to its increased stress resistance, we studied and compared the transcriptomes of the parental and the PEF-resistant variant using RNA sequencing. We identified a total of 147 genes differentially expressed in the parental strain and the PEF-resistant variant ($p < 0.05$), of which 22 were highly significant ($p < 0.001$) (see Supplementary Material). After applying the Bonferroni correction factor – which, despite reducing the number of true discoveries, reduces the number of false positives – six genes showed differential expression levels (>2 fold, $p < 0.05$) when comparing the parental and the PEF-resistant variant (Table 3).

Among these six genes, five of them were up-regulated in the PEF-resistant variant, and one of them was down-regulated. In addition, five of them have been shown to be RpoS-dependent. These results strongly suggest that the mutation we found led to a decrease in hnr-dependent RpoS proteolysis, thereby indicating that this PEF-resistant variant displays increased RpoS activity.

3.3. qRT-PCR analysis of RpoS activity

In order to verify the results obtained by RNAseq, three well-known RpoS-regulated genes (*katE*, *katN* and *otsB*) were used by means of qRT-PCR as reporters to quantify RpoS activity. Both, i.e., *katE* gene, encoding the HPII catalase, and *katN* gene, encoding a non-heme catalase, are considered to be RpoS-dependent in *S. enterica* (Chen et al., 1996; Ibañez-Ruiz et al., 2000; Robbe-Saule et al., 2001), and they also contribute to the prevention of oxidative stress (Visick and Clarke, 1997). RpoS is likewise involved in the transcription of the *otsBA* operon in *S.* Typhimurium (Balaji et al., 2005; Lévi-Meyrueis et al., 2014), which plays an important role in countering osmotic stress via regulation of the trehalose synthesis pathway. Under high-osmolarity conditions, trehalose serves as an osmoprotectant.

Fig. 1 shows the relative expression of the three genes under study ($2^{-\Delta\Delta Ct}$) in the PEF-resistant (SL1433-RS) variant as compared to the parental strain. As can be observed in the graph, the three RpoS-regulated genes were overexpressed in the PEF-resistant variant, especially *otsB* gene, confirming the results obtained by RNAseq. It should be noted that *S.* Typhimurium SL1344-RS also displayed a significantly higher catalase activity (often used as an indirect index of RpoS activity (see Schellhorn, 1995; Tanaka et al., 1997), as determined by Iwase et al. (2013) (data not shown).

Table 3
Genes with differential expression levels after applying the Bonferroni correction factor (>2 fold, $p < 0.05$) in the parental strain, *S.* Typhimurium SL1344, and the PEF-resistant variant as determined by RNAseq. Species in which these genes were shown to be RpoS-dependent are indicated in parentheses.

Gene	Fold change	RpoS-dependent?	Reference
SL1344_1197 (YhjQ)	30.50	Yes (*E. coli*)	Dong (2010)
SL1344_1443 (YmdF)	154.31	Yes (*S.* Typhimurium)	Oguri et al. (2019)
yciF	91.61	Yes (*S.* Typhimurium)	Beraud et al. (2010)
yciE	204.80	Yes (*S.* Typhimurium)	Beraud et al. (2010)
katN	159.04	Yes (*S.* Typhimurium)	Beraud et al. (2010)
zraP	−19.37	–	Appia-Ayme et al. (2012)

3.4. Further characterization of the stress resistance of S. Typhimurium SL1344-RS

In the article in which its isolation was reported (Sagarzazu et al., 2013), the resistant variant SL1344-RS displayed the same degree of heat resistance as the parental strain. However, the survival rate in acidic pH, especially in hydrogen peroxide and ethanol, was higher in this resistant variant (Sagarzazu et al., 2013). In the current study, we investigated whether the PEF-resistant variant was more tolerant to other hurdles as well. Following the procedure described in Guillén et al. (2020), we determined the SL1344-RS strain's resistance to osmotic stress, high hydrostatic pressure, and UV-C, and compared it with that the parental strain, *S.* Typhimurium SL1344. Survival curves were obtained by plotting the logarithm of the survival fraction versus the treatment time; we then applied the non-linear Geeraerd model (Geeraerd et al., 2000) to calculate the corresponding resistance parameters (N_0; S_l; K_{max}, N_{res}). The mean values obtained for these parameters and their standard deviation, together with the goodness-of-fit parameters,

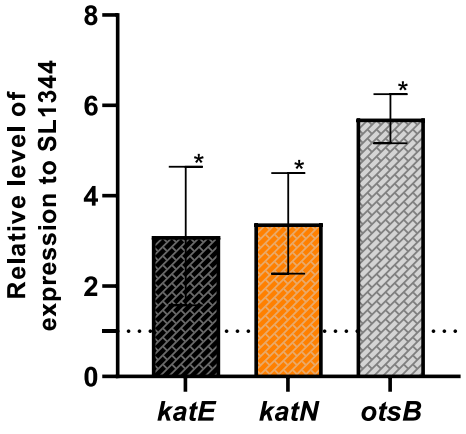

Fig. 1. Relative expression of the three genes studied ($2^{-\Delta\Delta Ct}$) in the PEF-resistant variant as compared to the parental strain. Error bars correspond to the standard deviation of the means (n = 3) and the asterisk (*) indicates statistically significant differences ($p < 0.05$) between the parental and the variant strain.

are included in Table S2. The *2D*-value parameter (the time required to inactivate the first 2 $\log_{10}$ cycles) was calculated from Equation (1) or (2) and used to establish meaningful comparisons between *S.* Typhimurium SL1344 and PEF-resistant variant SL1344-RS, according to the method described in Guillén et al. (2020).

As can be deduced from Fig. 2, the SL1344-RS variant showed a higher tolerance to osmotic medium (12.90 ± 0.252 *vs* 9.12 ± 0.468 h) and to UV-C (74.02 ± 2.749 *vs* 66.51 ± 0.431 s) ($p < 0.05$), but no significant differences ($p > 0.05$) in HHP resistance were found between the two strains. Hence, this PEF-resistant variant would not only be more resistant to PEF, acid, hydrogen peroxide and ethanol, as previously reported (Sagarzazu et al., 2013), but it is also more osmotic and UV-C resistant than its parental counterpart. This is plausible, considering the role of RpoS as a master regulator of the general stress response in *S. enterica* and the fact that RpoS has already been shown to be essential for this microorganism's optimal tolerance of desiccation, starvation, and acid (Lee et al., 1995; Loewen et al., 1998). The role of RpoS in *S. enterica* UV-C and PEF resistance has not yet been explored, but results obtained in the current study suggest that RpoS might contribute toward an increase in *S. enterica* resistance to those agents. On the other hand, it is not surprising that the two agents to which no change in resistance was observed were heat and HHP, since they share several cellular targets and there is a substantial overlap in microbial responses to them (Cebrián et al., 2016b). However, these results do not directly imply that RpoS activity has no influence on the resistance of *S. enterica* to these technologies. What is more, the major role played by RpoS in the resistance of *E. coli* to heat and HHP has been amply proven (Bhagwat et al., 2006; Charoenwong et al., 2011; Robey et al., 2001; Vidovic et al., 2012). Similarly, RpoS also seems to play a relevant role in the resistance of *S. enterica* to heat, at least under certain conditions (Cui et al., 2019). We can speculate that the difference in RpoS activity required to induce a significant change in resistance to heat and HHP could be greater than the difference observed between the two strains, or that once a RpoS activity threshold is reached, subsequent increases would not have a relevant effect on heat and HHP resistance. Other potential explanations may exist.

Further research is required to fully elucidate the role of RpoS in *S. enterica* resistance to all these agents. Similarly, new experiments will be necessary in order to determine which member/s of the rpoS regulon is/are responsible for the increase in resistance to each of these agents.

3.5. S. Typhimurium SL1344-RS growth rates in different media

Growth curves of SL1344-RS and of the parental strain were obtained in three different media: in TSB-YE, a nutrient-rich medium, in LB medium with iron limitation caused by the addition of DPY, and in a minimal medium containing gluconate as the sole carbon source. The selection of these growth media has been previously discussed in Guillén et al. (2022). The μ_{max} (1/h) values calculated in the three growth media for SL1344 and for SL1344-RS are shown in Fig. 3; growth parameters and goodness-of-fit parameters are included in Supplementary Table S3. None of the strains displayed a statistically significant lag phase (h) (different from 0; $p > 0.05$) in any of the three media tested. Significant differences between the two strains were only found in M9-Gluconate, which is the most restrictive medium, in such a way that the parental strain showed a higher maximum specific growth rate ($p < 0.05$) (1.424 ± 0.069 *vs* 1.230 ± 0.056 1/h for SL1344 and SL1344-RS, respectively).

These results are consistent with those reported for *E. coli* and *S.* Typhi, two microorganisms for which it has been observed that cells with a reduced RpoS activity can grow better in media with low levels of nutrients, and also seem to possess an advantage in competitive colonization of the intestine (Altuvia et al., 1994; Krogfelt et al., 2000; Sabbagh et al., 2010). In any case, it should be mentioned that the differences in growth rate were lower than 15%, thereby indicating that the cost of the acquisition of resistance for strain SL1344-RS was not very high or relevant.

3.6. Virulence capacity of S. Typhimurium SL1344-RS

The virulence capacity of the parental and PEF-resistant variant was

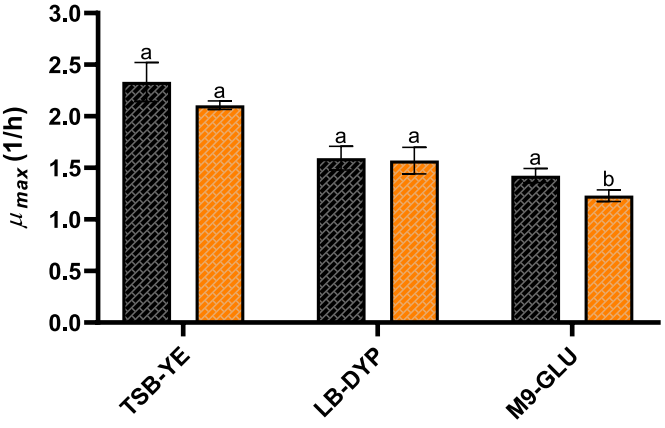

Fig. 3. Maximum specific growth rates (μ_{max} (1/h)) of *S.* Typhimurium SL1344 (SL1344, black) and PEF-resistant variant (SL1344-RS, orange), in TSB-YE, LB supplemented with 100 μM 2-2′dipyridyl (DPY) and M9-broth supplemented with 20 mM gluconate at 37 °C. Different letters indicate statistically significant differences ($p < 0.05$). Error bars correspond to the standard deviation of the means (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

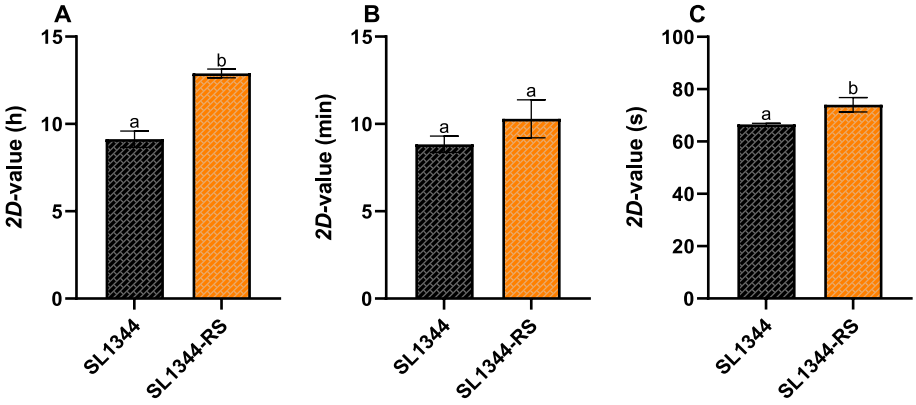

Fig. 2. *2D*-values of the parental *S.* Typhimurium SL1344 (SL1344) and PEF-resistant variant (SL1344-RS) to sodium chloride (30% w/v, A), to high hydrostatic pressure (300 MPa, B) and to UV-C (0.47 mW/cm^2, C). Different letters indicate statistically significant differences ($p < 0.05$). Error bars correspond to the standard deviation of the means (n = 3).

evaluated by determining the percentage of cells capable to adhere to and to invade Caco-2 cells (Fig. 4). Our results indicate that the PEF-resistant strain (SL1344-RS) displayed a higher capacity of adhesion to Caco-2 cells than the parental strain (SL1344), 5.21 *vs* 2.00%, respectively ($p < 0.05$), but no significant differences in terms of invasion ability were observed (0.19 *vs* 0.12%; $p > 0.05$). In any case, given the high variability of these assays, as already discussed in Mellor et al. (2009), these conclusions should be treated with caution.

These apparently contradictory results may be related to the highly complex role played by RpoS in the virulence of *S. enterica* (Guillén et al., 2021). RpoS seems to reduce the expression of certain virulence factors while inducing other ones: therefore, depending on the particular phenotypical trait and the level of expression/activity, the outcome can be completely different.

3.7. Static biofilm formation ability of S. Typhimurium SL1344-RS

The results of the static biofilm formation assay are shown in Fig. 5. In order to establish meaningful comparisons, we compared the AUC values calculated as described in the Materials and Methods section. Biofilm formation capacity was not altered by the development of PEF resistance in SL1344-RS, as no statistically significant differences ($p > 0.05$) were found between the resistant variant and the parental strain: the obtained AUC values were 2.56 ± 1.125 and 2.29 ± 0.799, respectively. It has been described that RpoS plays an important role in biofilm formation by regulating the central regulator CsgD (Simm et al., 2014). Nevertheless, it has also been shown that CsgD regulation is serovar-specific and may be partially independent of RpoS, since other sigma factors can maintain a certain level of biofilm formation after removal of RpoS (Feng et al., 2020; Römling et al., 2003). These results contrast with the higher capacity of adhesion to Caco-2 cells shown by SL1344-RS. However, although these processes are analogous, the involved surface characteristics and structures/metabolic pathways are not completely equivalent (Peng, 2016).

3.8. Antibiotic resistance of S. Typhimurium SL1344-RS

Finally, as can be observed in Table 4, *S.* Typhimurium SL1344-RS was more resistant to ampicillin, cephalexin, chloramphenicol, and oxytetracycline. Remarkably, moreover, it tolerated an eight-fold higher concentration of kanamycin than its parental strain. Only for two out of the seven tested antibiotics (nalidixic acid and sulfonamide) was the SL1344-RS strain just as resistant as the parental strain; consequently, it

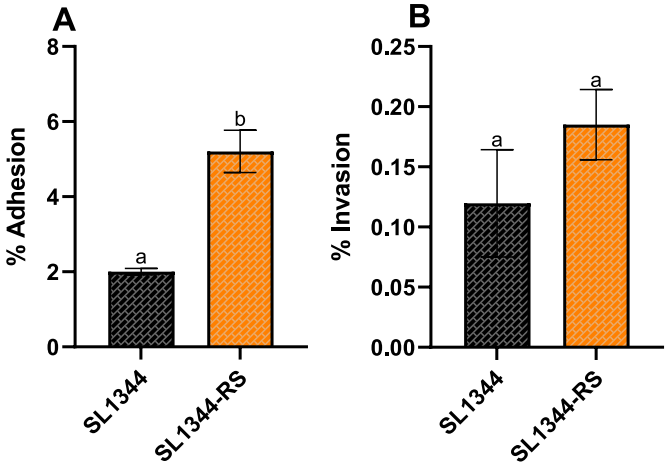

Fig. 4. Adhesion (A) and invasion (B) capacity to Caco-2 cells of *S.* Typhimurium SL1344 (SL1344) and its PEF-resistant variant (SL1344-RS). Different letters indicate statistically significant differences ($p < 0.05$). Error bars correspond to the standard deviation of the means (n = 3).

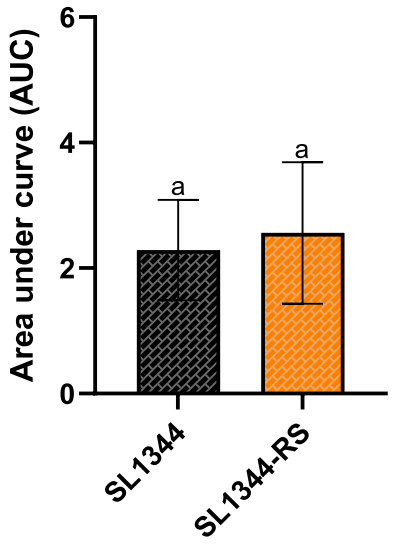

Fig. 5. Biofilm-forming ability of *S.* Typhimurium SL1344 (SL1344) and PEF-resistant variant (SL1344-RS). Values correspond to the Area under the Curve calculated as described in Material and methods. Different letters indicate statistically significant differences ($p < 0.05$). Error bars correspond to the standard deviation of the means (n = 3).

Table 4
Minimum inhibitory concentrations (MIC) of S. Typhimurium SL1344 (SL1344) and its PEF-resistant variant (SL1344-RS). Units in μg/ml.

Antibiotic	Strain	
	SL1344	SL1344-RS
Ampicillin	4	8
Cephalexin	8	32
Chloramphenicol	4	8
Kanamycin	8	64
Nalidixic acid	8	8
Oxytetracycline	1	4
Sulfonamide	4096	4096

was not more sensitive to any of them.

This increased antibiotic resistance of the strain displaying a higher RpoS activity (SL1344-RS) is consistent with previously published results: a link between RpoS activity and antibiotic resistance has already been demonstrated in other bacterial species (Gutierrez et al., 2013; Mathieu et al., 2016; Murakami et al., 2005). Several authors have also described that sub-MICs of antibiotics or repeated exposure to stress can lead to an increase in RpoS activity (Álvarez-Molina et al., 2020), which, in turn, triggers upregulation of several genes for antibiotic resistance, whereas, in the absence of rpoS (ΔrpoS), those cells did not tolerate the same concentration of antibiotic (Dersch et al., 2017; Mathieu et al., 2016).

4. Conclusions

Results obtained in this study indicate that the increased PEF resistance of the PEF-resistant *S.* Typhimurium variant SL1344-RS could be due to increased RpoS activity, caused by a mutation in the hnr gene. This increased RpoS activity also resulted in increased resistance to multiple stresses (acid, osmotic, oxidative, ethanol and UV-C, but not to heat and HHP), decreased growth rate in M9-Gluconate (but not in TSB-YE or LB-DPY), increased ability of adhesion to Caco-2 cells (but without significant changes in invasion ability), and enhanced antibiotic resistance (to six out of eight agents). This study provides a significant contribution to understanding the development of mechanisms of stress resistance in *S. enterica*, and highlights the crucial role played by RpoS in

this process. Since increased stress resistance had a fitness cost in M9-Gluconate, the medium designed to simulate intestinal conditions, further research should be carried out to determine precisely whether this PEF-resistant variant represents a higher, equal, or lower associated hazard than the parental strain in different environments.

Author contributions

Conceptualization, P.M., and G.C.; methodology, S.G., N.H., P.M., and G.C.; formal analysis, S.G., N.H. and G.C.; investigation, S.G. and L. N.; writing-original draft preparation, S.G.; writing-review and editing, L.N., N.H., P.M., and G.C.; supervision, G.C.; project administration, G. C.; funding acquisition, P.M., and G.C.

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Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data are included as supplementary material, in the links provided or will be made available upon request

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2023.104285.

References

Altuvia, S., Almirón, M., Huisman, G., Kolter, R., Storz, G., 1994. The dps promoter is activated by OxyR during growth and by IHF and sigma S in stationary phase. Mol. Microbiol. 13, 265–272. https://doi.org/10.1111/j.1365-2958.1994.tb00421.x.

Álvarez, I., Condón, S., Raso, J., 2006. Microbial inactivation by pulsed electric fields. In: Raso, J., Heinz, V. (Eds.), Pulsed Electric Fields Technology for the Food Industry: Fundamentals and Applications, Food Engineering Series. Springer, Boston, MA, pp. 97–129. https://doi.org/10.1007/978-0-387-31122-7_4.

Álvarez-Molina, A., Toro, M. de, Ruiz, L., López, M., Prieto, M., Alvarez-Ordóñez, A., 2020. Selection for antimicrobial resistance in foodborne pathogens through exposure to UV Light and nonthermal atmospheric plasma decontamination techniques. Appl. Environ. Microbiol. 86 https://doi.org/10.1128/AEM.00102-20.

Andrews, S., 2010. FastQC: A quality control tool for high throughput sequence data [WWW Document]. URL. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/. accessed December.14.21.

Arroyo, C., Cebrián, G., Condón, S., Pagán, R., 2012. Development of resistance in Cronobacter sakazakii ATCC 29544 to thermal and nonthermal processes after exposure to stressing environmental conditions. J. Appl. Microbiol. 112, 561–570. https://doi.org/10.1111/j.1365-2672.2011.05218.x.

Arroyo, C., Somolinos, M., Cebrián, G., Condón, S., Pagán, R., 2010. Pulsed electric fields cause sublethal injuries in the outer membrane of Enterobacter sakazakii facilitating the antimicrobial activity of citral. Lett. Appl. Microbiol. 51, 525–531. https://doi.org/10.1111/j.1472-765X.2010.02931.x.

Atshan, S.S., Shamsudin, M.N., Lung, L.T.T., Ling, K.H., Sekawi, Z., Pei, C.P., Ghaznavi-Rad, E., 2012. Improved method for the isolation of RNA from bacteria refractory to disruption, including S. aureus producing biofilm. Gene 494, 219–224. https://doi.org/10.1016/j.gene.2011.12.010.

Balaji, B., O'Connor, K., Lucas, J.R., Anderson, J.M., Csonka, L.N., 2005. Timing of induction of osmotically controlled genes in Salmonella enterica serovar Typhimurium, determined with quantitative real-time reverse transcription-PCR. Appl. Environ. Microbiol. 71, 8273–8283. https://doi.org/10.1128/AEM.71.12.8273-8283.2005.

Baranyi, J., Roberts, T.A., 2000. Principles and application of predictive modeling of the effects of preservative factors on microorganisms. In: Lund, B., Baird-Parker, T.C., Gould, G.W. (Eds.), Microbiological Safety and Quality of Food. Aspen Publishers, Gaithersburg, Maryland, pp. 342–358.

Barbosa-Cánovas, G.V., Góngora-Nieto, M.M., Pothakamury, U.R., Swanson, B.G., 1999. Biological principles for microbial inactivation in electric fields. In: Góngora-Nieto, M.M., Pothakamury, U.R., Swanson, B.G. (Eds.), Preservation of Foods with Pulsed Electric Fields, Food Science and Technology. Academic Press, US. https://doi.org/10.1016/B978-012078149-2/50004-0.

Battesti, A., Majdalani, N., Gottesman, S., 2011. The RpoS-mediated general stress response in Escherichia coli. Annu. Rev. Microbiol. 65, 189–213. https://doi.org/10.1146/annurev-micro-090110-102946.

Bhagwat, A.A., Tan, J., Sharma, M., Kothary, M., Low, S., Tall, B.D., Bhagwat, M., 2006. Functional heterogeneity of RpoS in stress tolerance of enterohemorrhagic Escherichia coli strains. Appl. Environ. Microbiol. 72, 4978–4986. https://doi.org/10.1128/AEM.02842-05.

Bleibtreu, A., Gros, P.-A., Laouénan, C., Clermont, O., Le Nagard, H., Picard, B., Tenaillon, O., Denamur, E., 2013. Fitness, stress resistance, and extraintestinal virulence in Escherichia coli. Infect. Immun. 81, 2733–2742. https://doi.org/10.1128/IAI.01329-12.

Carabetta, V.J., Mohanty, B.K., Kushner, S.R., Silhavy, T.J., 2009. The response regulator SprE (RssB) modulates polyadenylation and mRNA stability in Escherichia coli. J. Bacteriol. 191, 6812–6821. https://doi.org/10.1128/JB.00870-09.

Cebrián, G., Condón, S., Mañas, P., 2016a. Influence of growth and treatment temperature on Staphylococcus aureus resistance to pulsed electric fields: relationship with membrane fluidity. Innovat. Food Sci. Emerg. Technol. 37, 161–169. https://doi.org/10.1016/j.ifset.2016.08.011.

Cebrián, G., Mañas, P., Condón, S., 2016b. Comparative resistance of bacterial foodborne pathogens to non-thermal technologies for food preservation. Front. Microbiol. 7, 734. https://doi.org/10.3389/fmicb.2016.00734.

Cebrián, G., Raso, J., Condón, S., Mañas, P., 2012. Acquisition of pulsed electric fields resistance in Staphylococcus aureus after exposure to heat and alkaline shocks. Food Control 25, 407–414. https://doi.org/10.1016/j.foodcont.2011.10.048.

Charoenwong, D., Andrews, S., Mackey, B., 2011. Role of rpoS in the development of cell envelope resilience and pressure resistance in stationary-phase Escherichia coli. Appl. Environ. Microbiol. 77, 5220–5229. https://doi.org/10.1128/AEM.00648-11.

Chen, C.Y., Eckmann, L., Libby, S.J., Fang, F.C., Okamoto, S., Kagnoff, M.F., Fierer, J., Guiney, D.G., 1996. Expression of Salmonella typhimurium rpoS and rpoS-dependent genes in the intracellular environment of eukaryotic cells. Infect. Immun. 64, 4739–4743.

Chueca, B., Pagán, R., García-Gonzalo, D., 2015. Transcriptomic analysis of Escherichia coli MG1655 cells exposed to pulsed electric fields. Innovat. Food Sci. Emerg. Technol. 29, 78–86. https://doi.org/10.1016/j.ifset.2014.09.003.

Cui, X., Hu, C., Ou, L., Kuramitsu, Y., Masuda, Y., Honjoh, K., Miyamoto, T., 2019. Transcriptional analysis on heat resistance and recovery from thermal damage in Salmonella under high salt condition. LWT 106, 194–200. https://doi.org/10.1016/j.lwt.2019.02.056.

Dersch, P., Khan, M.A., Mühlen, S., Görke, B., 2017. Roles of regulatory rnas for antibiotic resistance in bacteria and their potential value as novel drug targets. Front. Microbiol. 8.

Dorman, M.J., Thomson, N.R., 2020. 'Community evolution' – laboratory strains and pedigrees in the age of genomics. Microbiology 166, 233–238. https://doi.org/10.1099/mic.0.000869.

El Zakhem, H., Lanoisellé, J.-L., Lebovka, N.I., Nonus, M., Vorobiev, E., 2006. The early stages of Saccharomyces cerevisiae yeast suspensions damage in moderate pulsed electric fields. Colloids Surf. B Biointerfaces 47, 189–197. https://doi.org/10.1016/j.colsurfb.2005.12.010.

Espina, L., Pagán, R., López, D., García-Gonzalo, D., 2015. Individual constituents from essential oils inhibit biofilm mass production by multi-drug resistant Staphylococcus aureus. Molecules 20, 11357–11372. https://doi.org/10.3390/molecules200611357.

Feng, Z., El Hag, M., Qin, T., Du, Y., Chen, S., Peng, D., 2020. Residue L193P mutant of RpoS affects its activity during biofilm formation in Salmonella Pullorum. Front. Vet. Sci. 7, 571361 https://doi.org/10.3389/fvets.2020.571361.

García, D., Gómez, N., Raso, J., Pagán, R., 2005. Bacterial resistance after pulsed electric fields depending on the treatment medium pH. Innovat. Food Sci. Emerg. Technol. 6, 388–395. https://doi.org/10.1016/j.ifset.2005.04.003.

Geeraerd, A.H., Herremans, C.H., Van Impe, J.F., 2000. Structural model requirements to describe microbial inactivation during a mild heat treatment. Int. J. Food Microbiol. 59, 185–209. https://doi.org/10.1016/S0168-1605(00)00362-7.

Geeraerd, A.H., Valdramidis, V.P., Van Impe, J.F., 2005. GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves. Int. J. Food Microbiol. 102, 95–105. https://doi.org/10.1016/j.ijfoodmicro.2004.11.038.

Golberg, A., Rae, C.S., Rubinsky, B., 2012. Listeria monocytogenes cell wall constituents exert a charge effect on electroporation threshold. Biochim. Biophys. Acta 1818, 689–694. https://doi.org/10.1016/j.bbamem.2011.11.003.

Guillén, S., Marcén, M., Fau, E., Mañas, P., Cebrián, G., 2022. Relationship between growth ability, virulence, and resistance to food-processing related stresses in non-typhoidal Salmonellae. Int. J. Food Microbiol. 361, 109462 https://doi.org/10.1016/j.ijfoodmicro.2021.109462.

Guillén, S., Marcén, M., Mañas, P., Cebrián, G., 2020. Differences in resistance to different environmental stresses and non-thermal food preservation technologies among Salmonella enterica subsp. enterica strains. Food Res. Int. 132, 109042 https://doi.org/10.1016/j.foodres.2020.109042.

Guillén, S., Nadal, L., Álvarez, I., Mañas, P., Cebrián, G., 2021. Impact of the resistance responses to stress conditions encountered in food and food processing environments on the virulence and growth fitness of non-typhoidal Salmonellae. Foods 10, 617. https://doi.org/10.3390/foods10030617.

Gutierrez, A., Laureti, L., Crussard, S., Abida, H., Rodríguez-Rojas, A., Blázquez, J., Baharoglu, Z., Mazel, D., Darfeuille, F., Vogel, J., Matic, I., 2013. β-lactam antibiotics promote bacterial mutagenesis via an RpoS-mediated reduction in replication fidelity. Nat. Commun. 4, 1610. https://doi.org/10.1038/ncomms2607.

Haskamp, V., Karrie, S., Mingers, T., Barthels, S., Alberge, F., Magalon, A., Müller, K., Bill, E., Lubitz, W., Kleeberg, K., Schweyen, P., Bröring, M., Jahn, M., Jahn, D., 2018.

The radical SAM protein HemW is a heme chaperone. J. Biol. Chem. 293, 2558–2572. https://doi.org/10.1074/jbc.RA117.000229.
Heinz, V., Alvarez, I., Angersbach, A., Knorr, D., 2001. Preservation of liquid foods by high intensity pulsed electric fields—basic concepts for process design. Trends Food Sci. Technol. 12, 103–111. https://doi.org/10.1016/S0924-2244(01)00064-4.
Hengge, R., 2011. Stationary-phase gene regulation in Escherichia coli. EcoSal Plus 4. https://doi.org/10.1128/ecosalplus.5.6.3.
Hengge-Aronis, R., 1996. Regulation of gene expression during entry into stationary phase. In: Neidhardt, F.C., Curtiss III, R., Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Riley, M., Schaschter, M., Umbarger, H.E. (Eds.), Escherichia Coli and Salmonella Typhimurium: Cellular and Molecular Biology. ASM Press, Washington, D.C., pp. 1497–1511
Ho, S.Y., Mittal, G.S., 1996. Electroporation of cell membranes: a review. Crit. Rev. Biotechnol. 16, 349–362. https://doi.org/10.3109/07388559609147426.
Ibañez-Ruiz, M., Robbe-Saule, V., Hermant, D., Labrude, S., Norel, F., 2000. Identification of RpoS (σ^S)-regulated genes in Salmonella enterica serovar typhimurium. J. Bacteriol. 182, 5749–5756. https://doi.org/10.1128/jb.182.20.5749-5756.2000.
Iwase, T., Tajima, A., Sugimoto, S., Okuda, K., Hironaka, I., Kamata, Y., Takada, K., Mizunoe, Y., 2013. A simple assay for measuring catalase activity: a visual approach. Sci. Rep. 3, 3081. https://doi.org/10.1038/srep03081.
Kinosita, K., Hibino, M., Itoh, H., Shigemori, M., Hirano, K., Kirino, Y., Hayakawa, T., 2012. Events of membrane electroporation visualized on a time scale from microsecond to seconds. In: Guide to Electroporation and Electrofusion. Elsevier Inc., pp. 29–46. https://doi.org/10.1016/B978-0-08-091727-6.50006-X
Kröger, C., Dillon, S.C., Cameron, A.D.S., Papenfort, K., Sivasankaran, S.K., Hokamp, K., Chao, Y., Sittka, A., Hébrard, M., Händler, K., Colgan, A., Leekitcharoenphon, P., Langridge, G.C., Lohan, A.J., Loftus, B., Lucchini, S., Ussery, D.W., Dorman, C.J., Thomson, N.R., Vogel, J., Hinton, J.C.D., 2012. The transcriptional landscape and small RNAs of Salmonella enterica serovar Typhimurium. Proc. Natl. Acad. Sci. USA 109, E1277–E1286. https://doi.org/10.1073/pnas.1201061109.
Krogfelt, K.A., Hjulgaard, M., Sørensen, K., Cohen, P.S., Givskov, M., 2000. rpoS gene function is a disadvantage for Escherichia coli BJ4 during competitive colonization of the mouse large intestine. Infect. Immun. 68, 2518–2524. https://doi.org/10.1128/IAI.68.5.2518-2524.2000.
Lago, M., Monteil, V., Douche, T., Guglielmini, J., Criscuolo, A., Maufrais, C., Matondo, M., Norel, F., 2017. Proteome remodelling by the stress sigma factor RpoS/σ^S in Salmonella: identification of small proteins and evidence for post-transcriptional regulation. Sci. Rep. 7, 2127. https://doi.org/10.1038/s41598-017-02362-3.
Lee, I.S., Lin, J., Hall, H.K., Bearson, B., Foster, J.W., 1995. The stationary-phase sigma factor sigma S (RpoS) is required for a sustained acid tolerance response in virulent Salmonella typhimurium. Mol. Microbiol. 17, 155–167. https://doi.org/10.1111/j.1365-2958.1995.mmi_17010155.x.
Lévi-Meyrueis, C., Monteil, V., Sismeiro, O., Dillies, M.-A., Monot, M., Jagla, B., Coppée, J.-Y., Dupuy, B., Norel, F., 2014. Expanding the RpoS/σ^S-network by RNA sequencing and identification of σ^S-controlled small RNAs in Salmonella. PLoS One 9, e96918. https://doi.org/10.1371/journal.pone.0096918.
Loewen, P.C., Hu, B., Strutinsky, J., Sparling, R., 1998. Regulation in the rpoS regulon of Escherichia coli. Can. J. Microbiol. 44, 707–717. https://doi.org/10.1139/cjm-44-8-707.
Mañas, P., Pagán, R., 2005. Microbial inactivation by new technologies of food preservation. J. Appl. Microbiol. 98, 1387–1399. https://doi.org/10.1111/j.1365-2672.2005.02561.x.
Marcén, M., Cebrián, G., Ruiz-Artiga, V., Condón, S., Mañas, P., 2019. Cellular events involved in E. coli cells inactivation by several agents for food preservation: a comparative study. Food Microbiol. 84, 103246 https://doi.org/10.1016/j.fm.2019.103246.
Mathieu, A., Fleurier, S., Frénoy, A., Dairou, J., Bredeche, M.-F., Sanchez-Vizuete, P., Song, X., Matic, I., 2016. Discovery and function of a general core hormetic stress response in E. coli induced by sublethal concentrations of antibiotics. Cell Rep. 17, 46–57. https://doi.org/10.1016/j.celrep.2016.09.001.
Mellor, G.E., Goulter, R.M., Chia, T.W.R., Dykes, G.A., 2009. Comparative analysis of attachment of Shiga-toxigenic Escherichia coli and Salmonella strains to cultured HT-29 and Caco-2 cell lines. Appl. Environ. Microbiol. 75, 1796–1799. https://doi.org/10.1128/AEM.02232-08.
Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., Wold, B., 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods 5, 621–628. https://doi.org/10.1038/nmeth.1226.
Murakami, K., Ono, T., Viducic, D., Kayama, S., Mori, M., Hirota, K., Nemoto, K., Miyake, Y., 2005. Role for rpoS gene of Pseudomonas aeruginosa in antibiotic tolerance. FEMS Microbiol. Lett. 242, 161–167. https://doi.org/10.1016/j.femsle.2004.11.005.
Österberg, S., del Peso-Santos, T., Shingler, V., 2011. Regulation of alternative sigma factor use. Annu. Rev. Microbiol. 65, 37–55. https://doi.org/10.1146/annurev.micro.112408.134219.
Pakhomova, O.N., Khorokhorina, V.A., Bowman, A.M., Rodaitė-Riševičienė, R., Saulis, G., Xiao, S., Pakhomov, A.G., 2012. Oxidative effects of nanosecond pulsed electric field exposure in cells and cell-free media. Arch. Biochem. Biophys. 527, 55–64. https://doi.org/10.1016/j.abb.2012.08.004.
Pavlin, M., Kotnik, T., Miklavčič, D., Kramar, P., Maček Lebar, A., 2008. Electroporation of planar lipid bilayers and membranes. In: Leitmannova Liu, A. (Ed.), Advances in Planar Lipid Bilayers and Liposomes. Academic Press, pp. 165–226. https://doi.org/10.1016/S1554-4516(07)06007-3.
Pavlin, M., Leben, V., Miklavcic, D., 2007. Electroporation in dense cell suspension–theoretical and experimental analysis of ion diffusion and cell permeabilization. Biochim. Biophys. Acta 1770, 12–23. https://doi.org/10.1016/j.bbagen.2006.06.014.
Peng, D., 2016. Biofilm Formation of Salmonella. In: Dhanasekaran, D., Thajuddin, N. (Eds.), Microbial Biofilms - Importance and Applications. InTech, London, pp. 231–249.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29, e45. https://doi.org/10.1093/nar/29.9.e45.
Ramos, S.J., Chiquirrín, M., García, S., Condón, S., Pérez, M.D., 2015. Effect of high pressure treatment on inactivation of vegetative pathogens and on denaturation of whey proteins in different media. LWT–Food Sci. Technol. 63, 732–738. https://doi.org/10.1016/j.lwt.2015.03.085.
Raza, K., Mishra, A., 2012. A novel anticlustering filtering algorithm for the prediction of genes as a drug target. Am. J. Biomed. Eng. 2, 206–211. https://doi.org/10.5923/j.ajbe.20120205.03.
Robbe-Saule, V., Coynault, C., Ibanez-Ruiz, M., Hermant, D., Norel, F., 2001. Identification of a non-haem catalase in Salmonella and its regulation by RpoS (σ^S). Mol. Microbiol. 39, 1533–1545. https://doi.org/10.1046/j.1365-2958.2001.02340.x.
Robey, M., Benito, A., Hutson, R.H., Pascual, C., Park, S.F., Mackey, B.M., 2001. Variation in resistance to high hydrostatic pressure and rpoS heterogeneity in natural isolates of Escherichia coli O157:H7. Appl. Environ. Microbiol. 67, 4901–4907. https://doi.org/10.1128/AEM.67.10.4901-4907.2001.
Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140. https://doi.org/10.1093/bioinformatics/btp616.
Römling, U., Bokranz, W., Rabsch, W., Zogaj, X., Nimtz, M., Tschäpe, H., 2003. Occurrence and regulation of the multicellular morphotype in Salmonella serovars important in human disease. Int. J. Med. Microbiol. 293, 273–285. https://doi.org/10.1078/1438-4221-00268.
Sabbagh, S.C., Forest, C.G., Lepage, C., Leclerc, J.-M., Daigle, F., 2010. So similar, yet so different: uncovering distinctive features in the genomes of Salmonella enterica serovars Typhimurium and Typhi. FEMS Microbiol. Lett. 305, 1–13. https://doi.org/10.1111/j.1574-6968.2010.01904.x.
Sagarzazu, N., Cebrián, G., Pagán, R., Condón, S., Mañas, P., 2013. Emergence of pulsed electric fields resistance in Salmonella enterica serovar Typhimurium SL1344. Int. J. Food Microbiol. 166, 219–225. https://doi.org/10.1016/j.ijfoodmicro.2013.07.001.
Sanz-Puig, M., Velázquez-Moreira, A., Torres, C., Guerrero-Beltrán, J.Á., Cunha, L.M., Martinez, A., Rodrigo, D., 2019. Resistance changes in Salmonella enterica serovar Typhimurium treated by high hydrostatic pressure and pulsed electric fields and assessment of virulence changes by using Caenorhabditis elegans as a test organism. Innov. Food Sci. Emerg. Technol., Innovations in Food Science and Technology at the Spanish National Research Council (CSIC) 51, 51–56. https://doi.org/10.1016/j.ifset.2018.04.002.
Schellhorn, H.E., 1995. Regulation of hydroperoxidase (catalase) expression in Escherichia coli. FEMS Microbiol. Lett. 131, 113–119. https://doi.org/10.1016/0378-1097(95)00215-Q.
Simm, R., Ahmad, I., Rhen, M., Le Guyon, S., Römling, U., 2014. Regulation of biofilm formation in Salmonella enterica serovar Typhimurium. Future Microbiol. 9, 1261–1282. https://doi.org/10.2217/fmb.14.88.
Somolinos, M., García, D., Mañas, P., Condón, S., Pagán, R., 2008. Effect of environmental factors and cell physiological state on Pulsed Electric Fields resistance and repair capacity of various strains of Escherichia coli. Int. J. Food Microbiol. 124, 260–267. https://doi.org/10.1016/j.ijfoodmicro.2008.03.021.
Sözer, E.B., Levine, Z.A., Vernier, P.T., 2017. Quantitative limits on small molecule transport via the electropermeome — measuring and modeling single nanosecond perturbations. Sci. Rep. 7, 57. https://doi.org/10.1038/s41598-017-00092-0.
Tanaka, K., Handel, K., Loewen, P.C., Takahashi, H., 1997. Identification and analysis of the rpoS-dependent promoter of katE, encoding catalase HPII in Escherichia coli. Biochim. Biophys. Acta 1352, 161–166. https://doi.org/10.1016/S0167-4781(97)00044-4.
Tarek, M., 2005. Membrane electroporation: a molecular dynamics simulation. Biophys. J. 88, 4045–4053. https://doi.org/10.1529/biophysj.104.050617.
Tsong, T.Y., 1991. Electroporation of cell membranes. Biophys. J. 60, 297–306. https://doi.org/10.1016/S0006-3495(91)82054-9.
van Iterson, M., Boer, J.M., Menezes, R.X., 2010. Filtering, FDR and power. BMC Bioinf. 11, 450. https://doi.org/10.1186/1471-2105-11-450.
Vidovic, S., Mangalappalli-Illathu, A.K., Xiong, H., Korber, D.R., 2012. Heat acclimation and the role of RpoS in prolonged heat shock of Escherichia coli O157. Food Microbiol. 30, 457–464. https://doi.org/10.1016/j.fm.2011.12.029.
Visick, J.E., Clarke, S., 1997. RpoS- and OxyR-independent induction of HPI catalase at stationary phase in Escherichia coli and identification of rpoS mutations in common laboratory strains. J. Bacteriol. 179, 4158–4163. https://doi.org/10.1128/jb.179.13.4158-4163.1997.
Weaver, J.C., Chizmadzhev, YuA., 1996. Theory of electroporation: a review. Bioelectrochem. Bioenerg. 41, 135–160. https://doi.org/10.1016/S0302-4598(96)05062-3.
Zhou, Y., Gottesman, S., 1998. Regulation of proteolysis of the stationary-phase sigma factor RpoS. J. Bacteriol. 180, 1154–1158.